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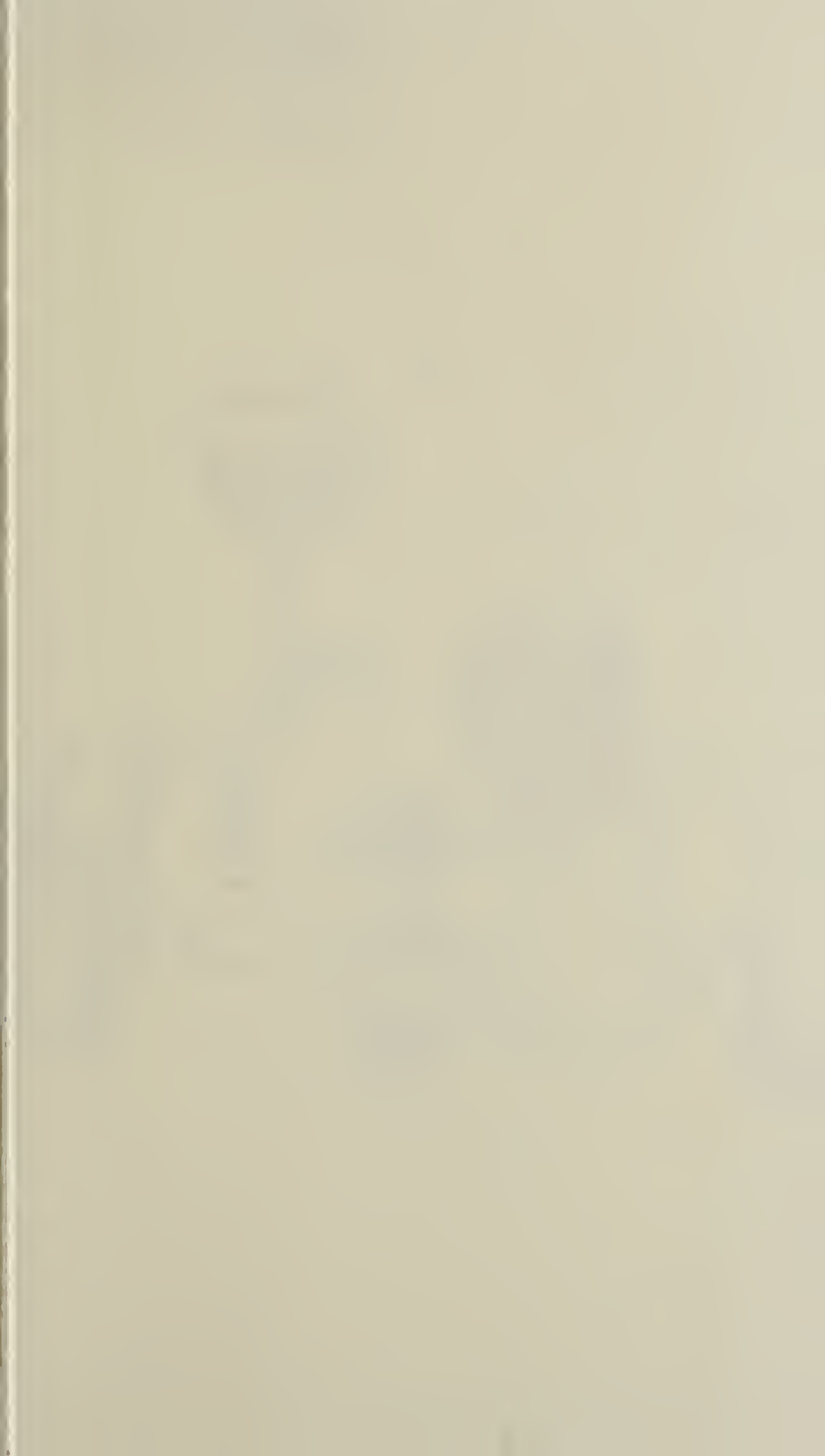
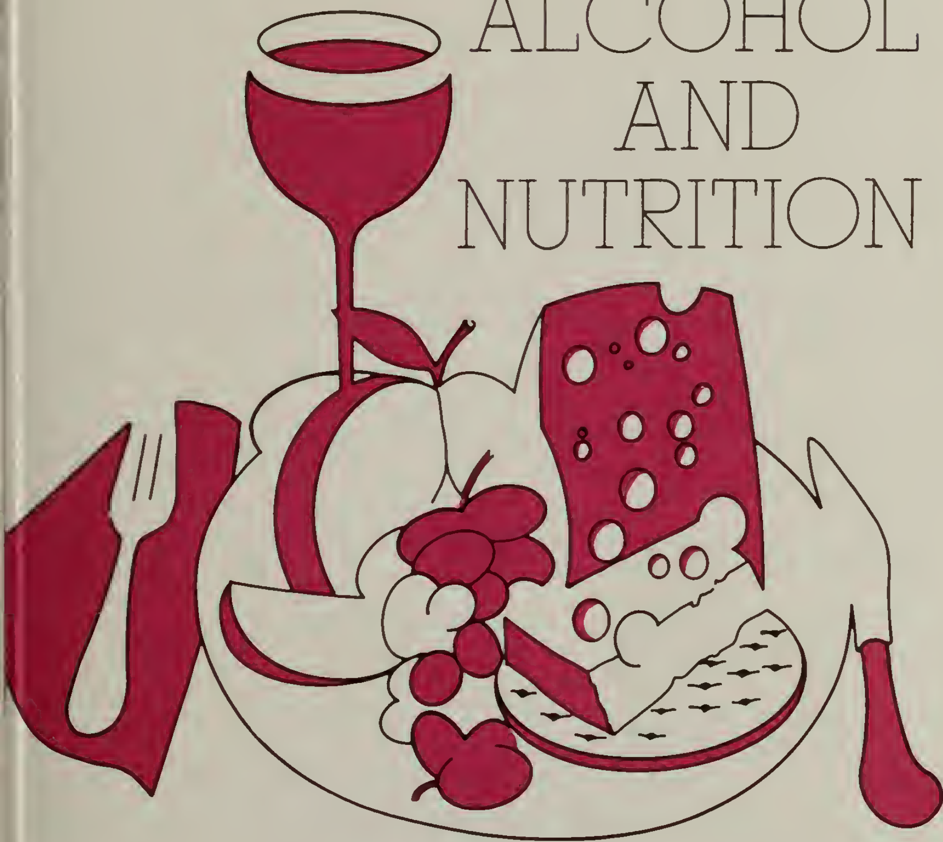


National Institute on Alcohol Abuse and Alcoholism

RESEARCH Monograph 2

ALCOHOL AND NUTRITION



U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE • Public Health Service • Alcohol, Drug Abuse, and Mental Health Administration

Research Monograph No. 2

ALCOHOL AND NUTRITION

Proceedings of a Workshop
September 26-27, 1977
Indianapolis, Indiana

Sponsored by:

Division of Extramural Research, NIAAA

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Foreword

This volume is the second in the series of research monographs recently begun by the National Institute on Alcohol Abuse and Alcoholism. This series sets forth current information on a number of topics relevant to alcohol abuse and alcoholism as reported through workshops in research, prevention, and treatment as well as through state-of-the-art reviews on selected subjects.

This monograph is also the second one based on a research workshop sponsored by NIAAA. The main purposes for supporting research workshops are to disseminate newly acquired knowledge, to uncover new research opportunities, to stimulate fresh ideas for study, and to attract the best scientists into alcoholism research. I believe that this research monograph series enhances the prospects that these purposes will be achieved. Much has already been accomplished by gathering together the group of scientists at the research workshop on "Alcohol and Nutrition": recent findings have been presented to and shared with other scientists in open forum; and discussions have yielded a critical examination of these findings and have suggested new studies as a logical extension of these data. It is too early to determine if the alcoholism research community has been increased as a result of this workshop. The publication of the proceedings of this workshop will further extend what already has been accomplished by providing a written record of the workshop and broadcasting this record far beyond the immediate time and place of the workshop itself.

Albert A. Pawlowski, Ph.D.
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Introduction

In September of 1977, the National Institute on Alcohol Abuse and Alcoholism held a workshop on the interrelations of alcohol and nutrition. This volume represents the proceedings of that workshop.

The importance of this subject to alcoholism and alcohol-derived diseases is clear. Alcohol is ingested primarily because of its psychopharmacologic effects. However, it exerts effects upon the nutritional health of the consumer through the contribution of its own caloric content and through its disruptive effects on gastrointestinal function, nutrient absorption, vitamin activation and elimination, mineral and fluid electrolyte balance, energy, and carbohydrate, lipid, and protein metabolism. The caloric content of alcohol is substantial—7.1 cal/gram. A consumer of 20 ounces of 86-proof beverage derives from this 1,500 calories, or one-half to two-thirds of his or her daily caloric needs. Economic factors and impaired appetite can further contribute to deficient nutrient intake. An added complication is the phenomenon of energy wastage: the observation that healthy, nonalcoholic individuals lose weight on an adequate diet which contains alcohol as 36 percent of total calories.

The nutrient value of the calories derived from alcoholic beverages is poor. Some investigators have termed these "empty calories," owing to the absence of significant protein, vitamin, or mineral content in such beverages.

This volume also addresses the subject of alcohol metabolism and important questions related to the effects nutrition may have in altering enzyme and co-factor levels in the pathways of alcohol oxidation.

An adequate understanding of the nutritional consequences of alcoholism is fundamental for knowledge development in all of the biological disciplines attacking the problems of alcohol abuse and alcoholism. As such, it is hoped that this volume will be useful to pharmacologists and behavorial scientists, as it will be to physiologists, biochemists, and nutritionists.

Kenneth R. Warren, Ph.D.
National Institute on Alcohol Abuse and Alcoholism

Summary

Section I: Effects of Alcohol on Nutrition and Gastrointestinal Functions

Dr. Smith emphasized that although overt nutritional exogenous deficiencies have nearly been eliminated in the United States, marginal and conditioned nutritional inadequacies are still prevalent. The traditional methods for assessing nutritional states are often insensitive, however, so new approaches must be developed to detect these marginal and conditioned deficiencies. Establishing "normal values" for these states is also an important problem. More precise guidelines for interpreting laboratory tests must be established.

Since the turn of the century, food consumption trends in the United States indicate a marked alteration in our intake of certain foods—we eat more refined sugars, processed foods, and fats and oils and less flour and cereal products. Fats and oils cannot be depended upon as major sources of fat-soluble vitamins, and they are extremely poor sources of trace elements. Data from the First Health and Nutrition Examination Survey (HANES I), conducted in 1971-72, indicate that a substantial segment of the American population has a substandard intake of certain nutrients—particularly iron, vitamin A, calcium, and trace elements such as zinc. Factors that produce conditioned nutritional inadequacies include dietary chelators, drug ingestion, and alcohol abuse. Alcohol abuse is the most common cause of vitamin and trace element deficiency in adults in the United States. Alcohol abuse also leads to inefficient use of energy.

Dr. Lieber reviewed the complex interaction between alcohol and nutrition. Alcohol abuse leads to primary malnutrition (i.e., deficient intake) and secondary malnutrition (i.e., deficient nutrient utilization). Alcoholic beverages provide "empty" calories because ethanol does not contain significant amounts of protein, vitamins, or minerals. A person who drinks 20 ounces of an 86-proof beverage consumes about 1,500 empty calories—one-half to two-thirds of the normal daily caloric requirement. Thus intake of other foods decreases, and daily nutrient ingestion becomes grossly imbalanced. In addition to this problem, economic

factors and impaired appetite (caused by gastrointestinal and liver disorders) also contribute to deficient nutritional intake.

Secondary malnutrition is also caused by multiple factors: ethanol-induced gastrointestinal damage, deficiency-induced maldigestion and malabsorption, decreased activation or increased inactivation of nutrients, and energy wastage. Recognition of energy wastage due to alcohol consumption is a new finding in experimental animals and in human studies. Human nonalcoholic subjects placed on an adequate diet, but who received 36 percent of their total calories in ethanol, consistently lost weight even though no decrease in absorption of energy-containing substances was observed. The mechanism responsible for energy wastage due to alcohol consumption is not yet known. Experimental data indicate that either the induction of the microsomal ethanol-oxidizing system (MEOS) or the activation of Na^{+}–K^{+} ATPase, or both, may be causally linked to energy wastage.

Dr. Lieber also reviewed the pathogenesis of alcoholic liver disease. In the baboon, chronic alcohol feeding in the presence of otherwise adequate diet produced the sequential development of fatty liver, alcoholic hepatitis, and cirrhosis.

Dr. Dietschy presented an elaborate formulation of the general types of transport involved in the movement of solutes across biological membranes and across the gut in particular. He discussed the characteristics of passive and active transport, with special emphasis on solute interaction, membrane polarity, and diffusion barriers (particularly the unstirred water layer). Alcohol could alter transport of a given substrate by altering the unstirred layer resistance, changing gut permeability, or affecting active transport.

Dr. Shanbour discussed the effects of alcohol on the function of the key digestive organs: stomach, pancreas, liver, and small intestine. Studies in her laboratory on isolated dog stomachs showed that 20-percent ethanol decreased gastric acid secretion by one-third, inhibited active transport of various ions, and decreased gastric mucosal adenosine triphosphate (ATP). Isolated rat pancreas preparations showed that ethanol inhibits bicarbonate and water output; this inhibition is associated with a fall in pancreatic ATP but not in cyclic adenosine monophosphate (AMP) content. Again it was postulated that the decrease in ATP may be the mechanism by which alcohol impairs pancreatic function. In the area of the liver, alcohol given orally (but not intravenously) increased hepatic cyclic AMP but did not alter ATP levels. Glucagon also stimulated hepatic cyclic AMP; this effect was potentiated by alcohol. Thus, oral alcohol may exert its effect on hepatic nucleotides via gut hormones (i.e., glucagon and secretin) or via enhanced

hepatic sensitivity to them. Finally, Dr. Shanbour commented on the effects of alcohol on the jejunum. Using an Ussing chamber, which neutralizes electrochemical gradients across the gut, it was shown that 3-percent ethanol decreased the active transport of Na^+, 3-*o*-methylglucose, and L-alanine to less than 50 percent of pre-ethanol values.

Section II: Effects of Alcohol on Mineral Metabolism

Dr. Vallee provided an overview of the rapidly developing field of zinc biochemistry. Zinc is a dietary essential. In recent years, several zinc deficiency syndromes have been defined in humans. These deficiencies include growth failure, hypogonadism, and acrodermatitis enteropathica; all are amenable to zinc therapy. Zinc deficiency also appears to be a problem in patients with alcoholism and/or cirrhosis; however, the benefit of zinc therapy has not yet been evaluated in controlled clinical trials with alcoholic patients.

There are presently 92 known zinc metalloenzymes and metalloproteins. The metalloenzymes participate not only in carbohydrate, protein, and nucleic acid metabolism, but also in alcohol metabolism. At the molecular level, zinc appears to function in two ways: as an essential component of enzymic catalysis and as a determinant in the structural configuration of certain nonenzymic macromolecules. Recent advances in zinc biochemistry include the characterization of human alcohol dehydrogenase isoenzymes (see later: Bosron and Li) and the finding that adequate zinc nutriture is required for the activities of DNA and RNA polymerases and reverse transcriptases (see later: Falchuk).

Dr. Falchuk reported on recent studies of the metabolic effect of zinc deprivation on *Euglena gracilis*. This eukaryotic organism exhibits growth arrest when the zinc content in the medium is decreased to $<10^{-7}$ M. A number of striking biochemical changes follow: cellular DNA content doubles, cell volume increases, protein content and ^{3}H-uridine incorporation into RNA both decrease, certain unusual proteins accumulate, and the cellular content of Mn, Mg, Ca, Fe, Ni, Cr, and Cu increases. By using synchronized cell populations and flow cytofluorometry, it is found that the biochemical processes essential for cells to pass from G_1 into S, from S to G_2, and from G_2 to mitosis depend on the presence of zinc; its deficiency blocks all three phases of the growth cycle of *E. gracilis*. Zinc is essential for the function of DNA polymerase, and it is also required for RNA metabolism. Whereas two DNA-dependent RNA polymerases, I and II (each

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contains two atoms of zinc per molecule of enzyme), are found in zinc-sufficient *E. gracilis*, a single, unusual RNA polymerase (also containing zinc) is present in the zinc-deficient organism. This major change in the RNA polymerases, induced by zinc deficiency, results in a doubling of the content and in a profound alteration in the base composition of the mRNA in the zinc-deficient cells. These findings in *E. gracilis* clearly illustrate the importance of zinc nutriture in the translation of information from the genome into proteins.

Dr. Prasad reviewed the clinical syndromes of zinc deficiency in man. The best documented syndromes are the hypogonadal dwarfs in Iran and Egypt, congenital acrodermatitis enteropathica, and acquired forms of acrodermatitis enteropathica (e.g., secondary to prolonged total parenteral alimentation). Zinc therapy in these instances results in dramatic cure.

The present understanding of zinc deficiency, conditioned by alcohol abuse and alcoholic liver disease, is fragmentary and somewhat speculative. The laboratory criteria for the diagnosis of zinc deficiency have not been unequivocally established. Plasma zinc, erythrocytic zinc, hair zinc, urinary zinc, and salivary zinc are useful indexes, but each has pitfalls.

Abnormal zinc metabolism, which occurs in patients with alcoholic cirrhosis, was first described by Dr. Vallee. The abnormal features include low serum zinc, decreased hepatic zinc, and, paradoxically, hyperzincuria. Although these observations have now been corroborated in many laboratories, whether low serum zinc and decreased hepatic zinc are pathognomonic of zinc deficiency is uncertain. Kinetic studies with ^{65}Zn in cirrhotics indicate a diminished pool size, but a careful metabolic balance study of zinc in cirrhotics has not yet been performed. The spectrum of alcoholic liver diseases varies physiologically and biochemically from minimal aberration of function to severe functional impairment. Yet there have been no studies of zinc metabolism in the alcoholic patient that address this point. At present, the critical test for zinc deficiency in humans is a definitive clinical response to zinc supplementation under controlled conditions. However, with the innumerable metabolic and clinical manifestations in liver disease, it has been difficult to define the specific abnormality benefited by zinc therapy.

The critical question is "How does zinc work?" Does it work in a general way and thereby improve the general well-being and, perhaps, the survival of alcoholics with and without liver disease? Or, does it work in a specific manner, such as by improving drug metabolism, correcting the metabolic defects in hepatic coma, or rectifying night blindness and aberrations in taste and smell?

Dr. Beard, who summarized the current state of knowledge about the effect of ethanol on Mg^{2+} metabolism, emphasized that careful balance studies are critical to further understanding of the interrelationship between ethanol and Mg^{2+} metabolism. Mg^{2+} in the body is compartmentalized in bone, skeletal muscle, and extracellular spaces. Mg^{2+} is ubiquitous in just about all known foodstuffs; deficiency due to dietary inadequacy is difficult to produce. About 30 to 40 percent of the Mg^{2+} ingested is absorbed by the gastrointestinal tract. Fractional Mg^{2+} absorption varies with intake; i.e., with increased intake, absorption decreases. Mg^{2+} is excreted mainly by the kidneys. Thus, to study the effect of ethanol on Mg^{2+} metabolism, its effect on the compartmentalization of Mg^{2+} must be defined.

What is the mechanism of the acute increase in serum magnesium and increased urinary excretion following the ingestion of alcohol? What are the mechanisms involved in producing the sudden reduction of serum Mg^{2+} following abstinence from alcohol? Finally, what would be the effect of given deficits or shifts in the distribution of Mg^{2+} on cellular intermediary metabolism?

The next area for discussion was opened by Dr. Knochel who presented information on the pathogenesis and the effect of phosphate depletion in the alcoholic patient. Hypophosphatemia, associated with the administration of nutrients without adequate phosphorus supplementation, may not occur until 4 or 5 days after admission to a hospital. Hypophosphatemia is not necessarily diagnostic of phosphorus deficiency because it may be the result of respiratory alkalosis without phosphorus deficiency. Moreover, severe phosphorus deficiency, as evidenced by a markedly lowered muscle phosphorus content, may exist in the face of a normal serum phosphorus concentration. Hypophosphatemia commonly occurs in association with a sharp rise in creatine phosphokinase activity in serum, and, in this instance, phosphorus deficit may exceed 500 mmol. The pathogenesis of phosphorus deficiency in the alcoholic patient is multifactorial; this deficiency stems from inadequate dietary intake vomiting and diarrhea, magnesium deficiency, deranged vitamin D metabolism, hypercalcitoninemia, and metabolic acidosis. In addition, administration of nutrients, infusion of fructose, acute respiratory alkalosis, and hyperinsulinism may compound phosphorus deficiency by producing acute hypophosphatemia. Phosphorus deficiency and hypophosphatemia may result in serious consequences: osteomalacia; myopathy; rhabdomyolysis; impairment of erythrocytic, leukocytic, and platelet function; hemolysis; renal tubular lesions;

neurological impairment; myocardial insufficiency; and hepatocellular dysfunction.

Dr. McDonald described her recent metabolic balance studies on the effect of ethanol on mineral metabolism in humans. Six healthy, male, nonalcoholic volunteers participated in these experiments, which consisted of one 3-day equilibrium period followed by four 18-day experimental periods. Each man served as his own control. In a randomized block design, wine, dealcoholized wine, ethanol, and deionized water were individually consumed with meals in each experimental period. Caloric intakes were adjusted for each experimental period to keep body weight constant. During the alcohol periods, the blood ethanol level of the subjects was 20 mg% 1 hour after a meal; no alcohol could be detected 3 hours postprandially. The effects of wine, dealcoholized wine, ethanol, and deionized water on Na^{+}, K^{+}, Ca^{2+}, P, Mg^{2+}, Zn^{2+}, and nitrogen excretion and balance are shown in table 1. These data indicate that wine and ethanol differ in their effects on mineral balance and that constituents in wine other than ethanol also affect mineral balance.

Table 1. The Effect of Wine, Dealcoholized Wine, Ethanol, and Deionized Water on Mineral Excretion and Balance*

Mineral Excretion and Balance		Wine	Dealcoholized Wine	Ethanol	Deionized H_2O
Na^{+}	Fecal				
	Urinary		↓		
K^{+}	Fecal				
	Urinary			↑	
	Balance			–	
Ca^{2+}	Fecal			↑	↑
	Urinary				
	Balance	–	–	more –	more –
P	Fecal			↑	↑
	Urinary			↑	
	Balance	–	+	–	–
Mg^{2+}	Fecal			↑	↑
	Urinary				
	Balance	–	–	–	–
Zn^{2+}	Fecal			↑	
	Urinary	↑		↑	
	Balance	+	+	–	–
Nitrogen, Urinary		↑		↑	

*↑, increased excretion; ↓, decreased excretion; –, negative balance; +, positive balance.

As shown in table 1, ethanol increases Mg^{2+} excretion in feces. However, the balance measurements in the studies performed by Dr. McDonald did not provide any definitive information on Mg^{2+} metabolism because all the subjects exhibited negative Mg^{2+} balance with all the drinking solutions.

Section III: Effects of Alcohol on Vitamin Metabolism

Dr. Halsted described the results of a study of the effects of prolonged (3 to 6 months) oral administration of alcohol, accounting for 50 percent of the total calories, to macaque monkeys. Effects on hepatic histology, serum markers such as serum glutamic oxaloacetic transaminase (SGOT) of hepatic injury, liver and erythrocyte folate levels, and intestinal folate absorption were analyzed. The liver showed evidence of damage, with increased fat deposition, early collagen in the space of Disse, and mitochondrial injury accompanied by increasing SGOT levels. Hepatic folate concentration and the quantity present as methylated folate decreased in the ethanol-fed animals, but gut mucosa and erythrocyte folate remained normal. There was no evidence of anemia. There was indirect evidence of impaired folate absorption in the ethanol-fed baboons. Dr. Halsted emphasized the need for further assessment of hepatic status and folate absorption as well as folate tissue levels in more prolonged states of controlled alcohol feeding to these subhuman primates. This animal model lends itself to various modifications of experimental design to assess the interrelationship of alcohol and nutritional deficiency.

Dr. Hillman discussed the possible mechanism(s) whereby alcohol acutely depresses serum folate. This phenomenon is unrelated to methodologic artifacts or the presence of folate binders in plasma. Using labeled folate (^{3}H-pteroyl-glutamic acid and ^{14}C methyltetrahydrofolic acid), it was shown that, normally, these compounds are taken up rapidly by the liver. The pteroylglutamic acid is reduced and methylated to methyltetrahydrofolate monoglutamate in liver and to some extent (15 to 20 percent) to the pentaglutamate form. On the other hand, the methyltetrahydrofolate is not incorporated into the hepatic folate pool and is not excreted in bile. The excreted methyltetrahydrofolate enters an enterohepatic cycle, which has a half-life of about 6 hours. Thus, enterohepatic circulation of folate is important in controlling serum folate levels.

In folate deficiency, Dr. Hillman reported, the hepatic storage of folate is decreased, and the enterohepatic cycle is increased.

Teleologically, this would tend to maintain the level of serum folate. Alcohol, on the other hand, causes a major shunting of pteroylglutamic acid into the pentaglutamate liver stores. This results in a major decrease in biliary folate output and might explain the lowering of serum folate with alcohol. The mechanism of this effect of alcohol is still uncertain.

Dr. Wagner discussed the normal uptake of folate by isolated rat hepatocytes and presented some preliminary data on the effect of alcohol on this process. Folate, in the form of 5-methyltetrahydrofolate—the major form of the vitamin in human plasma—is taken up by the liver by a saturable, concentrative process, but this is competitively inhibited by various folate analogs and by substances that interfere with energy production. At least 80 percent of the 5-methyltetrahydrofolate in liver is unchanged and unbound after 90 minutes of incubation. Increased uptake of folate with the administration of sodium azide could be explained by inhibition of efflux, resulting in greater hepatic net flux. Alcohol (40 mM added in vitro) stimulated folate uptake by the hepatocytes. This stimulation was noted only after 20 minutes of incubation and was greatest after 40 minutes. A metabolite of alcohol, possibly acetaldehyde, could be responsible for this effect. The increase in folate influx into liver cells with alcohol exposure could be due to inhibition of its efflux, but further studies are needed to establish this as fact.

Dr. Hoyumpa discussed the current status of intestinal transport of thiamine, a vitamin often deficient in alcoholics. He first characterized normal thiamine transport, which seems to be bimodal. At low concentrations ($<1.0\ \mu M$), which are probably in the physiological range of normal thiamine intake, transport appears to be saturable, inhibited by metabolic antagonists, sodium-dependent, and mediated by a carrier. At higher concentrations, thiamine transport is passive. Alcohol, given orally as a single 50 to 750-mg/100 g body weight dose or added in vitro at a concentration of 2.5 percent to gut sacs, inhibited the active (but not the passive) transport of thiamine. This effect appeared to be localized to the exit step of thiamine from the tissue across the gut serosa and correlated with a decrease in Na-K-dependent ATPase in the basolateral membrane of the intestine. Chronic oral alcohol administration over 6 to 8 weeks with blood alcohol levels under 100 Mg% had no effect on intestinal thiamine transport, the Na-K ATPase activity, or tissue levels of thiamine pyrophosphate. Addition of an acute dose of alcohol reproduced the findings described earlier and raised blood alcohol levels to about 185 mg%. Dr. Hoyumpa concluded by emphasizing the need for further

definition of intracellular events of intestinal thiamine transport such as phosphorylation and protein binding.

Dr. Lumeng discussed the problem of vitamin B_6 deficiency in chronic alcohol abuse and reviewed the present knowledge of the nature of this abnormality. In alcoholics, abnormally lowered serum folate is most prevalent, followed by low levels of vitamin B_6 and thiamine. The incidence of low plasma pyridoxal phosphate is greater than 80 percent in patients with alcoholic cirrhosis and about 50 percent in those with normal liver histology. Recent studies indicate that pyridoxal phosphate is the major B_6 vitamer in plasma and that the measurement of plasma pyridoxal phosphate is a reliable and sensitive indicator of vitamin B_6 undernutrition and vitamin B_6 storage. In the normal metabolism of vitamin B_6, the content of pyridoxal phosphate in tissues (e.g., erythrocytes and liver) is governed conjointly by protein binding and by hydrolysis of this coenzyme when it is synthesized in excess of the binding capacity.

Experimentally, it has been shown that ethanol oxidation lowers hepatic pyridoxal phosphate. The mechanism is mediated by acetaldehyde, which acts by displacing pyridoxal phosphate from protein binding, thereby increasing the availability of free pyridoxal phosphate for hydrolysis. The net effect of ethanol oxidation and acetaldehyde action is the promotion of pyridoxal phosphate degradation. It is important to remember that, in both acute and chronic liver diseases, the degradation of plasma pyridoxal phosphate is accelerated.

Dr. Rudman outlined normal carnitine formation, derived either from ingested carnitine or synthesized in the liver from lysine and methionine. He presented evidence from a nutritional survey as well as from metabolic balance studies that cirrhotics with severe liver disease may exhibit major carnitine deficiency; these data correlate with various indexes of poor nutrition as well as with tests of liver dysfunction such as serum bilirubin and prothrombin time.

Carnitine deficiency may be present in as many as 30 percent of hospitalized cirrhotics. Part of this deficiency is due to poor intake of dietary carnitine, as well as of its precursors lysine and methionine. However, even when adequate amounts of these two amino acids are provided, the diseased liver is unable to synthesize carnitine at a normal rate. Thus carnitine deficiency in cirrhotics may be due to anorexia with poor dietary intake, to a low protein diet deficient in carnitine and its precursor amino acids, or to impaired hepatic synthesis of carnitine. Dr. Rudman speculated that carnitine deficiency may have clinical relevance with regard to the

neurological and myopathic syndromes observed in some cirrhotics, although this has not yet been firmly established.

Section IV: Effects of Alcohol on Protein and Amino Acid Metabolism

Dr. Lieber and Dr. Hsu discussed the current status of plasma α-amino-n-butyric acid (AANB) and leucine levels as indexes of chronic alcoholism. In rats, baboons, and humans, chronic heavy alcohol consumption results in elevation of the AANB level in plasma and liver. Protein restriction leads to a decrease in plasma AANB level, so it is necessary to use other plasma amino acids as a reference base. In this regard, plasma leucine level is used because it reflects dietary protein intake. Although the plasma AANB/leucine ratio was used earlier, this relationship is not linear over the entire range of leucine values. Thus, the use of this ratio is now replaced by experimentally derived curves. Using these curves, one can detect approximately 80 percent of active alcoholics sampled within 7 days of drinking, with only a 2-percent false-positive result among controls. This test is more sensitive than measuring blood alcohol level. Moreover, blood alcohol level does not distinguish acute from chronic alcohol consumption. AANB is as sensitive as, but much more specific than, plasma γ-glutamyl transpeptidase activity.

Dr. Rothschild discussed the effect of ethanol and acetaldehyde on hepatic albumin synthesis in both the fed and fasted states. Acute ethanol administration results in disaggregation of the endoplasmic membrane-bound polysome, decreased urea formation, decreased albumin synthesis, and reduced synthesis of other proteins. In liver of fed animals, these effects of ethanol can be reversed by administration of a number of amino acids, as well as by administration of polyamines.

In liver from fasted animals, the combined stresses of starvation and ethanol cause more severe changes. Not only do the endoplasmic membrane-bound polysomes become disaggregated, but the free polysomes are also disaggregated. It is possible that the aggregated free polysomes are responsible for the synthesis of the pre- or propeptide, and the latter initiates attachment of the ribosome to the endoplasmic membrane to form the albumin destined for export. In liver from fasted animals, the addition of amino acids and polyamines is less likely to reverse the deleterious effects of ethanol.

Dr. Rothschild has also studied the effect of acetaldehyde on these steps in albumin synthesis, but acetaldehyde does not

reproduce the changes mediated by ethanol. Therefore, the deleterious effects of ethanol must be explained by metabolic sequences of alcohol oxidation other than the generation of acetaldehyde.

Section V: Alcohol Metabolism: Including the Effects of Chronic Alcohol Ingestion and Nutritional States

Dr. Cornell presented a paper on the rate-determining factors for ethanol oxidation in rats in vivo and in isolated rat hepatocytes. He pointed out that the rate of ethanol metabolism in rats in vivo is about 3 μmol/min/g liver. This rate is observed with isolated hepatocytes from fed and 48-hour starved rats, provided that lactate or pyruvate is also added as a substrate. In the absence of lactate or pyruvate, hepatocytes from starved rats oxidize only 0.75 μmol of ethanol/min/g and cells from fed rats exhibit a rate of 1.9 μmol/min/g. The effect of lactate and pyruvate is not due to increased ATP utilization for glucose synthesis; instead, lactate and pyruvate replenish intermediates required for the malate-aspartate hydrogen shuttle in the isolated cells. Dr. Cornell emphasized that, although the malate-aspartate shuttle may be rate limiting for ethanol oxidation in isolated hepatocytes under some conditions, this effect is probably not the case in vivo. Alcohol dehydrogenase is present in rat liver at 1.5 times the activity required to account for the rate of ethanol oxidation in vivo, suggesting that the level of alcohol dehydrogenase can be a major rate-determining factor. Dr. Cornell has determined the kinetic properties of the rat liver alcohol dehydrogenase and has calculated the rate of ethanol elimination based on the steady-state rate equation for an ordered bi-bi reaction mechanism. Indeed, the calculated rates are similar to those observed in vivo. Dr. Cornell therefore concluded that, in vivo, the rate of ethanol elimination in the rat is determined in a significant way by the amount of alcohol dehydrogenase in liver.

Dr. Bosron also presented data indicating that the level of alcohol dehydrogenase in liver is not in large excess. Additionally, hepatic alcohol dehydrogenase activity decreases rapidly with fasting; the amount of decrease is as much as 50 percent. The relationship of hepatic alcohol dehydrogenase activity and alcohol elimination in vivo during fasting is being investigated.

Dr. Kulkosky presented data indicating that the maximal ethanol intake in rats is determined by the hepatic alcohol dehydrogenase activity. Ethanol intake can be maximized by adding saccharin and NaCl to the alcohol solution; the maximal intake in

rats is about 7 to 8 g/kg/d. This is the same as the ethanol metabolic capacity of 8 g/kg/d calculated from Dr. Cornell's data.

Drs. Bosron and Li discussed the multiple molecular forms of human alcohol dehydrogenase (ADH). Of particular importance is the identification and isolation of a new molecular form of human alcohol dehydrogenase, Π-ADH. The human liver contains as many as 6 to 10 ADH molecular forms. The number and the amount of the individual forms in liver tissue vary among individuals and races, depending on genetic factors as well as on the health of the individuals and the manner in which tissue specimens are handled (i.e., biopsy, autopsy tissue from patients who died of sudden traumatic deaths, or autopsy tissue from patients who died of various illnesses). Biopsy and traumatic death-related autopsy samples exhibit high specific activity; they also contain the newly discovered molecular form of alcohol dehydrogenase, Π-ADH.

Π-ADH is the most anodic enzyme form among the ADH isozymes on starch gel electrophoresis. At concentrations of ethanol that produce moderate to severe intoxication, i.e., 30 to 100 mM, Π-ADH represents as much as 40 percent of the total alcohol-oxidizing capacity in human liver. Π-ADH has been purified by means of affinity chromatography, and this molecular form exhibits a number of unique properties: Π-ADH is unstable, is relatively insensitive to inhibition by 4-methylpyrazole, and exhibits a high K_M for ethanol. On the other hand, Π-ADH is similar to the other ADH molecular forms with respect to molecular weight, subunit composition, and zinc content.

The discovery of Π-ADH bears importantly on our understanding of normal human alcohol metabolism and its pathological derangements. The occurrence of this high K_M form of ADH indicates that the rate of alcohol oxidation in vivo should increase when blood ethanol concentrations rise to intoxicating levels in some individuals. Although the failure of pyrazole compounds to completely inhibit ethanol oxidation is frequently argued as functional evidence for non-ADH mediated pathways of ethanol metabolism, the presence of the pyrazole-insensitive Π-ADH in human liver indicates that the existence or lack of such alternate pathways in humans cannot be inferred conclusively from the effects of these compounds. Moreover, because both the molecular heterogeneity of liver ADH and alcoholism appear to be under genetic control, the question arises whether the presence or absence of Π-ADH or of any of the other molecular forms may prove to be the biochemical links to alcoholism. Finally, whether chronic alcohol abuse or malnutrition alters the relative distribution

and amount of П-ADH and other enzyme forms remains another pertinent question.

It should be emphasized that in alcohol metabolism, the rate of each component step may affect the overall rate, and there may be several slow steps. For example, in the steady-state reaction

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C,$$

for which each rate constant is 1.0, the overall rate constant (k_{obs}) for A → C is 0.5 [$k_{obs} = k_1 k_2/(k_1 + k_2)$]. If k_1 were increased to 10, the overall rate constant would increase to 0.91. Note that an increase in the overall rate constant of more than twofold requires that the rates of both steps be increased. Thus, the increase in the rate of one step and in the overall rate would not be linearly correlated, and it would be erroneous to conclude that neither the first nor second step had a rate-limiting role. In the overall metabolism of ethanol, it appears that the amount of hepatic alcohol dehydrogenase and the rate of turnover of NAD^+-NADH may both have rate-limiting effects (see also Dr. Cornell's discussion).

Dr. Salaspuro, in this workshop, presented a paper on accelerated ethanol metabolism after chronic alcohol consumption, with special reference to nonlinearity of blood ethanol elimination and associated redox changes. He also reviewed the present status of the microsomal ethanol-oxidizing system (MEOS). This system has been reconstituted recently with three microsomal components, cytochrome P-450, NADPH cytochrome c reductase, and lecithin. The K_M of this reconstituted system for ethanol is 10 mM. It requires NADPH; it is not active with an H_2O_2-generating system; and it is insensitive to catalase inhibitors. Thus, the existence of MEOS in the rat is well documented.

In baboons that are pair-fed isocaloric diets with and without ethanol, the rates of ethanol elimination are not linear. Even in the alcohol-naive animals, the alcohol elimination rates are biphasic—the rate at high ethanol concentrations (45 to 20 mM) is 10 percent higher than that at low ethanol concentrations (15 to 5 mM). In the alcohol-fed animals, after 2 months of alcohol feeding, the rate at high ethanol concentrations is 14 percent higher than that at low concentrations; after 24 months of alcohol feeding, the corresponding difference is 30 percent.

Similar data have been obtained with human subjects, i.e., after 4 weeks of alcohol ingestion, the alcohol elimination curve is nonlinear, and the increase in ethanol elimination rate occurs especially at high ethanol concentrations. In humans, either MEOS or

Π-ADH may be responsible for the nonlinear rates of ethanol elimination; however, Π-ADH has not been detected in baboons.

Dr. Salaspuro also reported the effect of chronic ethanol consumption in baboons on the change of $NAD^+/NADH$ ratio is evidenced by inhibition of the rate of galactose elimination. Acutely, ethanol administration inhibits galactose elimination by approximately 46 percent. However, in baboons that have consumed alcohol chronically for 24 months, the acute administration of ethanol results in less reduction in the $NAD^+/NADH$ ratio and in less inhibition of galactose elimination (only 21 percent). Based on these data, Dr. Salaspuro interpreted the results to indicate the following. (1) The rate-limiting factor of alcohol oxidation may be different between the naive animals and the animals fed ethanol chronically. The rate of NADH reoxidation is rate limiting in the naive animal; however, in the animals fed ethanol chronically, the level of hepatic alcohol dehydrogenase may become the major rate-determining factor in the alcohol dehydrogenase pathway for alcohol elimination. The switch to a more oxidized $NAD^+/NADH$ in chronic alcohol consumption may be due to a decrease in the amount of alcohol dehydrogenase or to an increase in the capacity to remove cytosolic reducing equivalents. (2) Depending on the limiting step in the alcohol dehydrogenase pathway, there may or may not be accumulation of reducing equivalents in hepatic cytosol after the acute administration of alcohol. (3) Consequently, the acute effects of ethanol on hepatic intermediary metabolism may be completely different in alcoholics and in animals fed alcohol chronically compared to those effects in controls. (4) Whether any of the acute metabolic effects of alcohol still occur in the chronic situation is unknown.

Section VI: Other Effects of Alcohol on Nutrition

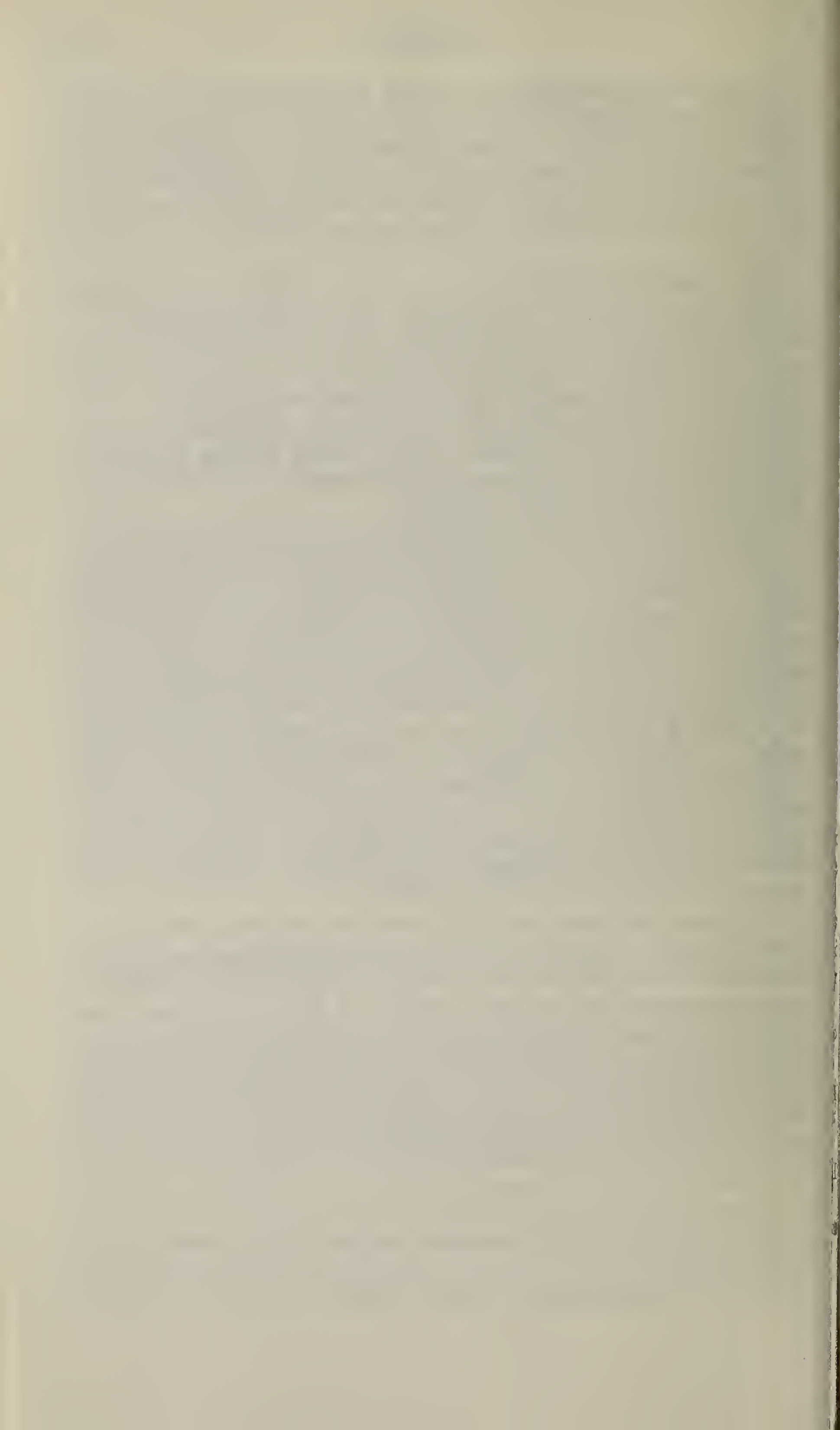
Dr. Hurley reviewed the history and the clinical characteristics of the fetal alcohol syndrome (FAS) in humans. Although alcohol per se may be the cause of FAS, Dr. Hurley suggested that nutritional disturbances, e.g., magnesium, zinc, or folate deficiencies, also could be teratogenic. Magnesium deficiency is known to be teratogenic for rats, resulting in increased fetal resorption rate, congenital anomalies, and a high neonatal mortality. Folate deficiency may have similar effects, presumably by inhibiting nucleic acid synthesis and by causing abnormal enzymic differentiation. Teratogenic effects have been observed both in rats and human beings. Finally, zinc depletion clearly induces congenital anomalies

in experimental animals, even when the deficiency is relatively modest and of brief duration. The mechanism of this effect appears to be impaired synthesis of nucleic acids. Dr. Hurley concluded that careful assessment of nutrient status, particularly that of magnesium, folate, and zinc, should be carried out in studies of the FAS syndrome both in humans and in appropriate animal models.

Dr. Lester summarized the role of hypoandrogenization and hyperestrogenization in mediating the feminizing effects of chronic alcohol abuse. The existence of hypoandrogenization in chronic alcoholism has been established. It is due to a direct effect of ethanol on the testis, to coexistent hypothalamic-pituitary suppression by alcohol, and to changes in hormonal metabolism produced by alcoholic liver disease. The net effect of these alterations is a decreased amount of testosterone available to the target tissues, leading to decreased spermatogenesis.

The genesis of hyperestrogenization in male alcoholics, on the other hand, remains more difficult to understand. The plasma level of estradiol in male alcoholics is normal. Normal estradiol levels in the presence of diminished testosterone levels may produce the hyperestrogenization effect, but this suggestion is not universally accepted. In this workshop, Dr. Lester presented a novel hypothesis for hyperestrogenization, based on dietary intake of a number of nonsteroidal estrogenic substances ubiquitous in plants. Although the amount of nonsteroidal estrogens in the normal human diet is insufficient to produce estrogenic effects in man, it is suggested that, in the presence of abnormal diet or altered metabolism of nonsteroidal estrogens in advanced liver disease, sufficient quantities of these dietary substances may accumulate to produce estrogenic effects.

To prove this hypothesis, Dr. Lester and his group have developed a receptor assay based on the differential displacement of radiolabeled estradiol by nonsteroidal estrogens from estradiol-binding proteins purified from male and female rat liver. In a survey of 15 sera from hard-core male alcoholics with liver disease, 3 sera contained significant amounts of nonsteroidal estrogens. These preliminary data therefore support the provocative hypothesis that nonsteroidal estrogens from an exogenous source may play a role in producing hyperestrogenization in alcoholics.



Recommendations for Research Needs

1. More sensitive tests must be developed to diagnose marginal and conditional deficiency states. In this regard, more stringent criteria must be employed in sample collection to define the normal values of these tests.

2. Further studies are needed to define the mechanism responsible for the energy wastage due to chronic alcohol consumption.

3. Further basic research must be performed to understand the transport mechanisms responsible for absorption of nutrients in the gastrointestinal tract. In studies dealing with the effects of ethanol on the kinetics of transport processes, particular attention should be given to the unstirred water layer.

4. More biochemical studies are needed to delineate the effect of ethanol on the cellular mechanisms responsible for the digestive, secretory, and absorptive functions of the gastrointestinal tract.

5. Research is needed to define the distribution and interorgan transport of various minerals and trace elements and the effect of ethanol.

6. Metabolic balance studies in humans should be conducted to define the effect of acute and chronic ethanol consumption on Zn^{2+}, Mg^{2+}, Ca^{2+}, Na^{+}, K^{+}, and P. In this regard, the role of congeners in alcoholic beverages should be further examined.

7. Studies are needed to explain the effect of ethanol on metabolism of trace elements, e.g., copper, cobalt, molybdenum, selenium, and manganese.

8. Further research must be performed to explain the effect of altered Zn^{2+} and Mg^{2+} metabolism in alcohol consumption on intermediary metabolism. The role of Zn^{2+} in DNA and RNA metabolism needs further delineation.

9. Further research should be conducted to define the effect of phosphorus deficiency on skeletal muscle metabolism.

10. Controlled clinical trials should be performed to define the potential benefits of Zn^{2+}, Mg^{2+}, P, and carnitine replacement in the management of the medical complications in alcoholic patients.

11. Further research should be done to delineate the role of the liver in folate and vitamin B_6 metabolism and the effect of ethanol.

12. Studies are needed to determine the effect of ethanol on thiamine metabolism in the intestine, liver, and brain.

13. Clinical evaluations should be conducted to compare plasma α-amino-n-butyric acid/leucine levels with other diagnostic indicators for chronic alcoholic intake, e.g., urinary glucaric acid.

14. Further research is needed to define the effect of ethanol and its metabolites on the ribosomal membrane complex for protein synthesis and on the golgi-microtubule system for protein secretion.

15. The multiple molecular forms of hepatic alcohol dehydrogenase should be isolated, purified, and characterized to understand the extent each form participates in ethanol elimination.

16. A subhuman primate model exhibiting the multiple molecular forms of hepatic alcohol dehydrogenase similar to that seen in humans and, in particular, showing Π-ADH, must be sought to facilitate further studies on the rate-determining factors for alcohol elimination.

17. The extent that non-ADH pathways participate in alcohol elimination in humans must be evaluated further.

18. Additional studies are needed to delineate differences in the acute and chronic effects of ethanol on hepatic metabolism.

19. Further research must be carried out to define the pathogenesis of the fetal alcohol syndrome. Specifically, the mechanism for transplacental transport of minerals and vitamins should be studied.

20. The hypothesis that dietary nonsteroidal estrogen compounds may be responsible for feminization in alcoholic males should be pursued.

Section I: Effects of Alcohol on Nutrition and Gastrointestinal Functions

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Marginal Nutritional States and Conditioned Deficiencies

J. Cecil Smith, Jr.*

Abstract

Optimal nutrition and overt nutritional deficiencies are poles apart. Between the extremes are the marginal or suboptimal nutritional states that may result from inadequate intake and conditioned deficiencies. The latter result from factors that interfere with the normal metabolism of nutrients, although the intake may be within the recommended range.

Factors that contribute to marginal and conditioned nutritional inadequacies are inadequate intake; loss of body fluids; malabsorption; dietary chelators; and ingestion of drugs, including alcohol. For many nutrients, the basic requirements for humans are yet unknown. Optimal levels of nutrients must be established. Traditional methods for assessing nutritional states are often insensitive to marginal or conditioned deficiencies.

Of equal importance is the definition of "normal" values as the basis for detecting a suboptimal or deficiency state. Likewise, more precise guidelines for interpreting laboratory tests must be established. Dietary surveys and clinical findings show that, in general, gross deficiencies with overt clinical signs and symptoms have nearly been eliminated in the United States. However, results of such surveys indicate that problems concerning suboptimal intakes of certain nutrients linger.

Trends in U.S. food consumption patterns indicate that intake of certain foods has changed markedly since 1900. Examples are the increase in consumption of sugars and the decrease in consumption of cereal products. Consumption of processed and refined foods also has increased sharply. These changes should be assessed with respect to their nutritional impact.

Chronic alcohol ingestion can result in both marginal and conditioned nutritional inadequacies. Indeed, alcoholism has been suggested as the most common cause of undernutrition and the chief cause of vitamin deficiency in adults in the United

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States. In addition, alcohol ingestion has been reported to result in inefficient utilization of energy. The mechanism by which alcohol results in energy wastage is unknown. A critical threshold level for daily alcohol ingestion has been suggested in regard to the development of liver disease.

Introduction

Present knowledge of nutritional status in humans encompasses a broad spectrum, ranging from frank, gross deficiency diseases with lesions and accompanied by clinical signs and symptoms, to an optimal state of sustained, excellent health. Between the poles—gross deficiency diseases and optimal status—are the areas commonly referred to as "marginal" (suboptimal nutritional states) and "conditioned" deficiencies. Unlike marginal deficiencies, which result from inadequate intake, conditioned deficiencies result from factors that interfere with the normal metabolism of nutrients.

Recent nutritional surveys within the United States have revealed few gross deficiencies with overt clinical signs and symptoms (51,52). Although the incidence of frank deficiency diseases has decreased in the United States, reports of marginal and conditioned deficiencies have increased. Selected factors that contribute to marginal and conditioned nutritional deficiencies are listed in table 1.

Human Nutrient Requirements: Basic and Optimal

Recommended dietary allowances (RDA's) have been established by the Food and Nutrition Board of the National Academy of Sciences for a variety of specific nutrients (33). The RDA's are the levels of intake of essential nutrients deemed adequate to meet the known nutritional needs of practically all healthy persons. However, Mertz (28) has differentiated between the basic and optimal requirements for specific nutrients. He defined the basic requirement as "that daily intake which allows the actual absorption into the organism of an amount sufficient to prevent deficiency disease." In contrast, the optimal requirement was defined as "that daily intake which allows an absorption of an amount sufficient to maintain in near-optimal function all biochemical and physiological mechanisms in which the element is involved, under the various stress conditions of life." Mertz recognized that practical difficulties often inhibit meeting the

Table 1. Factors Contributing to Marginal and Conditioned Nutritional Deficiencies

Anorexia (starvation)
Hemolytic anemias
Drug treatment
penicillamine
ethambutol
diuretics
oral contraceptives
antibiotics
Total parenteral nutrition
Losses in body fluids
sweat
urine
exudates (burns)
blood (parasites)
Malabsorption
steatorrhea
regional enteritis
jejunoileal bypass surgery
sprue
Pregnancy
Dietary chelators (native and additives)
phytate, fiber, pica (clay, starch), ethylenediamine tetraacetate (EDTA)
Changes in food supply and dietary consumption patterns
Alcohol consumption

optimal requirement (although it would be desirable), because optimal intake would assure prevention of marginal deficiencies. Furthermore, as indicated in table 2, requirements have yet to be established for many of the basic nutrients.

Evaluation Techniques for Assessing Nutritional Status

The detection of nutritional deficiencies presupposes establishment of accepted norms, considered to represent satisfactory, if not optimum, health and well-being. The major assessment methods traditionally have included nutritional history, physical examination, and laboratory procedures (13).

The nutritional history consists of records of dietary intake (including alcohol) and comparison with appropriate standards.

Table 2. Present State of Knowledge Regarding Nutritional Requirements

		INFANTS			CHILDREN			ADULTS			
		PRE-MATURE	0-6 MONTHS	6-23 MONTHS	PRE SCHOOL	SCHOOL AGE	ADOL-ESCENT	YOUNG	AGED	PREGNANT	LACT-ATING
TOTAL ENERGY		F	F	F	F	S	S	S	F	F	F
CARBOHYORATES	STARCH										
	SUGARS	F	F	F	F	F	F	S			
	FIBER						F	F			
TOTAL FAT			S								
ESSENTIAL FATTY ACIOS			F					F	F		
PROTEIN		S	F	F	F	F	F	S	F	S	F
AMINO ACIDS	ARGININE		S			F		S			
	HISTIOINE	F	S			F		S			
	LEUCINE, ISOLEUCINE	F	S			F		S			
	LYSINE		S			F		S	F		
	METHIONINE		S			F		S	F		
	PHENYLALANINE		S			F		S			
	THREONINE		S			F		S	F		
	TRYPTOPHAN		S			F		S	F		
	VALINE	F	S			F		S			
MINERALS	CALCIUM	S	S	S	F	F	F	S	F	F	F
	MAGNESIUM				F	F	F	F			
	IRON	S	S	S	S	S	F	S	S	S	S
	PHOSPHORUS				F	F				F	F
	SULFUR				F	F				F	F
	SODIUM				F	F		F	F		
	POTASSIUM	F	F	F	F	F		F	F		
	COPPER	F	F		F	F		F			
	MOLYBOENUM										
	MANGANESE										
	ZINC		F	F	F	F	F	S			
	CHROMIUM	F	F	F	F	F	F	F		F	F
	SELENIUM				F						
	NICKEL							F			
	VANAOIUM										
	CHLORINE										
	FLUORINE				F	S	F		F		
	IOOINE				F	F	F	F			
VITAMINS	VITAMIN A	S	S		F	F		S			
	VITAMIN O	S	S	F	F	F	F				
	VITAMIN E	F						F			
	VITAMIN K	F	F							F	
	THIAMIN	F	F		F	F	F	S	F	F	F
	RIBOFLAVIN	F	F		F	F	F	S	F	F	F
	NIACIN							F	F	F	F
	PYRIOOXINE		F					F		F	
	PANTOTHENATE							F			
	COBALAMIN		F					F			
	FOLIC ACIO	F	F					F	F	F	
	BIOTIN										
	CHOLINE										
	ASCORBIC ACIO	S	S		F	F	F	S	F	F	F

AS OF 1977 [ ] LITTLE OR NO OATA [F] FRAGMENTARY OATA [S] SUBSTANTIAL OATA

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Also included are determination of the quality and quantity of food components; estimation of energy requirements as influenced by lifestyle; notation of symptoms and complaints; and a family history of eating, living, and health patterns.

The physical examination includes anthropometric measurements—height, girth, skinfold, and derived indexes—and comparison with suitable standards. In addition, signs suggestive of

inadequate nutrition (e.g., dermatitis and poor healing) are noted, and functional tests are performed.

The laboratory tests rely heavily on accepted normal ranges. The specimens most often examined are plasma or serum, urine, and tissue (when available). Biochemical parameters often include postabsorptive or load tests such as glucose tolerance. A meal, containing oysters, providing five times the recommended dietary allowance for zinc, has recently been used as a zinc load to measure absorptive response and homeostatic mechanisms of that element (42).

Direct analyses for vitamins and minerals are often supplemented with data from appropriate assays of enzyme activity to assess biochemical alterations. Glutathione peroxidase (a selenometalloenzyme) is used to assess selenium status (9), and red blood cell carbonic anhydrase, a zinc-metalloenzyme, to assess zinc nutriture (17).

Are Normals Normal?

"Normal" populations are usually used as control groups for assessment of nutritional status. Populations are considered normal if they are ambulatory, can carry out routine daily activities, and are willing to donate a specimen for analysis. However, few populations escape some health problem. A recent survey (1976) by the U.S. Department of Agriculture questioned approximately 1,400 households from different regions and social levels (16). In more than 60 percent of all households, someone had health problems, either diagnosed by a physician or self-ascribed. The incidence of health problems in regard to a specific disease or condition is shown in table 3. The most common problems were obesity, hypertension, and allergies, which were reported, respectively, in 30 percent, 22 percent, and 20 percent of the households. In approximately 40 percent of all households, someone had changed a dietary pattern to meet an existing health problem.

Results of that survey were strikingly similar to those of an earlier study (1967) by the U.S. Public Health Service (53) in which those interviewed were part of the "normal" population who were not hospitalized. Respondents were asked whether they had experienced, within the past 12 months, any of 11 conditions, including asthma, stomach ulcer, hay fever. The percentages of those who experienced one or more chronic condition were 43 for ages 17 to 24; 59 for ages 25 to 44; 71 for ages 45 to 64; and 85 for age 65 and older. The complaints were not

Table 3. Percentage of U.S. Households Reporting Health Problems[a,b]

Conditions	Percentage Households Reporting Health Problem
Obesity	30
High blood pressure	22
Allergy	20
Heart disease	9
Kidney problems	8
Diabetes	7

[a]Based on approximately 1,400 households.
[b]Data from reference (16).

verified by physical examination. However, the responses indicated the incidence of suboptimal health.

Recently, an editorial in the *New England Journal of Medicine* asked, "What does a healthy control control?" (6). The writers stressed that in some studies the control fluids or tissues used to assess a specific disease are obtained from so-called "normals," usually young and healthy persons—i.e., coauthors, other hospital personnel, or medical students. The editorial pointed out that if blood or tissue parameters of a patient with a certain disease differ from corresponding parameters of healthy controls, a mistake might be committed if that comparison were used as evidence that the deviation from normal is specific for that certain disease. "If such conclusions are to be valid, control material should also be obtained from patients who suffer sicknesses different in nature but comparable in acuteness and severity." Thus, the editorial suggested the use of two control groups, one consisting of healthy subjects; the other, subjects suffering from a disease different from that of the group under study.

Recruiting such double control groups would be nearly impossible, especially in a noninstitutional setting. Perhaps a more practical approach for assessing nutritional deficiencies would be to develop optimal controls. That is, a range of normal values might be developed from a population clinically verified to be in optimal health on the basis of appropriate criteria, including sustained freedom from disease and ideal function of the biochemical, physiological, and mental processes. Longevity could also be included.

An alternate, and perhaps supplemental, approach would be to develop "negative" control values representing measurements from individuals documented to have a primary deficiency of a specific

nutrient. Thus, deficient as well as optimal values would be delineated so that marginal and conditioned deficiencies could be readily identified by comparison. Specifically, the marginal values would be those that fall between the deficient values at the low extreme and the optimal values at the high extreme.

By use of a similar scheme, an attempt has been made to develop guidelines for the interpretation of vitamin A plasma concentrations in humans (19). In brief, plasma levels of less than 20 μg retinol/100 ml were interpreted as "less than acceptable (at risk)." Specifically, values of 10 to 19 μg retinol/100 ml were termed "low" with medium risk. Plasma retinol concentrations of <10 μg/100 ml were classified as "deficient" with high risk. Similar classifications were also suggested for vitamin C, thiamin, riboflavin, vitamin B_6, folic acid, vitamin B_{12}, and vitamin E (tentative) (19). Guidelines for interpreting biochemical indexes used in evaluating protein and calorie adequacy were reported (19).

"Deficient" levels of nutrients have generally been arbitrarily established by use of a cutoff at two standard deviations below the mean of a "normal" population. The incidence of unacceptable or deficient values, therefore, can be altered by changing the normal range. That source of variability was noted by Lowenstein (26) regarding the cutoff point for hemoglobin normality.

Dietary Surveys and Clinical Findings as Techniques for Assessing Nutritional Adequacies

Dietary surveys may identify inadequate intakes of specific nutrients. Each decade, the U.S. Department of Agriculture (USDA) conducts the Household Food Consumption Survey of the food and nutrient intake of individuals in the United States and compares nutrient intakes with recommended dietary allowances. The 1965-66 survey (49) indicated that calcium and iron were the minerals for which intake was most often below the allowance. Other nutrients ingested in inadequate quantities included vitamin A, thiamin, riboflavin, and ascorbic acid. Inadequate intakes of certain nutrients were especially common in households with incomes under $3,000 (1965-66 poverty level). Results of a 10-state nutrition survey (52) indicated that problems were associated with the following nutrients:

> *Iron.* Iron deficiency anemia, as evidenced by low levels of hemoglobin, was widespread within the population surveyed. Low hemoglobin levels in the total population appeared to be due largely to inadequate iron intake.

Protein. Despite apparently adequate protein intakes, marginal protein nutriture was found among pregnant and lactating women, as evidenced by low serum albumin levels. Present standards for protein requirements in pregnancy may not be adequate.

Vitamin A. Low-income Spanish Americans and a large proportion of young people had low serum levels of vitamin A.

Vitamin C. Low levels of vitamin C were more frequent among males than females. The prevalence of poor vitamin C status increased with age.

Riboflavin. Riboflavin level was low among blacks and young people in all ethnic groups.

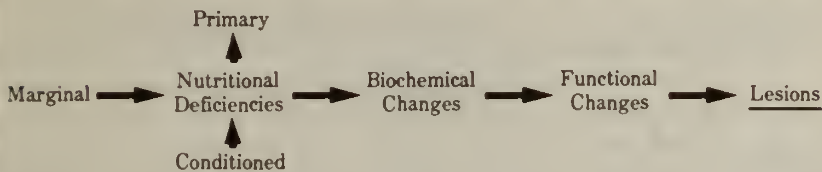
Data from the First Health and Nutrition Examination Survey (HANES I), conducted in 1971 and 1972, indicate that many people in the United States have suboptimal intakes of certain nutrients (51). On the basis of mean dietary intake, iron was the nutrient most frequently found to be below the RDA standard in certain population groups. Specifically, 95 percent of children aged 1 to 5 years and females aged 18 to 44 had iron intakes below the RDA. In low-income groups, 56 percent of the adults, white and Negro, aged 60 and older, consumed substandard levels of iron. Only for males aged 18 to 44 were mean iron intakes above the recommended allowance. Negro females aged 18 to 44 had calcium intakes 20 to 23 percent below the standard. White females aged 18 to 44 in the low-income group had mean vitamin A intakes 18 percent below the standard.

Biochemical tests confirmed the intake data. More than 10 percent of all adults had low hematocrit values. Nearly 42 percent of low-income Negro adults, aged 60 and older, had low hematocrit and low hemoglobin values.

The HANES I study concluded, "There is evidence of a deficiency with respect to the nutrient iron based on both the dietary intake and biochemical data" (51). The study illustrates the use of two parameters, dietary intake and biochemical data, to assess nutritional status and to detect marginal or conditioned nutritional deficiencies. Lowenstein (26) discussed the preliminary results of the HANES I study with regard to early signs of nutritional deficiency detected by biochemical parameters. "Early" detection may prevent the development of full blown lesions (with signs and symptoms) that might be irreversible. The stages of nutritional inadequacies are depicted in figure 1.

Both marginal and conditioned states are characterized by an inadequate supply of essential nutrients at the metabolic level.

Figure 1. Classification of Nutritional Deficiencies and Their Effects



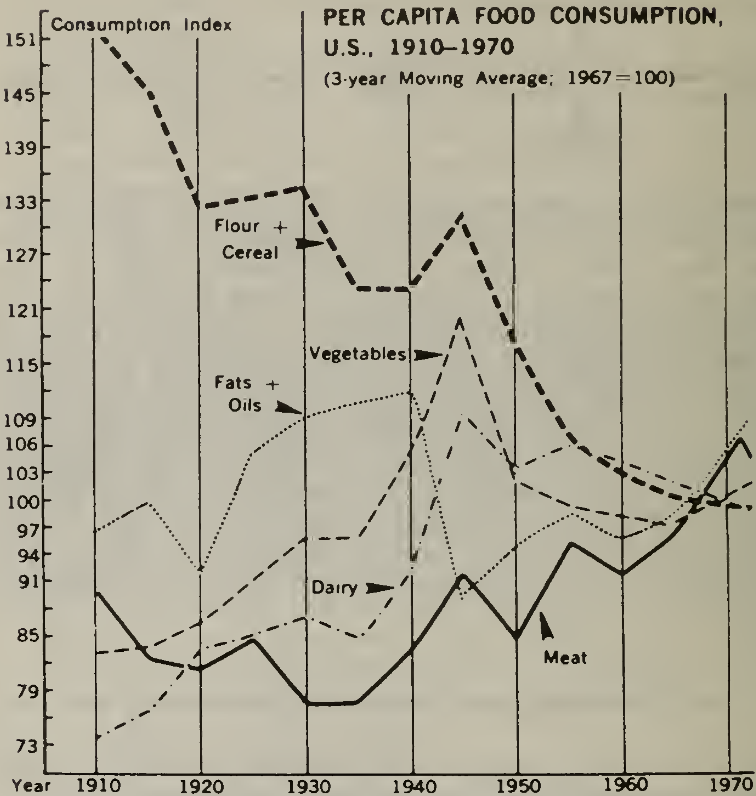
Marginal deficiencies are frequently detected by comparing the actual intake of nutrients with the recommended dietary allowance. Thus dietary history, including nutrient intake as determined by calculation or preferably by actual analysis, should be primary in assessing nutritional adequacy. Examples of a marginal or inadequate intake of a specific nutrient resulting in manifestation of a disease include the discovery that a lactose-free diet, prepared for an infant with acrodermatitis enteropathica, supplied inadequate quantities of zinc (29). Calculation of the zinc content first indicated this inadequacy. The patient was given a zinc supplement and recovered completely within a short time. Thus, a single observation regarding nutrient insufficiency resulted in the development of an accepted treatment of a potentially fatal disease.

Consumption Trends of Nutrients

For the U.S. population, food consumption patterns have changed markedly since the beginning of the 20th century, as shown in figure 2 (27). The major trends in the per capita consumption included a marked decline for flour and cereals and an increase for meats. The pattern of consumption of fats and oils has been erratic since 1910. It increased from 1920 to 1940, decreased sharply during World War II, then gradually increased since 1945. The intake of dairy products has shown a net increase since 1910, but a trend toward decreased intake began after World War II. Similarly, vegetables are consumed at a rate exceeding that near the turn of the century, but after a striking increase from 1935 to 1945, the trend has since been downward.

How do these trends affect nutrient intake? In general, the changing food consumption pattern has resulted in a decreased intake of fiber (roughage), vitamins, and essential trace elements. Major factors contributing to the decrease in the intake of specific essential nutrients include the following. (1) There was a marked

Figure 2. Major Trends of Food Consumption in the United States During This Century[a]



[a]Data from reference (27).

increase in the consumption of naked (empty) calories, such as those contributed by refined sugars. (In 1975, 90 lbs of refined sugar per capita was available for consumption, representing approximately 450 kcalories per day (48).) (2) There was increased consumption of processed and refined foods, i.e., white flour largely devoid of vitamins and minerals without enrichment, compared to the whole grain. (3) There was increased intake of naked calories from fats and oils.

Fats and oils may provide fat-soluble vitamins (A, D, E, and K), but they cannot be depended on as major contributors of these vitamins. Essential fatty acids can be provided by diets with a much lower fat content than the typical American diet, in which

40 to 45 percent of the calories are supplied by fat. Fats and oils are extremely poor sources of trace elements. Deficiencies of the trace minerals may be produced in animals, even though they have a diet containing 15 percent fat or oil. The fat or oil the animals are fed is usually identical to that consumed by humans and, in fact, is often purchased at the local supermarket.

Schroeder (38) compared the trace mineral contribution of various commercial animal diets with a typical human diet, as shown in table 4. The results indicate that on a unit weight basis the human diet provided the least amount of minerals. Thus, in general, the change in the food consumption pattern, coupled with increased refining and processing and introduction of "synthetic" foods into our food supply, has resulted in a trend of decreased intake of several essential nutrients, especially the trace elements.

It must be stressed that the problem with our present diet concerns nutritional quality, not quantity. The USDA reported that per capita food consumption reached a record high in 1976 and exceeded the previous high in 1972 by 1.5 percent (47). The short-term trend has been more food consumption; but even high intake may fail to overcome marginal inadequacies of specific essential nutrients.

The potentially marginal inadequacies of typical American diets are illustrated by a recent study from our laboratory (3). Although an institutional meat-containing diet, exceeding 3,100 calories, provided more than twice (129 g) the recommended protein intake, the zinc level (14.6 ± 4.5 mg, mean ± S.D.) was slightly lower than the recommended 15 mg (33). A vegetarian diet, which included dairy and egg products, provided essentially the

Table 4. Essential Trace Elements in Human, Rat, and Dog Diets, ppm (dry)[a]

Element	Human	Rat	Dog	Human/Dog (Percent)
Iron	30.60	197.0	200.0	15.3
Zinc	30.60	30.3	178.3	17.2
Manganese	4.62	54.4	59.95	7.7
Copper	6.15	15.1	17.05	36.0
Cobalt	0.10	0.37	0.48	2.1
Fluorine	0.59	65.0	50?	0.91
Iodine	0.12	1.17	2.25	5.3
Chromium	0.12	0.17	4.24	2.8

[a]Data from reference (38).

same levels of calories and protein as the meat diet, but even less zinc (12.2 ± 1.2 mg). In addition, the copper level of the meat diet was lower (0.9 ± 0.8 mg) than the 2 mg usually suggested as an adequate daily allowance (2).

Recently, Wolf et al. (58) have measured the daily intakes of zinc and copper from self-selected diets of individuals who were not institutionalized. Mean daily intake was 8.6 mg for zinc and 1.0 mg for copper. Calculated values indicated that iron levels were inadequate. Wolf and associates analyzed diets as actually consumed by the individuals in their normal environment, rather than as offered in an institution (3).

In *Dietary Goals for the United States*, the Senate Select Committee on Nutrition and Human Needs recently recommended changes in consumption patterns (7). In general, the recommendations appear justifiable, but apparently the committee took little account of the bioavailability of nutrients from the various classes of foods. Thus, it is possible that marginal inadequacies could result. If the population follows the recommendation to reduce the consumption of "meat" with an increase of poultry and fish, its total meat intake will probably decrease. But meat is a good source of most trace elements and vitamins, so the overall intake of these essential nutrients might be reduced.

In addition, the source of trace elements is important regarding bioavailability. For example, most trace elements are not only higher in concentration in meats than in fruits and vegetables but also are present in meats in a more readily available form. Thus, a decrease in meat consumption with a concomitant increase in fruit and vegetable consumption may cause serious inadequacies of bioavailable nutrients, e.g., trace elements.

On the other hand, decreased consumption of sugar and foods high in sugar would be nutritionally beneficial. Replacing the empty calories of sugar with complex carbohydrates would increase the nutritional value of the diet as well as decrease the caloric density.

Bioavailability Versus Total Quantity of Nutrient

Measurement of food intake by nutritional surveys does not accurately assess adequacy. The quality of the food also must be assessed with regard to its bioavailability. Just as the protein efficiency ratio indicates the quality of protein, bioavailability indicates the quality of other specific nutrients from various food sources. It is not sufficient to determine only the nutrient

composition of a food. The nutrients must be available for absorption and utilization.

The trace elements are more concentrated and, in general, more highly available in foods of animal origin than in foods of plant origin (10). For example, the zinc and iron deficiencies evident in developing countries such as Egypt and Iran, where whole grain or whole meals are the main dietary staple, have often been the result of poor availability rather than of insufficient total intake (35). Analyses of the daily diet of the zinc-deficient Iranian dwarfs indicated that intake was adequate. The major source of zinc, however, was wheat bread prepared without leavening. That bread, by virtue of its fiber and phytate content, further decreased the availability of zinc (34).

Chronic Alcohol Ingestion and Nutritional Inadequacies

Alcoholism, which costs American society a staggering $25 billion annually, ranges in degree of severity from illness to death (50). About 7 percent of the adult population are alcohol abusers. There may be as many as 10 million persons whose drinking has created some problem for themselves or associates. Alcohol is believed to account for 40 percent of U.S. traffic fatalities. Clinical risks associated with heavy drinking include heart, liver, and neurological disease as well as nutritional wastages. Excessive use of alcohol has been implicated as a factor in the development of specific cancers of the throat, mouth, and esophagus and remains the fourth most common cause of adult deaths, especially among those between the ages of 35 and 55.

Alcoholism may be the most common disease of undernutrition in adults in the United States (12). It may produce conditioned deficiencies by interfering with absorption and utilization of nutrients. A marginal deficiency may also result from inadequate food intake because alcohol often either replaces other food and/or causes nausea and vomiting. Anorexia may result and further decrease intake.

Chronic ingestion of alcohol, which is high in calories, can markedly change the composition of the diet. In a recent study of 92 alcoholic men (36), the average number of calories supplied by alcohol (1,384 cal/day) was more than one-half those supplied by food (2,505 cal). In that study, the alcoholics ingested an average of nearly 3,900 calories per day, well above the recommended allowance, but 40 percent of the alcoholics were underweight and only 10 percent were obese.

In another study of 3,000 alcoholic subjects (21), 69 percent had evidence of liver disease. Dietary deficiency occurred during periodic bouts of excessive alcoholic intake in 40 percent, extended prolonged dietary deficiency alternating with a marginal or normal diet during periods of abstinence was present in 25 percent, and continuous dietary deficiency (except when hospitalized) was present in 35 percent of the patients. The nutritional aspects of alcohol consumption have been reviewed comprehensively by Sinclair (41).

Difficulty in Detecting Nutritional Inadequacies in Alcoholics

There are few sensitive techniques for early detection of inadequacies in alcoholics. Routine laboratory studies were made of 490 alcoholics aged 15 to 69 years treated as outpatients. All had developed addictive drinking patterns after 5 to 8 years of excessive alcohol consumption (15). Routine laboratory studies showed only slightly increased serum glutamate oxaloacetate (SGOT) in about one-third of the patients. There was no relationship between SGOT and intensity or duration of alcoholism. Other laboratory tests showed no definite changes.

In another study of 3,000 alcoholic patients (21), conventional liver function tests were of limited value in detecting early phases of liver injury or determining the activity or severity of liver disease. The serum glutamic pyruvic transaminase activity often was normal. Empirical flocculation tests (where reactivity depends on abnormalities of serum proteins) were not reliable. The sulfobromophthalein (SBP) test frequently failed to detect subclinical liver disease. Only one test, the indocyanine green clearance, provided an index of functional capacity of the liver.

A possible technique for detecting and assessing the degree of alcoholism has recently been reported by Shaw et al. (40). The method is based on an apparent alteration of the ratio of the plasma amino acids, α-amino-n-butyric acid (A) and leucine (L). The A:L ratio in 42 alcoholic patients was double that in either normal controls or patients with nonalcoholic liver disease. There was a positive correlation between plasma A:L ratio and mean daily alcohol intake. This technique is more sensitive in the detection and assessment of the degree of alcoholism than blood alcohol concentration or blood enzyme alterations.

Signs and symptoms of marginal or conditioned inadequacies in alcoholism are often absent. Halsted et al. (11) found evidence that folate deficiency in combination with alcohol ingestion

induced a functional abnormality of the small intestine as evidenced by a decreased uptake of folic acid. However, no morphologic changes were observed. Apparently functional abnormality precedes detectable structural alteration in alcoholic folate deficiency.

Likewise, a deleterious effect of marginal or conditioned deficiency may not become evident until an accumulative threshold has been reached. For example, a recently reported large-scale study of alcohol consumption and blood pressure involving 83,947 men and women aged 15 to 79 years suggested that alcohol consumption was associated with an increase in blood pressure. However, this effect did not become statistically significant until the subjects ingested three or more drinks daily (18).

Methods for Detecting Conditioned Deficiencies in Alcoholics

In a recent study involving 119 hospitalized alcoholics with liver disease (5), plasma zinc levels were low in 82 percent. Further evidence of a conditioned zinc deficiency in alcoholic liver disease was exhibited by the decreased tissue zinc concentration in the liver. This was in contrast to normal concentrations of copper, magnesium, calcium, and manganese, as shown in table 5 (54). In addition, hyperzincuria in alcoholic liver disease patients has been reported in numerous studies (43,44,45,55).

Another study (5) indicated that nearly 60 percent of the alcoholics had low plasma vitamin A levels. The work of Patek and Haig (31) nearly 40 years ago indicated that patients with cirrhosis of the liver (the majority had a history of alcoholism) had an abnormal visual dark adaptation ("night blindness"). The vision

Table 5. Zinc and Other Element Concentrations in Livers of Autopsied Patients[a]

Element	Noncirrhotic[b] μg/g (dry)	Cirrhotic[b] μg/g (dry)	Significance P
Zinc	288 ± 100	99 ± 37	$<.001$
Copper	22 ± 5.1	24 ± 11	NS
Magnesium	531 ± 159	362 ± 162	NS
Calcium	126 ± 30	118 ± 50	NS
Manganese	5.2 ± 1.4	5.0 ± 1.1	NS

[a]Data from reference (54).
[b]Mean ± S.D.

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problem "tended to persist in the presence of nutritious diet, rich in vitamin A." Thus, they added, ". . . patients with cirrhosis of the liver may be deficient in vitamin A. It is also evident that the deficient state is not attributable to inadequate intake of the vitamin in their food." That condition, therefore, is an excellent example of a conditioned deficiency caused by alcohol ingestion that became evident in spite of adequate intake.

Abnormalities in zinc and vitamin A metabolism in alcoholic liver disease patients have recently been reviewed (4). Alterations in the plasma, urinary excretion, and absorption of several other nutrients as a result of chronic alcohol ingestion are summarized in table 6.

Alcohol, Diet, and Disease

Although accumulating experimental data show that heavy alcohol consumption without dietary inadequacy can result in

Table 6. Alterations in Metabolism of Nutrients as a Result of Chronic Alcohol Ingestion

Plasma or Serum	
	↓ Zinc
	↓ Calcium (transient)
	↓ Vitamin A and retinol binding protein (RBP)
	↓ Glucose
	↑ Cholesterol, phospholipids, and triglycerides
	↓ Thiamine
Absorption	
	↓ Thiamine
	↓ B_{12}
	↓ Folic acid
	↓ Glucose
	↑ Iron
Urinary Excretion	
	↑ Mg
	↑ K
	↑ Zn

liver damage leading to cirrhosis (24,25), there are still questions remaining regarding the role of diet in the development of cirrhosis in alcoholics. A recent study (30) concerned the nutritional practices of 304 hospitalized alcoholics: 195 were classified as cirrhotic, 40 as precirrhotic, and 69 as noncirrhotic. The subjects were all diagnosed as "chronic alcoholics" based on their histories of alcohol intake and other signs. The precirrhotics and noncirrhotics were differentiated on the bases of histories, clinical findings, and histological study of liver biopsies. Subjects with frank cirrhosis were well documented by the usual clinical parameters.

Dietary histories were usually obtained before diagnoses were established. An effort was made, based on patient recall, to ascertain the typical diet for at least 2 years prior to onset of the presenting illness to avoid changes of diet that may have resulted from the illness. There was no significant difference among the groups regarding the mean total daily caloric intake—3,544, 3,231, and 3,394—or the calories from alcohol—1,821, 1,875, and 1,864—for the noncirrhotics, precirrhotics, and cirrhotics, respectively. However, the daily mean caloric intake from protein sources was higher for the noncirrhotics (288) when compared to the precirrhotics (196) and cirrhotics (201). The authors concluded that a severe dietary deficiency may not be necessary to provoke cirrhosis in an alcoholic. They suggested, "It is conceivable that dietary deficiency at a near threshold level through a period of years could make a critical difference in the pathogenesis of human cirrhosis." The authors also observed that a high alcohol intake may interfere with absorption and utilization of essential nutrients in the diet.

Recent studies by Ristic et al. (36) indicated that a large percentage (40 percent) of the chronic alcoholics were underweight even though they had a high daily caloric intake averaging nearly 3,900 calories—well above the level necessary to maintain body weight in normal nondrinkers. Apparently energy utilization was inefficient because 36 percent of the calories were derived from alcohol. Earlier, the studies of Pirola and Lieber (32) concerning the energy cost of the metabolism of ethanol indicated that 2,000 calories from alcohol (added to a basal 2,000 caloric diet) resulted in essentially no body weight gain over a 44-day period, whereas 2,000 calories from chocolate caused, in the same subject, a gain of almost 3 kg in less than 30 days.

The mechanism by which alcohol results in energy wastage is unknown. Thus, there is no doubt that conditioning factors, including alcohol ingestion, can cause a wastage of specific nutrients, including energy.

Alcoholic liver damage is produced only by constant alcohol intake. The duration and quantity of alcohol intake are of decisive importance for the development of cirrhosis. Alcoholic fatty liver may be the only noticeable alteration for many years and is reversible within a few weeks of discontinuing ethanol intake. Chronic-abuse alcoholic hepatitis may develop (progressing from alcoholic fatty liver) and can quickly develop into cirrhosis if ethanol intake continues. In contrast, if ethanol intake is discontinued, alcoholic hepatitis of the liver heals with benign fibrosis (46).

Lelbach (22) has suggested that overindulgence in alcohol for more than 22 years results in a 50-percent probability of cirrhosis; apparently the resistance of the liver to alcohol damage varies among individuals. The susceptibility to liver damage by alcohol also varies according to sex, independent of nutritional state and type of diet (46). Based on morbidity data from 1,960 males and 627 females regarding the effect of the quantity of alcohol on the development of cirrhosis, Pequignot [cited by Thaler (46)] noted an increase in cirrhosis morbidity when more than 60 g of pure alcohol per day were consumed by men or more than 20 g were consumed by women (table 7). The data were based on a smaller sample for females than for males. The high susceptibility of women for liver cirrhosis was not explained. Such a large difference, however, cannot be attributed to body weight alone. The amount suggested as a "critical threshold" for men (60 g of pure alcohol) may be supplied by approximately either 4 bottles of beer, 1 bottle of wine, 1/2 l of vermouth or sherry, or 5 single whiskies; for women the critical level of 20 g of ethanol is supplied by either 1-1/2 bottles of beer, 1/4 l of wine, 3 glasses of dessert wine, or a weak double whisky. Beyond those critical levels, the

Table 7. Alcohol and Calorie Content of Popular Beverages and the Critical Threshold for Men and Women[a]

Beverage	Alcohol Content vol %	Pure Alcohol in 1,000 ml g	Calories per 1,000 ml	Critical Threshold[b] (liters)	
				Males	Females
Beer	4	31	400	2[b]	0.7[b]
Wine	10	79	700–1,200	0.7	0.25
Dessert wine	15	118	1,600	0.5	0.16
Hard liquor	38	300	2,500	0.2	0.07

[a]Data from reference (46).
[b]Volume (liters) supplying the critical threshold of 60 g of pure alcohol for men and 20 g for women.

morbidity of cirrhosis multiplies with increasing amounts of ethanol. In contrast, if ethanol consumption stops, even in early stages of alcoholic cirrhosis, life expectancy is only slightly lower than that of the average population.

Compared to matched controls of the same age, sex, and profession, patients with calcifying pancreatitis drank more alcohol (mean intake 2 g/kg/day) and their intakes of dietary fat and protein were greater (37). The mean duration of alcohol consumption before onset of the symptoms was 17 years in men. There was a linear relationship between the mean daily consumption of ethanol and the relative risk for developing chronic pancreatitis. Thus the possibility of toxicity of ethanol begins with the smallest dose. The log of the risk approximately doubles for every 40 g of ethanol per day.

Experimental Animal Studies

Providing 20-percent alcohol as the sole liquid delayed the onset of liver necrosis in rats deficient in vitamin E and selenium (23). After 5 to 6 weeks, the animals given alcohol tended to have less fat in their livers than controls supplemented with vitamin E, selenium, or both. The authors suggested that vitamin E and selenium status may affect the progression of alcoholic liver disease.

Huber and Gershoff (14) reported that the metabolism of alcohol was inhibited in zinc-deficient rats. Specifically, the oxidation of ethanol (and retinol) was significantly decreased as indicated by lowered activity of alcohol dehydrogenase in the retina and liver of zinc-deficient pups and in the retina (but not the liver) of zinc-deficient adult male rats (table 8). These data suggest that zinc deficiency may inhibit alcohol oxidation. Thus it may be speculated

Table 8. Alcohol Dehydrogenase Activity in the Liver and Retina of Zinc-Deficient Male Adult Rats and Zinc-Deficient Pups[a,b]

Group	Tissue	Control	Zinc Deficient	P
Male adult rats	retina	26 ± 4.0	15 ± 0.5	.02
(n = 10)	liver	125 ± 5	115 ± 10	NS
Pups at weaning	retina	40 ± 5	10 ± 0.8	.001
(n = 10)	liver	150 ± 11	117 ± 6	.02

[a]Data from reference (14).
[b]mμmoles NADH/min/retina; substrate ethanol.

that the conditioned zinc deficiency resulting from alcoholism may in turn cause an inhibited ethanol oxidation with further deleterious effects. In addition, Wang and Pierson have reported alterations in zinc metabolism in rats as a result of alcohol ingestion (57). Concentrations of zinc in plasma and liver were decreased significantly as early as 2 weeks after the substitution of 20-percent ethanol for drinking water. The zinc content decreased significantly in liver subcellular fractions; the mitochondrial fraction was affected most severely.

Van Theil et al. (56) have recently reported that ethanol inhibits the in vitro oxidation of retinol in testicular homogenates. The alcohol dehydrogenase activity in the material was markedly decreased as the alcohol concentration was increased. Preliminary studies also showed that chronic ethanol feeding to pair-fed rats produced germinal cell injury in the alcohol-fed animals but not in the isocaloric control. The authors suggested that ethanol inhibits the oxidation of retinol to the bioactive retinal in the testis, and that a conditioned or "relative vitamin A deficiency" may be a factor in the development of sterility in chronic alcoholics.

Increased Nutrient Requirement Resulting from Alcoholism

A recent symposium concerning the impact of infection on nutritional status revealed that losses of certain nutrients (e.g., protein, calories, and iron) were excessive during acute infections (1). Therefore, increased intakes of those nutrients were recommended. The recommended intake of protein during convalescence was 200 percent of minimum requirements (8,39).

The concept of increased requirements due to conditioning factors might well apply to chronic alcoholism. Indeed, as Leevy and Baker (20) indicated, "Alcoholism is the chief cause of vitamin deficiency among civilized people with adequate food supplies." They further suggested that this deficiency is in part due to increased vitamin needs imposed by the alcohol. They cautioned, however, that vitamin supplements alone would probably not prevent vitamin deficiency syndromes because a variety of other nutrients is required for the catalytic function of vitamins. Those authors, therefore, believe that an adequate food intake should be provided each day when there is no ethanol in the intestine or blood to interfere with absorption of the vitamins.

Summary

In this paper, I have discussed the complex and multiple factors that contribute to marginal and conditioned nutritional deficiencies. The problems of establishing nutrient requirements and assessing nutrient status also have been highlighted. An adequate intake of all nutrients in proper balance is in itself difficult to achieve. Even if an adequate intake were assured, interfering factors that affect bioavailability and utilization of nutrients may result in conditioned deficiencies and marginal nutritional states. Alcoholism is one such conditioning factor that may alter the metabolism of several nutrients; other such factors have been identified; and still others, undoubtedly, have yet to be identified. Of equal importance is the definition of optimal nutritional status and the identification of the segments of our population that would benefit from change or supplementation of diets, or both.

Last, nutritional status should be viewed not as an absolute and static state, but as a state that ranges from frank deficiency to optimal nutrition, with a wide area lying between the extremes. Optimizing the nutritional status of individuals would be a major contribution toward sustained excellent health. Thus, nutritional status literally affects *every body*!

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Alcohol-Nutrition Interactions*

Charles S. Lieber

Abstract

Alcohol abuse can lead to malnutrition because ethanol represents nutritionally "empty" calories; that is, calories not associated with substantial amounts of vitamins, minerals, or proteins. Ethanol also acts on the gastrointestinal tract and interferes with digestion and absorption. Moreover, it impairs the utilization of various nutrients.

Calories derived from ethanol are also not fully utilized in the body. Although alcoholism remains one of the major causes of nutritional deficiencies in affluent societies, the incidence of the malnourished alcoholic is decreasing while mortality from alcoholic liver diseases continues to rise. However, though florid nutritional deficiencies may be relatively rare, the impact of more subtle nutritional alterations produced by alcohol remains to be explored. Nevertheless, chronic alcohol abuse can lead to the development of liver injury, including cirrhosis, when the diet contains all the required nutrients in recommended amounts.

Introduction

Alcohol and nutrition interact at many levels (see table 1). Ethanol may directly alter the level of nutrient intake through its effect on appetite or its displacement of food in the diet, or by its deleterious effects at almost every level of the gastrointestinal tract. These changes result in disturbances of digestion and absorption. Furthermore, through its effect on various organs, especially the liver, ethanol may alter the transport, activation, catabolism, utilization, and storage of almost every nutrient studied. Therefore, alcoholism remains one of the major causes

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of nutritional deficiency syndromes in our society. Furthermore, ethanol is directly toxic to many tissues of the body, and this effect may be potentiated by concomitant nutritional deficiencies. Thus, because of its widespread use and multiple effects, ethanol has a major impact on overall nutritional status.

Table 1. Alcohol–Nutrition Interactions

1. Primary Malnutrition (deficient intake)
   — "empty" calories
   — economic factors
   — impaired appetite secondary to GI-liver disorders

2. Secondary Malnutrition (deficient nutrient utilization)
   — ethanol-induced GI damage (maldigestion-malabsorption)
   — deficiency-induced intestinal dysfunction
   — energy wastage
   — decreased activation or increased inactivation of nutrients

Effects of Alcohol Abuse on the Gastrointestinal Tract

Ethanol may affect the stomach in a number of ways. Acid secretion may be increased as a result of direct stimulation, vagal effects, or gastrin release (Chey, 1972). Higher acid levels may secondarily increase absorption of iron (Charlton et al., 1964). In addition to stimulating acid secretion, ethanol disrupts the mucosal barrier (Davenport, 1969) and is an accepted cause of acute gastritis. This mechanism may be one of the ways by which alcohol diminishes dietary intake. Alcohol may also impair gastric emptying (Barboriak and Meade, 1970). Chronic ethanol administration first results in increased mean daily acid secretion and then gradually decreases it (Chey et al., 1972). The role of alcohol in the genesis of duodenal and gastric ulcer and chronic gastritis remains unsettled (Lorber et al., 1974).

Alcohol has also been shown to be directly injurious to the small intestine (Rubin et al., 1972). Acute administration of ethanol (1 g/kg) p.o. results in endoscopic and morphologic lesions in the duodenum (Gottfried et al., 1976). Previous failure to observe such lesions may have been due to the transient and patchy

nature of these lesions (Pirola et al., 1969). Experimentally, such lesions are related to the concentration of ethanol used, with the greatest damage resulting from those solutions with the highest concentration of ethanol (Baraona et al., 1974). These correlations are shown in figure 1.

Acute administration of ethanol may impair the absorption of many nutrients and experimentally results in alterations in mucosal enzymes (Israel et al., 1969; Hillman, 1974; Baraona et al., 1974). Studies with oral and intravenous alcohol have revealed an inhibition of type I (impeding) waves in the jejunum and an increase in type III waves (propulsive waves) in the ileum (Robles et al., 1974). These changes have been proposed as a possible mechanism of the diarrhea observed in binge drinkers. Ingestion of ethanol has recently been demonstrated to result in release of secretin from the duodenum (Straus et al., 1975).

Figure 1. Varying Concentrations of Ethanol on Rat Small Intestine[a]

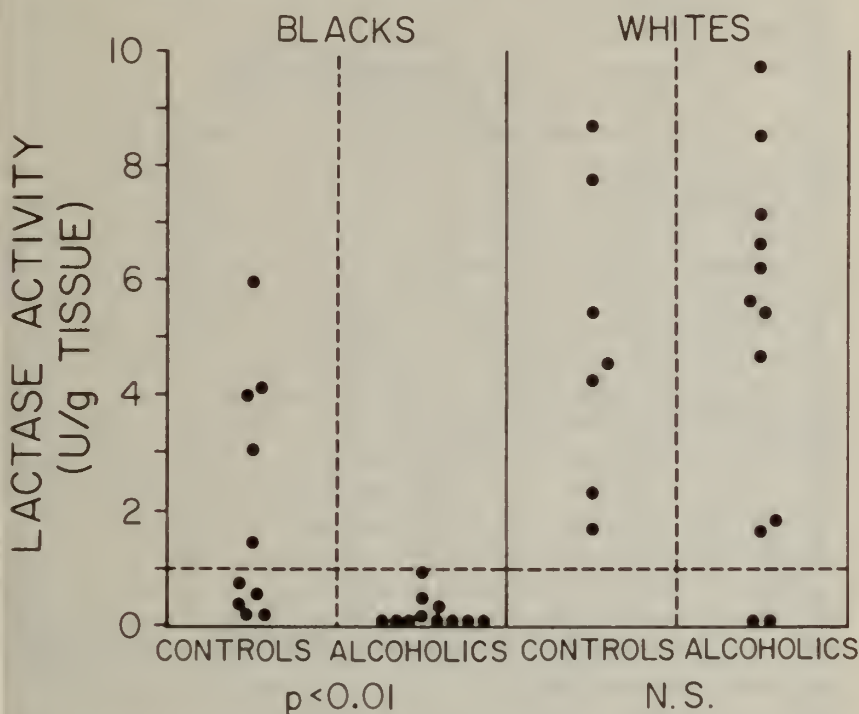
Varying the concentration of ethanol administered intragastrically (in a dose of 3 g/kg) affects the severity of damage in the proximal 20 cm of rat small intestine. Each animal is represented by a dot.

[a]From Baraona et al., 1974. Copyright 1974 by The Williams & Wilkins Co.

The effect of chronic ethanol consumption on intestinal function is complicated by the concomitant effects of nutrition. Indeed, malnutrition itself may lead to intestinal malabsorption (James, 1968; Mayoral et al., 1967); folate depletion, which is common in alcoholics, has been especially implicated in this regard (Winawer et al., 1965; Halsted et al., 1971, 1973; Hermos et al., 1972). Impaired absorption of folate, thiamine, B_{12}, xylose, and fat have been described in alcoholics; they recover after withdrawal from alcohol and institution of a nutritious diet (Lindenbaum and Lieber, 1971; Tomasulo et al., 1968; Roggin et al., 1969; Mezey et al., 1970; Halsted et al., 1971). The absorption of water and electrolytes from the jejunum was studied in 10 alcoholics using a triple-lumen tube perfusion system (Krasner et al., 1976). The mean rate of absorption of water in the alcoholic subjects (50.0 ± 2.3 ml/hr) was significantly lower ($p < 0.001$) than the mean value in 14 healthy control subjects (205 ± 15.9 ml/hr). Significant reduction in Na^+ and Cl^- absorption was also demonstrated in the alcoholic subjects. These results indicate that alcoholics, after acute alcohol abuse, may have a functional impairment of water and electrolyte absorption from the jejunum. This dysfunction may, in part, account for symptoms such as diarrhea that may be present (Krasner et al., 1976). However, generally the biochemical evidence of malabsorption correlates poorly with intestinal symptoms in the alcoholic (Lindenbaum and Lieber, 1975). Food intolerance, particularly of lactose, secondary to defective intestinal digestion, could contribute to the production of these symptoms. Low lactase activity in adulthood exists in a majority of the world's population (Bayless et al., 1971). Furthermore, location of lactase on the villi (Nordström et al., 1968) makes those structures vulnerable to the corrosive effect of luminal toxins such as alcohol (Baraona et al., 1974, 1975). Indeed, disaccharidase activities often decrease with intestinal injury (Herbst et al., 1970; Berchtold et al., 1971; Giannella et al., 1971).

To ascertain whether or not alcohol ingestion affects intestinal disaccharidase activities and influences the incidence of symptomatic lactose intolerance, lactase activity and lactose tolerance were studied in alcoholics and nonalcoholics. Two human population groups with genetically determined low and high intestinal lactase levels, namely blacks and whites of Northern European origin, were observed (Perlow et al., 1977). After an overnight fast, biopsies of the jejunum were obtained with a Quinton Multipurpose Suction Biopsy Tube positioned fluoroscopically at the level of the ligament of Treitz. When measured within 10 days of

Figure 2. Comparison of Jejunal Lactase Activity (Per Oral Biopsies) in Black and White Males, With and Without a History of Recent Alcoholism[a]



Comparisons were made of jejunal lactase activity per oral biopsies, between 21 black and 19 white males of similar nutritional status, with and without a history of recent alcoholism. All black alcoholics had lactase activity lower than 1 U/g, but only 50 percent of nonalcoholic blacks were deficient ($p < 0.01$). The difference among whites was not significant.

[a]From Perlow et al., 1977. Copyright 1977 by The Williams & Wilkins Co.

alcohol withdrawal, sucrase activity was decreased by 33 percent in the alcoholics. Lactase activity was less than 1 U/g in 100 percent of the black and 20 percent of the white alcoholics as compared to 50 percent of the black and none of the white controls. The results are shown in figure 2. Lactase activity was virtually absent in 45 percent of the black alcoholics.

A second jejunal biopsy following an additional 2-week period of alcohol abstinence exhibited significant secondary increases in the activities of both disaccharidases. Oral administration of lactose (1 g/kg body weight) resulted in significantly lower blood

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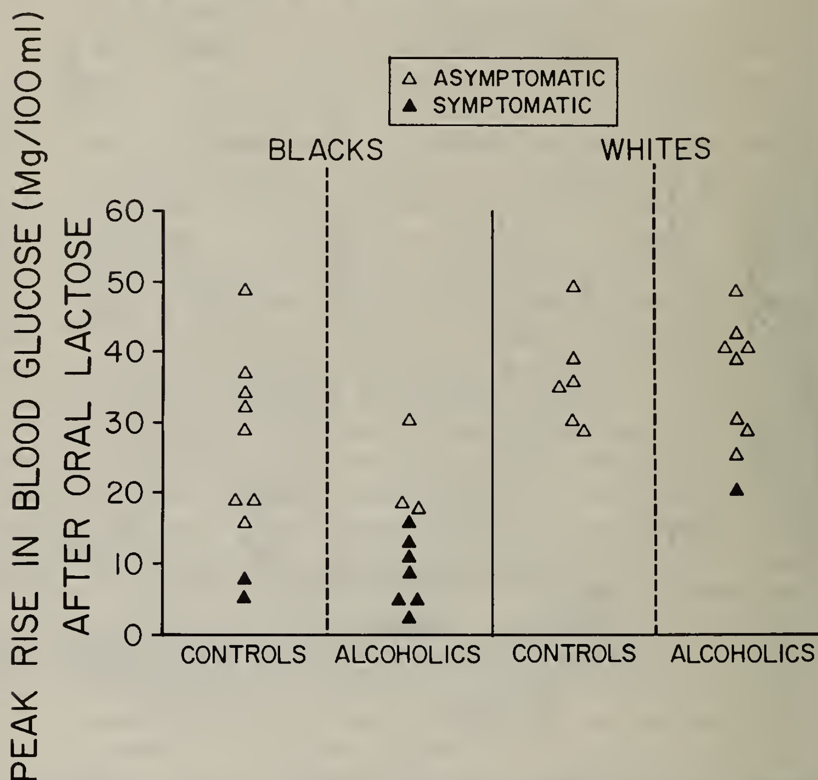
Figure 3. Correlation Between Peak Blood Glucose Concentration and Severe Symptoms of Intolerance After Administration of Lactose[a]



This figure shows the correlation between the peak rise in blood glucose concentration and the incidence of severe symptoms of intolerance after administration of lactose. A strikingly increased incidence of severe lactose intolerance was found in the group of black alcoholics.

[a]From Perlow et al., 1977. Copyright 1977 by The Williams & Wilkins Co.

glucose concentration and higher incidence of adverse effects in alcoholics, mainly among the blacks. These results are shown in figure 3.

The mechanism for the disaccharidase depression in alcoholics has not been fully elucidated. Because these effects were observed in alcoholics without nutritional deficiencies, the reduction in disaccharidase activity appears to be an effect of chronic alcohol ingestion per se.

Ethanol administration to human volunteers for 3 to 6 days has been reported to inhibit glycolytic and gluconeogenic intestinal

enzyme activities (Greene et al., 1974). Similar depressions of intestinal disaccharidase as well as alkaline phosphatase activities have been produced in rats ingesting alcohol either acutely or chronically with nutritionally adequate diets (Baraona et al., 1974). The dose of ethanol given these rats resulted in ethanol concentrations within the intestinal lumen comparable to those found in human subjects after drinking (Halsted et al., 1973).

Evidence of gut injury and subsequent regeneration of intestinal epithelium has been found in rats fed ethanol-containing diets (Baraona et al., 1974) and in humans endoscoped after controlled alcohol administration (Gottfried et al., 1976). The concomitant improvement in disaccharidase activities and increase in mitotic activity after alcohol abstinence (Perlow et al., 1977) are consistent with the possibility of regeneration. However, it is intriguing that the time required for the disaccharidase activities to improve was considerably longer than that expected for the epithelium to regenerate. It is possible that these effects may not merely reflect villus cell desquamation, but also may reflect altered cell renewal or maturation.

The low values of lactase activity in the jejunal biopsies of alcoholics were also accompanied by poor lactose absorption, as measured by the rise in blood glucose concentration after an oral load. Apparently the alteration produced by alcohol was not restricted to the upper segment of the jejunum, but was sufficiently extended to impair total lactose absorption. This effect was again more apparent in the black alcoholics. Lactose malabsorption was associated with increased incidence of colic, diarrhea, and a dumping-like syndrome severe enough to require medical attention. Thus, disaccharidase deficiency in alcoholics may not be only an indication of the damaging effect of alcohol on the intestinal epithelium, but the association may also lead to increased morbidity from primary lactase deficiency, a rather common disorder.

As little as 3 g of lactose has been shown to produce symptoms in individuals with low intestinal lactase activities (Bedine and Bayless, 1973). Thus, milk intolerance may be unmasked or exaggerated in those populations where both alcoholism and genetically determined low lactase levels are common. In view of the likelihood of significant milk intolerance in alcoholics, the common practice of liberal milk supplementation for "nutritional restoration" or the treatment of gastritis or ulcer-associated symptoms should be reconsidered, particularly in ethnic groups (such as blacks) with preexisting low lactase activities.

The acute and chronic effects of alcohol on small intestine function may be potentiated by concomitant alterations in pan-

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creatic function, bile salts, and small intestine flora. However, in patients with cirrhosis, steatorrhea (fecal fat greater than 30 g/24 hrs on a 100 g fat/d diet) is relatively uncommon and in one series was present in only 9 percent of cases (Linscheer, 1970).

Portal hypertension has also been postulated as a cause of malabsorption (Losowsky and Walker, 1969). Finally, specific therapeutic interventions, such as neomycin, may by themselves cause malabsorption (Faloon, 1970). Chronic pancreatitis may lead to pancreatic insufficiency in the alcoholic and may contribute to steatorrhea and malabsorption. Acute pancreatitis may result in diminished dietary intake and severe fluid and electrolyte disturbances. Both acute and chronic pancreatitis may cause alterations in glucose tolerance.

Acute and chronic alcohol administration as well as alcoholic liver disease have been noted to alter bile salt metabolism. Acutely, administration of ethanol intravenously or into the jejunum decreases intraluminal bile salts (Marin et al., 1973). Chronic ethanol feeding in the rat prolongs the half excretion time of cholic and chenodeoxycholic acid, increases the pool size slightly, and decreases daily excretion (Lefevre et al., 1972).

Alcohol, Malnutrition, and the Pathogenesis of Alcoholic Liver Injury

The question of the respective roles of alcohol and malnutrition in the pathogenesis of liver disease seen in the alcoholic (fatty liver, alcoholic hepatitis, and cirrhosis) is significant—both for the prevention and the treatment of the disease. The resolution of this question has been exceedingly difficult for several reasons: the unreliability of alcoholic populations, the variability of disease expression, the difficulty of accurate nutritional evaluation, and the long-term course of pathogenesis.

Malnutrition has been proposed as the predominant factor producing liver injury for several reasons. Each gram of ethanol provides 7.1 cal, which means that 20 oz (586 ml) of 86-proof (43 percent v/v) beverage represents about 1,500 cal, or one-half to two-thirds of the normal daily caloric requirement. Therefore, alcoholics have a much reduced demand for food to fulfill their caloric needs. Because alcoholic beverages do not contain significant amounts of protein, vitamins, and minerals, the intake of these nutrients may become readily borderline or insufficient.

Economic factors may also reduce the consumption of nutrient-rich food by alcoholics. In addition to acting as "empty" or

"naked" calories, alcohol can result in malnutrition by interfering with the normal processes of food digestion and absorption (Lindenbaum and Lieber, 1975). For all these reasons, deficiency diseases readily develop in the alcoholic. In rodents, severely deficient diets result in liver damage even in the absence of alcohol. Extrapolation from these animal results to humans led to the belief that in alcoholics, the liver disease is due not to ethanol but solely to the nutritional deficiencies. Thus, given an adequate diet, alcohol is merely acting by its caloric contribution and is not more toxic than a similar caloric load derived from fats or starches (Best et al., 1949). This opinion prevailed, despite some statistical evidence gathered both in France (Pequignot, 1962) and Germany (Lelbach, 1967), which indicated that the incidence of liver disease correlated with the amount of alcohol consumed rather than with deficiencies in the diet. A major challenge to the concept of the exclusively nutritional origin of alcoholic liver disease arose from an improvement of the method of alcohol feeding to experimental animals. Indeed, when the conventional alcohol feeding procedure is used, namely when ethanol is given as part of the drinking water and when the diet is adequate, rats usually refuse to take a sufficient amount of ethanol to develop liver injury.

This aversion of rats to ethanol was counteracted by the introduction of the new technique of feeding ethanol as part of a nutritionally adequate, totally liquid diet (Lieber et al., 1963; Lieber et al., 1965; DeCarli and Lieber, 1967). Using this procedure, ethanol intake was sufficient to produce a fatty liver despite an adequate diet. This technique is now widely adopted for the study of the pathogenesis of the fatty liver in the rat. In addition to the fatty liver, ethanol dependence developed in these rats, as witnessed by typical withdrawal seizures after cessation of alcohol intake (Lieber and DeCarli, 1973).

Having established an etiologic role for ethanol in the pathogenesis of the experimental fatty liver, the question of its importance for the development of human pathology remained. To determine whether ingestion of alcohol, in amounts comparable to those consumed by chronic alcoholics, is capable of injuring the liver even in the absence of dietary deficiencies, volunteers (with or without a history of alcoholism) were given a variety of nondeficient diets under metabolic ward conditions, with ethanol either as a supplement to the diet or as an isocaloric substitution for carbohydrates (Lieber et al., 1963; Lieber et al., 1965; Lieber and Rubin, 1968). In all these subjects, ethanol administration resulted in fatty liver development that was evident by both morphologic examination and by direct measurement of the lipid

content of the liver biopsies. These findings revealed a rise in triglyceride concentration up to 15-fold.

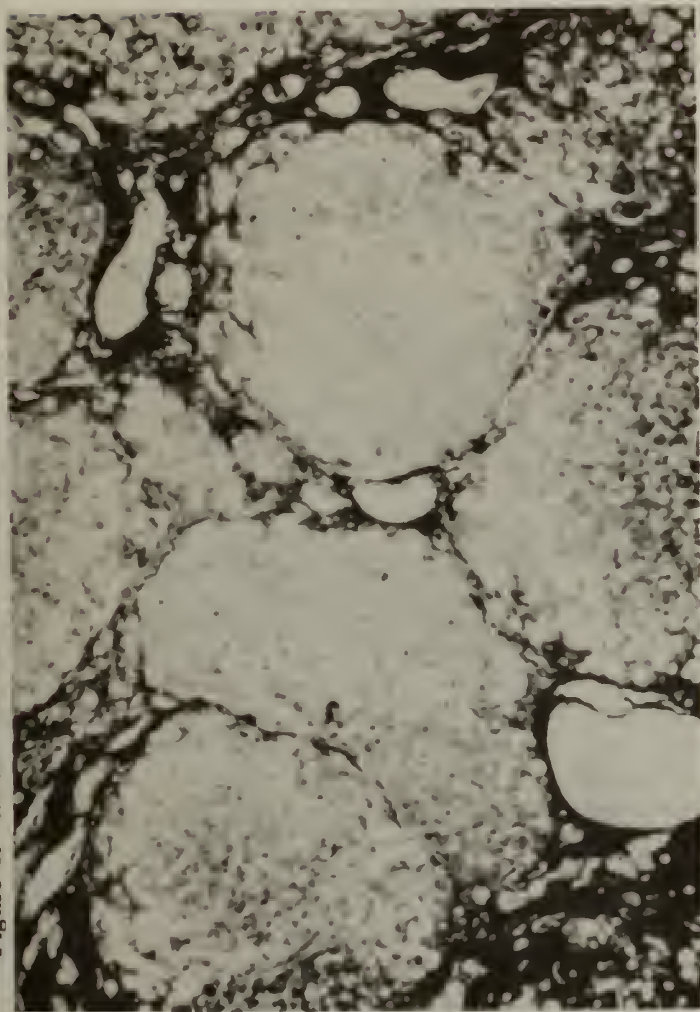
The etiological role of alcohol per se (in the absence of dietary deficiency) in the pathogenesis of alcoholic liver injury has now been extended from the fatty liver to the full spectrum of liver disease, including cirrhosis. This result was achieved by using an experimental model developed in the baboon (Lieber and DeCarli, 1974), in which the sequential development of all the liver lesions seen in the human alcoholic was reproduced. Of 23 baboons fed ethanol, all developed fatty liver, 5 progressed to mild hepatitis, and 6 had cirrhosis. An example is shown in figure 4. Maintenance of a nutritionally adequate regimen despite the intake of inebriating amounts of ethanol (50 percent of total calories) was achieved by incorporation of the ethanol in a totally liquid diet. On ethanol withdrawal, signs of physical dependence such as seizures and tremors developed. Ultrastructural changes of the mitochondria and the endoplasmic reticulum were already present at the fatty liver stage and persisted throughout the cirrhosis. The lesions were similar to those observed in alcoholics and differed from the alterations produced by choline and protein deficiencies. At the fatty liver stage, some "adaptive" increases in the activity of microsomal enzymes (aniline hydroxylase and the microsomal ethanol oxidizing system) were observed; the increases tended to disappear as hepatitis and cirrhosis developed. Fat accumulation was also more pronounced in the animals with hepatitis, as compared with those with simple fatty liver (an 18-fold compared to a 3- to 4-fold increase in liver triglycerides).

The demonstration that these lesions can develop despite an adequate diet indicates that in addition to correction of the nutritional status, control of alcohol intake is mandatory for the management of patients with alcoholic liver injury. Also, ethanol per se must be considered a direct etiological agent in the pathogenesis of alcoholic liver injury, independent of dietary factors (Lieber et al., 1975). This finding, however, does not preclude the possibility that dietary factors may contribute to and potentiate the alcohol effect, which had previously been shown to be the case in rats (Lieber et al., 1969). No similar studies are available for humans.

The Nutritional Value of Alcoholic Beverages

Ethanol liberates 7.1 kcal/g, but does not provide equivalent caloric food value when compared to carbohydrate. As shown in

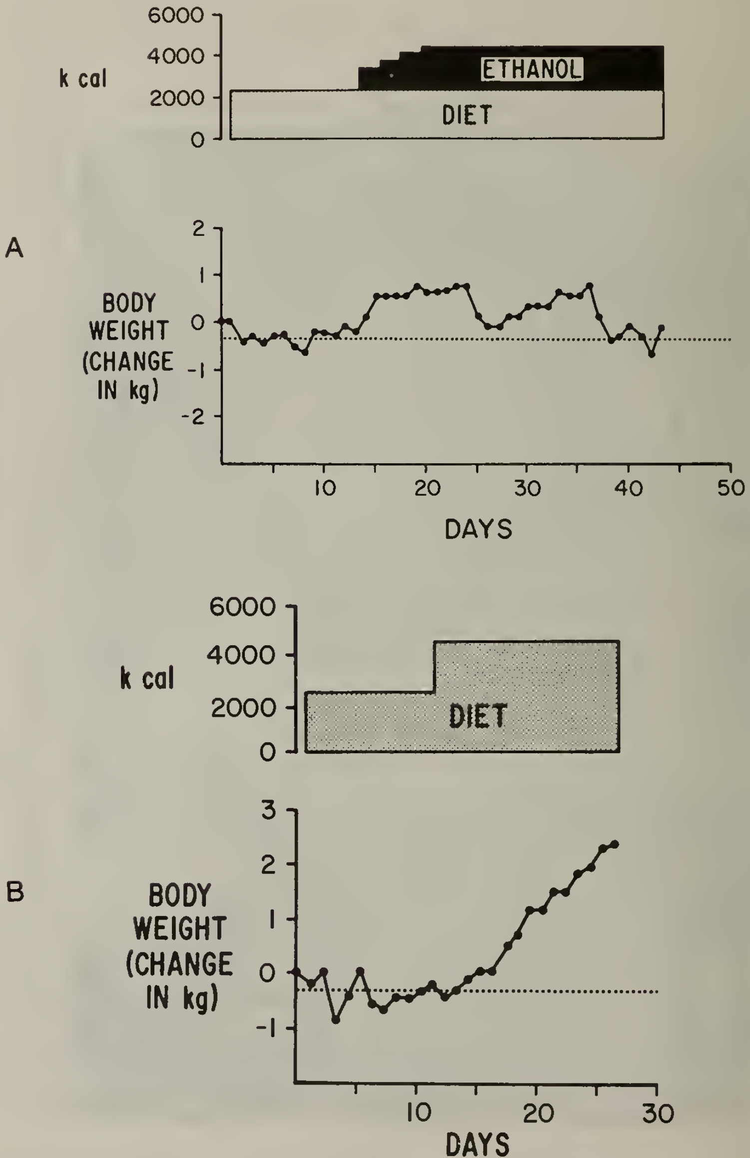
Figure 4. Liver Nodules of a Cirrhotic Baboon Fed Alcohol for 4 Years[a]



Fat is regularly distributed through liver nodules, surrounded by connective tissue septa. The slide is tinted with chromotrope-aniline blue and magnified 60 times.

[a]From Lieber and DeCarli, 1974. Copyright 1974 by S. Karger (Basel).

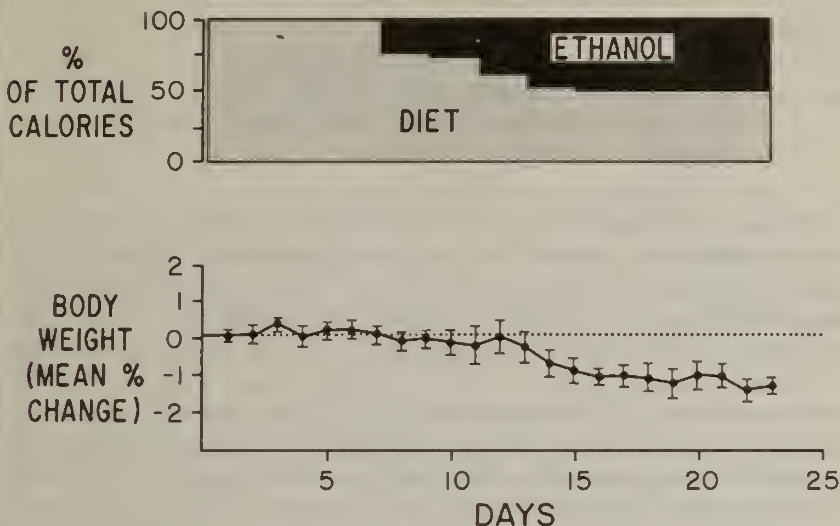
Figure 5. Effect on Body Weight of 2,000 Kcal Ethanol and Chocolate[a]



The same subjects were administered ethanol and chocolate at different times. The dotted line represents the mean change during the control period.

[a]From Pirola and Lieber, 1972. Copyright 1972 by S. Karger (Basel).

Figure 6. Body Weight Changes After Isocaloric Substitution of Carbohydrate for Ethanol[a]



Body weight changes after isocaloric substitution of carbohydrate (50 percent of total calories) by ethanol were recorded for 11 subjects (means ± standard errors). The dotted line represents the mean change in weight in the control period.

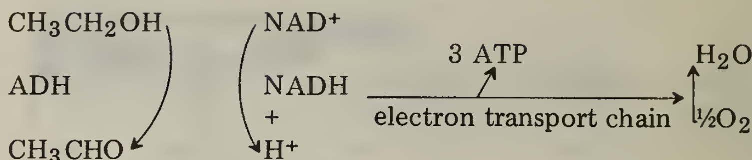
[a]From Pirola and Lieber, 1972. Copyright 1972 by S. Karger (Basel).

figure 5, gain in body weight is significantly lower with ethanol than with isocaloric amounts of carbohydrate (Pirola and Lieber, 1972). Isocaloric substitution of ethanol for carbohydrate as 50 percent of total calories in a balanced diet (as shown in figure 6), results in a decline in body weight (Pirola and Lieber, 1972). One interpretation of this lack of weight gain with ethanol, compared to other sources of dietary calories, is the possibility that chronic alcohol intake increases the energy requirements of the body. If this were the case, a higher rate of oxygen consumption should be reflected. Indeed, in rats fed alcohol as part of their totally liquid diet, oxygen consumption was slightly but significantly higher than that of animals pair-fed the isocaloric diet containing carbohydrates instead of ethanol (Pirola and Lieber, 1976).

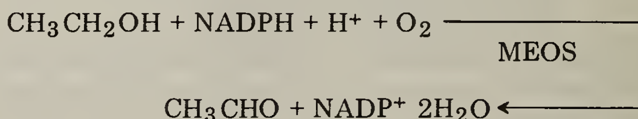
Among the many mechanisms that could be postulated to account for an inefficient use of ethanol calories, one involves the energy wastage secondary to an induction of liver microsomal pathways.

Efficient utilization of the calories of ethanol would be anticipated from a consideration of its major metabolic pathway, which

involves the hepatic cytosolic enzyme alcohol dehydrogenase (ADH).



From an energy point of view, this process appears to be an economical one, because the associated production of NADH supplies the electron transport chain with hydrogen equivalents and yields high-energy phosphate bonds. In addition to the ADH pathway, ethanol is also metabolized via a hepatic microsomal ethanol-oxidizing system (MEOS) (Lieber and DeCarli, 1970). The exact quantitative significance of the enzyme system in vivo remains uncertain, but studies indicate that it could normally involve 20 to 25 percent of the oxidation of ethanol (Lieber and DeCarli, 1972) and much more after chronic ethanol consumption —particularly at high ethanol concentrations (Matsuzaki et al., 1977). The potential importance of this mechanism on the body's caloric balance lies in the fact that, in contrast to the ADH pathway, MEOS results in the loss of chemical energy from both the substrate and the cofactor (NADPH) without any known effective coupling to ATP synthesis.



Presumably, the chemical energy is dissipated as heat and, insofar as it exceeds the body's thermoregulatory needs, represents an inefficient use of ingested calories. It is of interest that similar considerations apply to the oxidation of other drugs and endogenous substrates (such as steroids) by hepatic microsomal drug-metabolizing enzymes. These oxidations have the following general formula:

$$RH + NADPH + H^+ + O_2 \longrightarrow ROH + NADP^+ + H_2O$$

The proposed hypothesis (Pirola and Lieber, 1976) is that the inefficiency of microsomal drug-metabolizing enzymes could be of quantitative significance in the energy balance of the body during the repeated intake of drugs, especially ethanol.

The hypothesis is in keeping with other animal studies in which metabolic rates were increased by the administration of ethanol and barbiturates in doses known to induce hepatic microsomal enzymes. Thus, pretreatment with barbiturates enhanced oxygen consumption in rats tested under various conditions—in the absence of drugs, during hexobarbital anesthesia, and after the administration of aminopyrine (Pirola and Lieber, 1975).

There are, of course, many metabolic pathways in the body that are not effectively linked to ATP synthesis. These pathways contribute to the net wastage of calories to give a less-than-optimal overall efficiency of the body. In this respect, the microsomal drug-metabolizing enzyme system is unique in its extraordinary versatility and in its ability to be induced by a wide variety of agents.

Another major theory for the explanation of the hypermetabolic state produced by ethanol is an increased utilization of ATP by the Na^+-K^+-activated ATPase after chronic ethanol feeding. Israel et al. (1975) reported that in liver slices, ouabain, an inhibitor of the Na^+-K^+-activated ATPase, can completely block the extra ethanol metabolism elicited by chronic ethanol treatment. Dinitrophenol increased the rate of ethanol metabolism in the livers of the treated animals only in the presence of ouabain.

The theory that increased utilization of ATP by the Na^+-K^+-activated ATPase (and the resultant lowering of the phosphorylation potential) is responsible for the metabolic adaptation that occurs in rats after chronic ethanol treatment, and that a situation develops that is very similar to that found after administration of thyroid hormones or epinephrine, is intriguing. Certainly, the influence of ethanol consumption on hormonal actions deserves further investigation. To date, few studies have been performed to confirm these observations.

Under the conditions used by Israel et al. (1975), ethanol consumption did not result in liver changes comparable to those seen in human alcoholic liver injury; for instance, no fatty liver was observed. Under conditions that mimic the clinical situation with development of fatty liver, chronic ethanol consumption was not found to be associated with increased ATPase activity (Gordon, 1977), and the increase in the rates of ethanol metabolism after chronic ethanol consumption could not be abolished by ouabain (Cederbaum et al., 1976). These findings indicate that the theory of enhanced ATPase activity may not be applicable to the situation that normally prevails after chronic alcohol consumption.

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Discussion of Papers by Smith and Lieber

Dr. McDonald: Dr. Li has asked me to mention that Dr. Margen and I, in our experiment with humans at the University of California in Berkeley, observed a similar weight loss in nonalcoholic human volunteers who were placed on an adequate diet. This was not one of the purposes of the experiment; but when nonalcoholics were on an alcohol phase of the experiment, they consistently lost weight. We also observed no decrease in absorption in energy-containing substances. So this would confirm what Dr. Lieber has just said. If the MEOS can be blamed for the wasted calories, it would seem that even less than 36 percent of the total calories as alcohol would induce this system, because our subjects were receiving 22 to 25 percent of the calories as alcohol.

Dr. Lieber: Here is a very important point, because we never went down to that low level. I guess we were anxious to have a nicer effect, perhaps; that is why we used a larger amount. It is important to know that even this relatively modest intake is asserting a similar effect.

Dr. Vallee: This is not a question at all, but rather a comment on Dr. Smith's discussion. It is the implication that, by measuring metal contents in sera, one can establish criteria regarding nutritional status. I am sorry I did not hear about your new indexes for measurement of alcohol intake—which I surely hope you will tell us about—but I think that deserves a particular comment.

The amount of zinc which is around is distributed into multiple systems. Of the 92 zinc-containing enzymes, none is found in serum. Alcohol dehydrogenase was never detected in serum, so whatever moiety of that enzyme has anything to do with what we are talking about, it is not likely to be found there. As a matter of fact, I am unaware of a zinc transport protein, and I am unaware of a zinc absorption protein in the sense in which that is known about copper. I know Dr. Hurley is working on something. But we have tried for 30 years, and other people have, and we have not been able to find it.

So when there is a change in zinc concentration in serum, I have not the faintest notion what that means in these terms. In spite of the fact that I reported some myself in the past and attempted just like anybody else to try to understand what that could mean, I

have the excuse now, which I had then, too, that I did not know anything about it. I do not know any more. However, I think it is about as wise to do zinc analyses, to assault that question in serum, as it would be to do carbon, hydrogen, nitrogen, oxygen, or sulfur determinations, on serum. It will tell you something, but I don't know what.

Dr. Schenker: Dr. Vallee, perhaps you would like to tell us which tissues are the most sensitive to changes in zinc content for picking up a zinc-deficiency state, say of first marginally, and then subsequently severe, deficiency.

Dr. Vallee: None that I know of. That does not mean there aren't any.

Dr. Schenker: That is interesting, because it was my understanding that other workers have shown that bone and testes might be more sensitive to changes due to inadequate zinc intake than say, liver or other tissues. Perhaps I am wrong in that.

Dr. Vallee: You were asking a very specific question, and I gave you a very specific answer. I did not say that zinc deficiency would not manifest in certain tissues in a certain manner and preferentially in some of their metabolic behavior. Dr. Falchuk discussed this extensively in *Euglena gracilis* and showed you how difficult it is to run it down—where you might ultimately find evidence of a lesion. What is the limiting step of a reaction, as you saw, is extraordinarily difficult to establish. It is true that certain organs seem to be preferentially affected, but analysis of zinc content in tissues is probably too gross a measurement for the diagnosis of zinc-deficiency state.

Dr. Lester: I would like to ask Charles Lieber a question. You have emphasized the importance of alcohol in the production of various forms of pathology, as against changes in nutritional status. Specifically, with liver disease, you have shown beautifully that alcohol per se can produce a variety of forms of liver disease without deprivation of calories or other nutrients. My question is, can any of the phenomena of alcoholism, histologic or biochemical, be modified in the presence of either caloric deprivation or deprivation of other specific nutrients?

Dr. Lieber: Well, there are certainly circumstances where adding insult to injury and adding malnutrition to alcohol would aggravate the situation. In 1969, as a matter of fact, I had a paper in the *Journal of Lipid Research* which demonstrated that adding protein malnutrition to alcohol would accelerate the development of a fatty liver. So in that particular case, one looks at fatty liver as an end point, and the combination of protein malnutrition and alcohol certainly had much more striking effects than malnutrition alone or alcohol alone.

But that was with fatty liver, and the question is, what about cirrhosis? The situation is much more complicated, and I am not sure that at this point we have an answer. Under certain circumstances, certainly malnutrition by itself, severe malnutrition in experimental animals, can result in severe liver damage, including some cirrhotic changes. Under other circumstances, protein deficiency, for instance, may protect against the action of known cirrhogenic agents. Carbon tetrachloride cirrhosis, for instance, can be prevented by a low protein diet. There has been a puzzling observation published in Scandinavia where they claim that the incidence of cirrhosis in "skid row" alcoholics may be lower than that in better nourished alcoholics. Of course, you know that childhood cirrhosis in India occurs in the well-to-do families rather than in the poor ones.

So I think it is a complex question. We need more data, and we are trying to get them now. We are trying to study in our baboon the relationship between protein malnutrition and alcohol in the development of cirrhosis. Unless we have an experimental answer, I would not want to give you a theoretical answer because I could imagine a situation where excess protein might be detrimental. As we have studied recently, alcohol impairs the secretion of protein from the liver, and results in protein accumulation in the liver. We feel that this protein accumulation might, under certain circumstances, be detrimental to the liver. So under those conditions, excess protein might conceivably not be beneficial. On the other hand, of course, protein deficiency by itself has some damaging effects. Because of the complexity of the situation, I would not want to give you a black and white answer that affects the whole field of liver disease. Only in terms of fatty liver can I unequivocally say yes; that is, protein deprivation potentiates the injurious effect of alcohol and we have demonstrated that experimentally.

Determinants of Absorption from the Gastrointestinal Tract*

John M. Dietschy

This paper deals with two aspects of alcohol metabolism: (1) the mechanism of absorption of ethanol and other longer chain length alcohols and (2) the possible ways in which alcohol ingestion might alter the rates of absorption of other nutrients across the gastrointestinal tract. In order to approach both of these subjects, it is necessary to review the general features of the various mechanisms that determine the rates of solute transport across the intestine.

The Two General Types of Transport Involved in the Movement of Solutes Across Biological Membranes

Many complex types of transport systems have been described, but only two are probably important to explain the movement of most molecules into and out of many types of tissues. The first of these is usually designated as "simple passive diffusion" and involves the movement of individual solute molecules across the lipid-protein matrix of the cell membrane. Because such movement occurs across an "infinite" number of sites in the membrane and is driven by the chemical activity of the molecule in the pericellular perfusate, the rate of such movement is usually a linear function of the concentration[1] of the solute molecule to which a given cell is exposed. As shown in panel I of figure 1, the rate of movement (J) of the solute molecule from the outside of the cell into the cytosolic compartment is equal to the product of the

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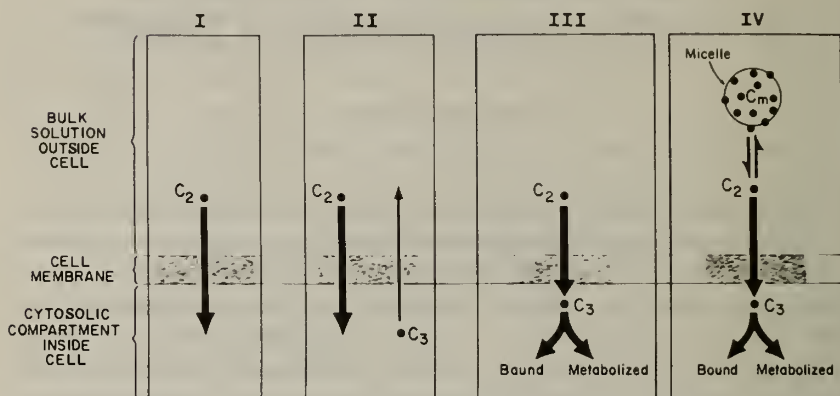
[1] As with all transport phenomena, the velocities of the various processes to be described depend on the chemical activities of the solutes in solution and not, strictly speaking, on their chemical concentrations. For the sake of convenience, however, in this review the terms "concentration" or "monomolecular concentration" should be understood to mean chemical activity in the specific sense.

Figure 1. Several Experimental Situations Encountered During the Monomolecular Movement of a Solute Molecule Across a Biological Membrane



In this diagram, C_2 represents the concentration of the solute molecule in the solution perfusing the outside of the cell membrane; C_3 represents the concentration of the molecule in the aqueous phase in the cytosolic compartment inside the cell membrane. C_m in panel IV represents the concentration of the solute molecule within the micellar phase, and K is a conventional partitioning coefficient dictating the relationship between C_m and C_2 such that $K = C_m/C_2$.

concentration of the molecule in the bulk solution (C_2) and the passive permeability coefficient (P) for that particular solute crossing that particular membrane.

$$J = (P)(C_2) \quad (1)$$

The passive permeability coefficient describes the amount of solute that crosses 1 cm^2 of the cell membrane per unit time per unit concentration of the solute to which the membrane is exposed and so has units such as $nmol/cm^2/sec/(nmol/cm^3)$, which reduces to the conventional units used for P of cm/sec.

When this value is multiplied by the concentration term, J describes a flux rate with the units of mass of solute moving across 1 cm^2 of membrane per unit time, e.g., $nmol/cm^2/sec$. However, in nearly all experimental systems used to study intestinal transport, the membrane surface area is unknown, and the rate of movement is normalized to some other parameter of cell mass, such as mg of protein, g wet weight, or cm length. Under these conditions, the experimentally determined flux rates and passive permeability coefficients are designated J_d and P_d, respectively, and have units such as nmol/g tissue/sec (J_d) and nmol/g

tissue/sec/(nmol/cm^3)(P_d). In such measurements, it is tacitly assumed that the surface area of the cell membranes across which transport is occurring has a constant relationship to the parameter of tissue mass utilized and, further, that this relationship does not change under different experimental conditions.

This relationship can be designated as S_m, so that J_d/S_m equals J and P_d/S_m equals P. Thus, for example, if a flux rate of 10 nmol/g tissue/sec has been experimentally determined (J_d), and if S_m is known to equal 100 cm^2/g tissue, then a flux rate of 0.1 nmol/cm^2/sec can be calculated. However, values for S_m are seldom known for experimental preparations, so most transport rates necessarily must be expressed as J_d. The corresponding passive permeability coefficients also must be normalized to the same parameter of tissue mass (P_d) and so will not have the conventional units of cm/sec.

Finally, it should be emphasized that P or P_d describes the ability of a particular solute to penetrate a particular biological membrane and has meaning independent of knowledge of C_2. In contrast, the magnitude of J or J_d has meaning only when one also knows the solute concentration at which the measurement was obtained.

The second type of transport important in the absorption of solutes involves the binding and membrane translocation of molecules by sites on the cell membrane. Because such a process involves the interaction of solute molecules with a finite number of transport sites on the cell membrane, the kinetics of uptake may be described by the following relationship.

$$J = \frac{(J^m)(C_2)}{K_m + C_2} \tag{2}$$

Here J^m is the maximal velocity of transport the system can achieve and K_m defines the concentration of the solute molecule at the aqueous-membrane interface (C_2) at which half the value of J^m is achieved. Again, both J and J^m should ideally be expressed as the amount of solute transported per unit time per cm^2 of surface area. However, as discussed above, this definition is not usually possible in most systems of importance to the study of intestinal transport; so these two velocity terms again must be normalized to some other parameter of tissue mass, and the terms J_d and J_d^m must be substituted for J and J^m, respectively, in equation 2.

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Monomolecular Diffusion of Molecules Through an Infinite Number of Sites on the Cell Membrane

Effect of Solute Interactions With Other Molecules in the Cytosolic Compartment and in the Bulk Phase on Monomolecular Diffusion Rates

As is evident from equation 1, the rate of molecular diffusion is determined essentially by only two factors—the concentration of the solute molecule in the perfusate bathing the cell membrane and the passive permeability coefficient for the molecule. Solutes may interact with other molecules in the bulk perfusate and/or cytosolic compartment and markedly alter their monomolecular concentrations in solution; these interactions profoundly affect the rate of transmembrane movement. As shown in panel I of figure 1, the rate of movement of the solute from the bulk solution into the cell equals the product $(C_2)(P_d)$. However, as the molecule diffuses into the cell, the concentration of the solute in the cell water begins to increase so that there is movement of the molecule out of the cell at a rate equal to $(C_3)(P_d)$,[2] as shown in panel II. Thus, at any point in time, the net flux of the solute into the cell is given by the following expression.

$$J_d^{net} = (C_2 - C_3)(P_d) \tag{3}$$

If the solute is not bound within the cytosol, metabolized, or transported out of the cell, then C_3 must eventually equal C_2 and J_d^{net} must equal 0. Using a radiolabeled molecule, one can still demonstrate the two unidirectional fluxes under this circumstance, even though net movement has ceased. If, however, the solute is rapidly bound to a receptor molecule in the cytosol, is metabolized, or is transported out, then C_3 is maintained lower than C_2 and there is a continuous net movement of the molecule to the cell at a rate described by equation 3.

Finally, it should also be pointed out that the direction of net movement can be reversed if the solute molecule is also being generated within the cell. For example, in the fed state, the concentration of fatty acid outside the adipocyte is higher than that inside so that there is net entry of lipid into the cell. With stimulation of the hormone-sensitive lipase within the cell, however, C_3

[2] In this formulation, the cell membrane is assumed to behave symmetrically with respect to passive permeability so that the same value of P can be utilized regardless of the direction of molecular diffusion. Most experimental data suggest that this assumption is correct.

greatly exceeds C_2, and there is net movement of fatty acid out of the cell.

Interactions of the solute with molecules in the bulk perfusate also can markedly influence the rate of both unidirectional and net solute movement. For example, in vivo steroid hormones are usually bound to carrier proteins in plasma; fatty acids are largely bound to albumin. In in vitro experiments, steroids and fatty acids are commonly added to the perfusate using various proteins, solvents, or detergents to increase their "solubility." In such cases, however, the term "solubility" is misleading: Although the total amount of the solute dispersed in the aqueous phase may be high, the actual amount of the solute in true solution and available for reaction with the membrane (C_2) may still be exceedingly low. Furthermore, the interactions between the solute and the carrier molecule are often complex and, if not taken into consideration, may interject marked artifacts into the interpretation of the kinetics of the uptake process. An example of this is shown in panel IV, where it is assumed that a solute molecule is "solubilized" in the bulk perfusate by using detergent-like bile acids that form micelles and that the ratio of the concentration of the solute in the micelle (C_m) and in the aqueous phase (C_2) can be defined in terms of a conventional partitioning coefficient (K). Under these conditions, the following relationship would be true.

$$KC_2 = C_m \tag{4}$$

This equation can be rewritten to yield

$$K\left(\frac{M_w}{V_w}\right) = \left(\frac{M_m}{V_m}\right) \tag{5}$$

where M_w and M_m are the masses of the solute molecule in the water and micellar phases, respectively, and V_w and V_m represent the volumes of the aqueous and micellar phases, respectively.[3] M_m equals the total mass of solute in the system (M_t) minus that in the water phase (M_w), so the expression $M_t - M_w$ can be substituted for M_m in equation 5 and, after rearranging terms, the following expression is obtained.

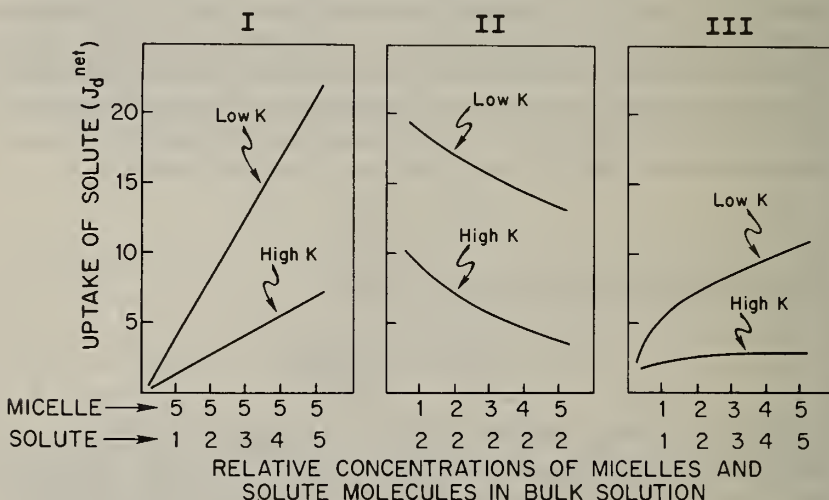
$$\frac{M_w}{V_w} = \frac{M_t}{KV_m + V_w} \tag{6}$$

[3]The volume of the micellar phase can be calculated from the concentration of detergent in the perfusate and its appropriate partial specific volume.

From this expression, the rate of uptake of the solute can be calculated because J_d^{net} is proportional to the term M_w/V_w; i.e., to C_2 (assuming for the purposes of this illustration that C_3 is 0).

The curves shown in figure 2 have been derived by this equation to illustrate the effect of altering the relative concentration of the detergent and solute on rates of intestinal uptake of the solute molecule. In panel I, the concentration of the detergent is kept constant while that of the solute is increased. As is evident, under this circumstance, J_d^{net} increases in a linear relationship to the total concentration of the solute in the perfusate, but the magnitude of the uptake rate is markedly dependent on K. When

Figure 2. Theoretical Relationship Between the Rate of Uptake of a Solute Molecule and the Concentration of That Molecule in the Perfusate



Here the solute is partially dissolved in a detergent micelle. Panel I illustrates the situation where the concentration of micelles is kept constant while the concentration of the solute is progressively increased. The opposite situation is illustrated in panel II: The concentration of solute is kept constant while the concentration of micelles is increased. Panel III illustrates the situation in which the concentration of both the micelles and solute is increased in parallel so that the molar ratio between these two components of the solution remains constant.

In each case, the results are shown for two experimental situations where the partitioning coefficient for the solute molecule into the micellar phase is either high or low. In this diagram, the units of solute uptake and concentration are arbitrary relative values. These illustrations, however, are based on specific calculations as given in reference (1).

the total concentration of the solute is kept constant and the concentration of the detergent is increased, the rate of uptake declines in a curvilinear fashion and, again, the absolute value of J_d^{net} is markedly influenced by K.

Of particular importance is the set of curves shown in panel III, where the concentrations of both the solute and the detergent have been increased in parallel so that the ratio between the two is constant. In this circumstance, the relationship between J_d^{net} and the total concentration of solute superficially resembles a "saturable" kinetic curve. Thus, the point to be emphasized is that when uptake rates of solutes are measured from a solution containing other molecules with which the solute can interact, the observed values of J_d may be determined as much by events within the bulk solution as by the kinetic characteristics of the transport system in the biological membrane under study. Under such circumstances, it is nearly impossible to interpret the meaning of relative rates of uptake of different solutes or of the kinetic characteristics of the uptake process unless appropriate mathematical or experimental corrections can be made to distinguish the true concentration of the molecule in solution (C_2) from the total concentration of the molecule (C_t) in the perfusate (1).

Effect of Membrane Polarity on Rates of Monomolecular Diffusion

The second major factor influencing the rate of molecular diffusion of lipids across cell membranes is the passive permeability coefficient for the solute (eq. 1). The value of P is unique for a given solute passing through a given membrane and is determined by the polar characteristics of both the solute and the membrane. Thus, for a particular solute molecule, P can be described by the following expression (2,3).

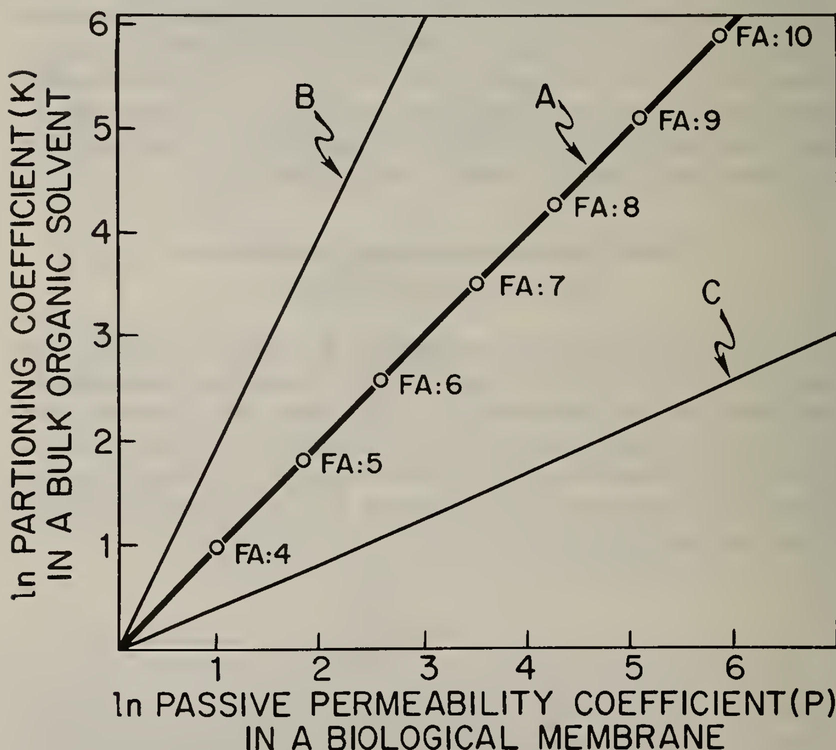
$$P = K_m \left(\frac{D_m}{d_m} \right) \tag{7}$$

Here K_m, D_m, and d_m represent, respectively, the partitioning coefficient for the solute molecule between the lipid phase of the cell membrane and the aqueous phase of the perfusate, the diffusion coefficient for the solute in the membrane, and the effective thickness of the membrane (2). The partitioning coefficient is the overwhelmingly important term in determining P because d_m is relatively constant for most biological membranes; for most

solutes, D_m varies over a narrow range and the values of K_m may vary over a range as great as 10^{10} (2,3). Thus, the values of P vary directly with the value of K_m for a particular cell membrane.

Recognition of this relationship has provided a useful way to assess and compare the "functional" polarity of membranes from different biological systems. For example, as illustrated in figure 3, the logarithms of the passive permeability coefficients for a series of fatty acids in a particular cell membrane have been plotted against the logarithms of the partitioning coefficients of these

Figure 3. Comparison of Passive Permeability Coefficients (P) for a Series of Fatty Acids With the Partitioning Coefficients of These Same Molecules Between Bulk Buffer Solution and a Bulk Organic Solvent



The logarithm of K is plotted in arbitrary units on the vertical axis; the logarithm of P is shown on the horizontal axis. Line A represents the situation in which the polarity of the bulk solvent and that of the membrane lipids are identical; line B represents the situation where the organic solvent is less polar; and line C represents the situation where the solvent is more polar.

same fatty acids into three different bulk organic solvents. As illustrated by line A, the addition of each $-CH_2-$ group to the solute has the same relative effect on increasing the movement of a molecule across the membrane or in increasing its partitioning into the bulk solvent; hence, the "effective" polarity of the solvent and cell membrane must be approximately the same.

In contrast, lines B and C, respectively, show the results obtained with two solvents that are either less or more polar than the cell membrane. For example, in the case of example B, the addition of each $-CH_2-$ group to the fatty acid chain has greater effect in two ways on increasing ln K than in increasing ln P. The $-CH_2-$ group principally undergoes hydrophobic interactions with components of the solvent and the cell membrane, so it follows that, in this case, the membrane behaves as a more polar structure than does the bulk solvent.

Comparisons of this type have been made between a number of different solvents and a variety of different cell membranes. In general, the membranes of most mammalian cells have been found to be relatively polar structures. Thus, in a number of instances, the membranes have been shown to behave in a manner similar to a bulk solvent such as isobutanol rather than as a very nonpolar solvent such as diethyl ether, benzene, or triglyceride (2,4,5,6).

Although they are useful, comparisons such as these are cumbersome to undertake and require that a number of different measurements of K and P be made using various homologous series of solute molecules. More recently, a second method commonly has been employed to describe the effective polarity of biological membranes. This method involves determining the manner in which the addition of a particular substituent group to any solute molecule alters the rate of movement of that solute across a biological membrane or alters the partitioning of the solute into a bulk organic solvent (1,2,3,6,7). In both instances, the following equation describes the relationship between the partitioning of a solute between a cell membrane (K_m) or a bulk solvent (K) and the aqueous phase of the perfusate and several thermodynamic parameters (2,3).

$$K_m \text{ or } K = e^{-\Delta F_{w \to 1}/RT} \tag{8}$$

Here $\Delta F_{w \to 1}$ is the free energy change associated with the movement of one mole of solute from the perfusate to the membrane (or solvent), R is the gas constant, and T is the absolute temperature.

It is difficult to obtain absolute values of $\Delta F_{w \to 1}$ for a solute, but the manner in which this thermodynamic parameter is changed

by the addition of a substituent group to the solute can be experimentally measured. Thus, the change in $\Delta F_{w\to 1}$, i.e., the incremental free energy change ($\delta\Delta F_{w\to 1}$), brought about by the addition of the substituent group s to the solute, is given by the following two expressions.

$$\delta\Delta F^{s}_{w\to 1} = -RT \ln\left(\frac{P^{s}}{P^{\circ}}\right) \quad (9)$$

$$\delta\Delta F^{s}_{w\to 1} = -RT \ln\left(\frac{K^{s}}{K^{\circ}}\right) \quad (10)$$

Equation 9 yields the incremental free energy change associated with the addition of group s to a solute, based on the measurement of the passive permeability coefficients for the solute with (P^{s}) and without (P°), the substituent group. Equation 10 gives the same value for substituent group s based on measurements of the effect of this group on partitioning of the solute into a bulk solvent.

In practice, $\delta\Delta F_{w\to 1}$ can be measured for nearly any substituent group, such as $-CH_2-$, -OH, $-NH_2$ and -COOH. The addition of a polar group, such as a hydroxyl function capable of hydrogen bonding with water molecules in the bulk perfusate, generally reduces the passive permeability coefficient or the partitioning coefficient obtained with a given solute. It therefore yields a positive value for $\delta\Delta F_{w\to 1}$.

In contrast, the addition of a nonpolar substituent group, such as the methylene group which is forced out of the aqueous phase by entropy effects and which undergoes hydrophobic interactions within the membrane or bulk solvent, generally increases the values of P and K and gives a negative value for the incremental free energy change.[4] At 37°C, the value of RT is approximately 616 cal/mol, so a nonpolar substituent group that increases P or K by a factor of 5-, 25-, or 125-fold is associated with a $\delta\Delta F_{w\to 1}$ value of approximately −1,000, −2,000 and −3,000 cal/mol, respectively. Conversely, a polar group that reduces P or K by a factor of 0.20, 0.04, or 0.008 would yield $\delta\Delta F_{w\to 1}$ values of +1,000, +2,000 and +3,000 cal/mol, respectively.

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[4]A detailed discussion of the intermolecular forces that provide the thermodynamic explanation for the effect of various substituent groups on permeability and partitioning coefficients is beyond the scope of this paper. For a more detailed discussion of these aspects, see references (2) and (11).

From this discussion, it is apparent that measurement of $\delta\Delta F_{w\to 1}$ values for various substituent groups provides a sensitive method for characterizing the effective polarity of a particular cell membrane and for comparing it to the membranes of other cell types and to various bulk solvents. Such data are now available for a number of different tissues and solvents; representative values for the hydroxyl and methylene groups are summarized in figure 4.

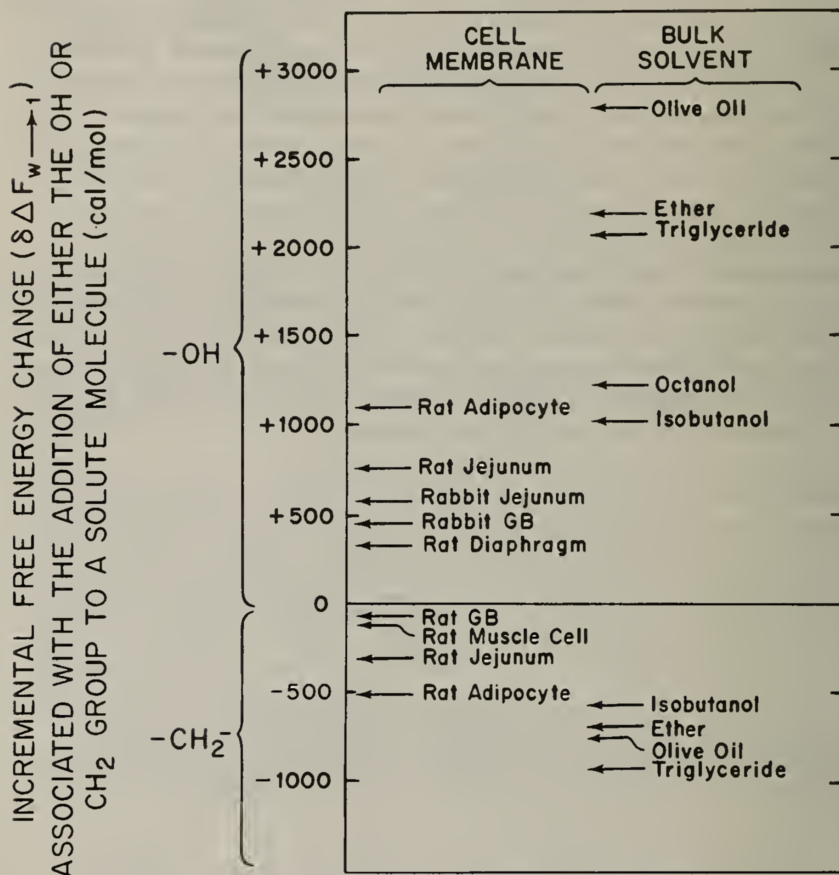
The addition of the hydroxyl function decreased the permeability coefficient for solute movement across the membranes of the muscle cell, adipocyte, gall bladder, and intestine by a factor that varied from 0.14 to 0.61. These values correspond to $\delta\Delta F^{OH}_{w\to 1}$ values of approximately +300 to +1,200 cal/mol. In contrast, this substituent group reduced partitioning of the solute into very nonpolar solvents such as triglyceride, ether, and olive oil by factors ranging from 0.035 to 0.011, yielding $\delta\Delta F^{OH}_{w\to 1}$ values varying from +2,070 to +2,800 cal/mol. These data indicate that the membranes of at least this group of cells behave as relatively polar structures (perhaps because they are relatively hydrated) and not as very hydrophobic "lipid" membranes.

This view is supported by the results obtained with the methylene group, also shown in figure 4. Again, the addition of this substituent group has much less of an effect in increasing the passive permeability coefficients ($\delta\Delta F^{CH_2}_{w\to 1}$ are all less than −550 cal/mol) than one would expect if the cell membranes behaved as a very nonpolar structure analogous to the nonpolar solvents. Thus, data obtained in a number of different laboratories for many different cell membranes suggest that these membranes (particularly the intestine) behave as relatively polar structures—at least with respect to the manner in which they affect the rate of transmembrane, monomolecular solute diffusion (2,4,5,6,7,8,9).

Dependency of Maximal Rates of Monomolecular Diffusion on the Polarity of the Cell Membrane

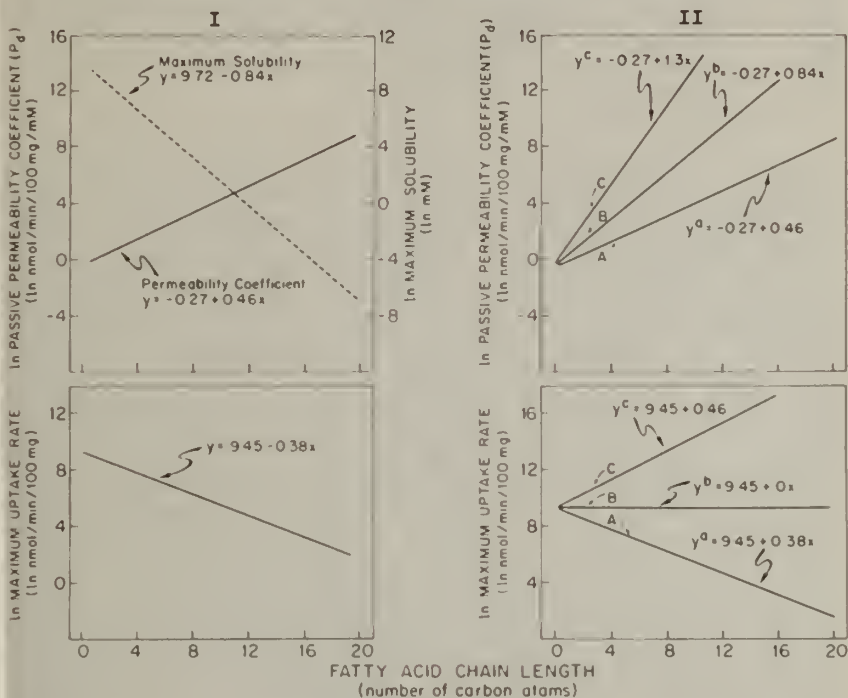
The recognition that intestinal cell membranes behave as relatively polar structures has profound implications with regard to the absorption of many solutes from the gastrointestinal tract, particularly those that have limited solubility, such as lipids and sterols. These implications can best be understood by examining a specific set of data taken from the literature (1); they are shown in figure 5. In this example, the passive permeability coefficients for the intestinal uptake of a homologous series of saturated fatty acids were experimentally determined. It was found that P increased

Figure 4. Incremental Free Energy Changes ($\delta\Delta F_{w\rightarrow 1}$) Associated With the Addition of Either the -OH or $-CH_2-$ Groups to a Solute Molecule



The addition of the hydroxyl function decreases the rate of membrane permeation and is associated with positive values; the addition of the methylene group enhances the rate of membrane permeation and is associated with negative values. The effect of the addition of these two substituent groups on the permeation of various probe molecules across the cell membranes of a variety of tissues is shown in the first column of data, and the effect of the addition of these same groups to the partitioning of various probe molecules into bulk organic solvents is shown in the second. The data presented in this figure are based on observations from a number of different laboratories, as summarized in reference (10).

Figure 5. Effect of the Relative Polarity of a Solute Molecule on Its Maximum Rate of Transmembrane Movement



The panels in column I represent actual experimental data obtained on the uptake of fatty acids of various chain lengths into the intestinal mucosal cell (1). The upper panel shows the logarithm of the passive permeability coefficients and the maximum aqueous solubilities of the homologous series of saturated fatty acids. The lower panel shows the logarithm of the maximum uptake rate, which equals the passive permeability coefficient times the maximum solubility for each individual fatty acid.

The two panels in column II illustrate the effect of altering the polarity of the biological membrane. Curves A, B, and C show the theoretical results obtained where the membrane is made progressively less polar so that the addition of each $\text{-}CH_2\text{-}$ group to the fatty acid chain increases the passive permeability coefficient by a factor of 1.58 (A), 2.32 (B), and 3.67 (C). These values would correspond to $\delta\Delta F_{w\rightarrow 1}$ values of −283, −517, and −801 cal/mol for the $\text{-}CH_2\text{-}$ group in these three respective situations. The results shown in this figure are based on experimental data in which the permeability coefficients and uptake rates were normalized to 100 mg dry weight of intestinal tissues.

by a factor of 1.58 for each $-CH_2-$ group added to the fatty acid chain. Thus, as shown by the solid line in the upper panel (panel I), the logarithm of P plotted against the fatty acid chain length has a slope of +0.46. In this same study, the maximum solubility of each of these fatty acids was determined in the perfusate and, as shown by the dashed line, maximum solubility decreased by a factor of 0.43 (slope of the semilogarithmic plot of −0.84) for each $-CH_2-$ group added to the fatty acid.

The rate of uptake of any of these fatty acids is equal to the product of the concentration of the fatty acid in the perfusate and its appropriate passive permeability coefficient (eq. 1), so it follows that the maximum rate of uptake must equal the product of the maximum solubility of each fatty acid in the perfusate times its passive permeability coefficient. When such values are calculated for each fatty acid (as seen in the lower solid line of panel I), the maximum rate of uptake decreases by a factor of 0.68 (slope of the semilogarithmic plot of −0.38) for each CH_2 group added to the fatty acid chain. Thus, for this homologous series of fatty acids, the highest rates of transport are seen with the more polar members of the series under circumstances where the concentration of each fatty acid in the perfusate has been elevated to its limit of solubility. This relationship derives from the fact that as $-CH_2-$ groups are added to the fatty acid chain, maximum solubility decreases out of proportion (slope of −0.84) to the increase in the passive permeability coefficient (slope of +0.46). This latter relationship, in turn, results from the cell membrane behaving as a relatively polar structure; in this instance, $\delta\Delta F_{w\rightarrow 1}^{CH_2}$ equals only −283 cal/mol.

The dependency of the maximal monomeric uptake rates on membrane polarity is illustrated by the set of curves shown in panel II. The membrane is made progressively less polar so that $\delta\Delta F_{w\rightarrow 1}^{CH_2}$ is increased from the experimentally determined value of −283 cal/mol (curve A) to −517 (curve B) and −801 (curve C) cal/mol. In curve B, the membrane has been made less polar to the extent that the addition of a $-CH_2-$ group results in an exactly equal incremental increase in P and decrement in solubility so that the maximum uptake rate becomes independent of chain length (curve B, lower panel II). Only when the membrane is made even less polar are higher rates of uptake observed with the fatty acids of longer chain length (curve C).

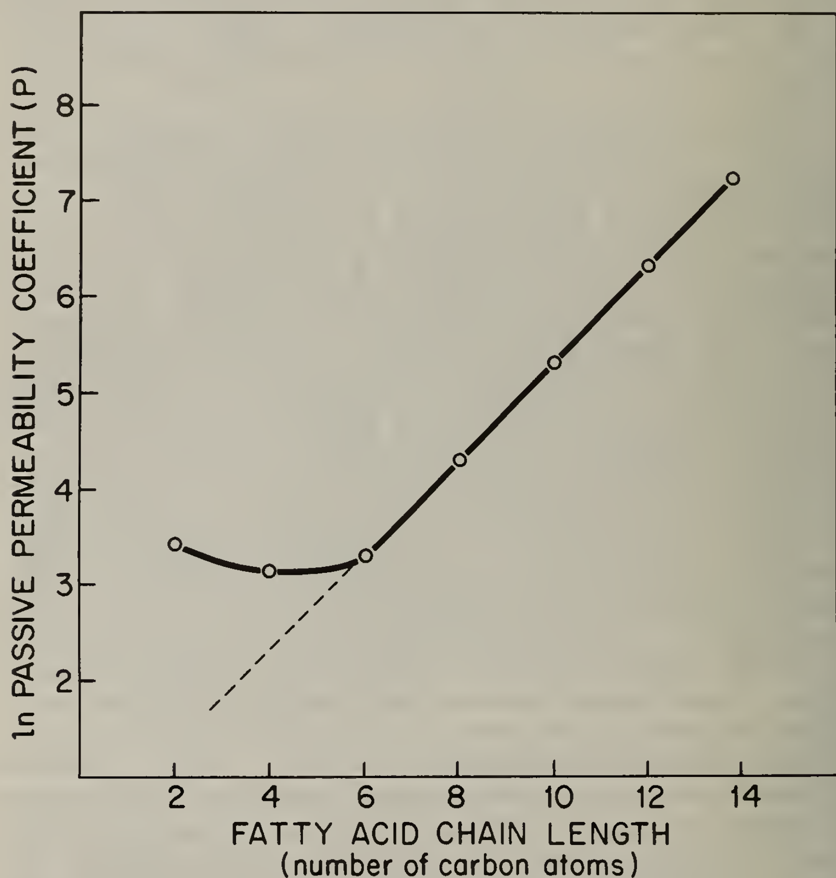
Anomalous Behavior of the Monomer Diffusion of Polar, Small Molecular Weight Solutes Across Biological Membranes

In figures 3 and 5, it has been assumed that the permeability coefficients for a homologous series of fatty acids follow a regular and predictable pattern based on the number of methylene groups in the fatty acid chain. In these instances, the addition of each $-CH_2-$ group increases P by a constant amount so that ln P is a linear function of the number of carbon atoms in the fatty acid.

A similar relationship can be seen for steroids where, for example, ln P varies as a linear function of the number of hydroxyl groups added to the sterol nucleus. Although such behavior has been described for the penetration of many different classes of solutes across biological membranes, it has also been consistently reported that, for any homologous series of molecules, the smaller molecular weight, more polar members of the series have anomalously high permeability coefficients. For example, researchers have reported data such as those shown in figure 6 in membranes of the intestine, gall bladder, and adipocyte (5,6,10). For the fatty acids with more than six carbon atoms in the chain, there is a linear relationship between ln P and chain length. This relationship does not hold, however, for the shorter chain length fatty acids, which typically manifest much higher passive permeability coefficients than would be extrapolated from the behavior of the higher molecular weight members of the series. Such anomalously high rates of monomolecular diffusion have been reported in nearly all biological membranes that have been studied for many types of small molecular weight molecules, including short chain length alcohols and fatty acids, urea, methylurea, formamide, and acetamide (2,4,5,6,8,9,10,11,12).

Such behavior has been attributed in the past to carrier-mediated diffusion or to aqueous "pores" within the cell membrane, but current data suggest that these high permeability coefficients are due to an inherent property of biological membranes that allows small molecules to pass relatively more rapidly between the structural components of the membranes than do larger molecular weight solutes (3). It should be emphasized that if the permeability coefficients for the saturated monohydroxy alcohols in the intestine were plotted in figure 6, they would form a second line—identical to that shown for the fatty acids—but displaced upward by an amount dictated by the $\delta\Delta F_{w-1}$ associated with the substitution of the -OH group on the molecule in place of the -COOH function. Thus, ethanol is one of those smaller molecular

Figure 6. Relationship of the Passive Permeability Coefficient to the Chain Lengths of Various Saturated Fatty Acids



This diagram illustrates that the shorter chain length, more polar members of this homologous series have higher passive permeability coefficients than would be expected from the linear extrapolation of the results obtained with those fatty acids containing six or more carbon atoms.

weight compounds associated with an anomalously high passive permeability coefficient.

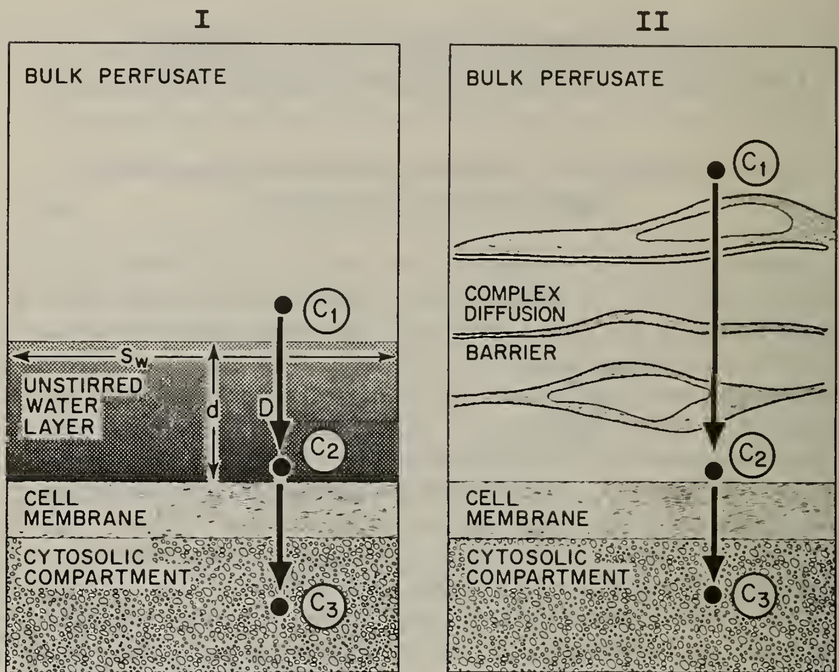
Effects of Diffusion Barriers on Rates of Movement of Lipids Across Biological Membranes

General Principles of Molecular Movement Across Diffusion Barriers in Biological Systems

Nearly all of the discussion thus far has been based on equation 1—the rate of diffusion of a solute across a membrane is determined by the concentration of the molecule in the perfusate and its passive permeability coefficient. This simple situation is probably never encountered in biological systems under either in vitro or in vivo conditions; the concentration of the solute molecule measurable in the bulk solution perfusing a particular tissue or cell preparation is usually not the same as the concentration of the solute molecule "seen" by the cell membrane. There is usually a diffusion barrier, be it simple or complex, interposed between the cell surface and the bulk perfusion medium.

The simplest situation is shown diagrammatically in panel I of figure 7 and involves the movement of a solute molecule from the bulk phase of the perfusate across a single cell membrane into the cytosolic compartment. However, interposed between the bulk perfusate and the membrane surface are layers of water that are not subject to the same gross mixing that takes place in the bulk perfusate and through which diffusion is the sole means for molecular movement (13). Obviously, there is no sharp demarcation between such "unstirred water layers" and the bulk solution of the perfusate; however, functional dimensions for these layers can be measured experimentally and such values are of critical importance in dealing with unstirred layer effects in any membrane transport system (6,14). Thus, in figure 7, C_1, C_2, and C_3 represent the concentrations of the solute in the bulk perfusate, at the aqueous-membrane interface, and just inside the cell membrane, respectively. S_w denotes the functional surface area of the unstirred water layer, d equals its functional thickness, and D is the diffusion coefficient for the specific solute. In this formulation, S_w is similar to the S_m term described earlier, but it represents the functional surface area of the unstirred water layer overlying a particular amount of tissue or cells. This term must be normalized to the same parameter of tissue mass utilized in the J_d term, so it

Figure 7. Major Parameters of Diffusion Barriers in Biological Systems



Panel I represents the simplified situation in which a solute molecule is moving from a bulk perfusate into the cytosolic compartment of a cell. In so doing, it must cross two diffusion barriers, the unstirred water layers outside the cell and the cell membrane itself. C_1, C_2, and C_3 represent the concentration of the solute molecule in the bulk perfusate, at the aqueous-membrane interface and in the cytosolic compartment, respectively. S_w and d represent the effective surface area and the effective thickness, respectively, of the unstirred water layer; D is the diffusion coefficient for the solute molecule.

Panel II represents the more complex situation, where the solute molecule must move from the bulk perfusate, e.g., serum within a capillary, to a target cell, e.g., an adipocyte, through a complex diffusion barrier made up of many different tissue spaces and cell membranes. In this situation, the values of S_w and d will be profoundly affected by the actual anatomical pathway the solute molecule must follow through the diffusion barrier.

commonly has units such as cm^2/g of tissue or cm^2/cm length of intestine.

In the situation shown in panel I, the net movement of solute from C_1 to C_3 is given by the following expression.

$$J_d^{net} = (C_1 - C_2)\left(\frac{S_w D}{d}\right) = (C_2 - C_3)P_d \qquad (11)$$

The first term in this equation describes the net rate of movement of the solute across the unstirred water layer, and the second term gives the net flux of the molecule across the cell membrane. In the steady state, these two flux rates must be equal, and C_2 may assume any value between the limits of C_1 and 0. This value can be calculated from the following expression.

$$C_2 = C_1 - \left(\frac{dJ_d^{net}}{S_w D}\right) \qquad (12)$$

The term $dJ_d^{net}/S_w D$ essentially represents the resistance encountered by the solute in crossing the unstirred water layer. The higher this resistance is, the lower the value of C_2. This resistance term is complex, however, and is determined by the physical dimensions of the unstirred water layer (d/S_w), by the diffusivity of the solute molecule in the aqueous phase (D), and by the net velocity of solute transport across the system (J_d^{net}).

In many physiological situations, both in vivo and in vitro, the diffusion barrier overlying a particular tissue essentially consists entirely of such unstirred water layers. This is probably the case, for example, in epithelial membranes such as intestine, gall bladder, choroid plexus, and bladder and when isolated cells are studied under in vitro conditions. Under both in vivo and in vitro conditions, the unstirred water layers overlying the surface of the intestine commonly vary in thickness from approximately 100 to 800 μM, depending on the rate of mixing of the bulk phase. It is seldom possible to reduce this thickness to less than 75 μM, even with the most vigorous mixing that can reasonably be employed under in vitro conditions (4,6,15). On the other hand, the thickness of the unstirred water layers surrounding individual cells suspended in an incubation medium is probably considerably less than 10 to 20 μM (10,16).

In many other tissues, the diffusion barriers are much more complex (panel II). For example, any solute that must move from capillary blood to a target tissue such as an adipocyte or muscle cell must necessarily pass through a complex series of cell membranes and aqueous spaces. The total resistance encountered during this diffusion process equals the sum of the resistances encountered in diffusing through each membrane in series, i.e., $(1/P_d)^1 + (1/P_d)^2 + (1/P_d)^3 + \ldots$, plus the sum of the resistances encountered in diffusing through each aqueous space, i.e., $(d/S_w D)^1 + (d/S_w D)^2 + (d/S_w D)^3 + \ldots$.

Furthermore, if the solute has limited solubility in the blood, then the rate at which it can be delivered to the capillaries of the tissue also may be limited; this limitation can be taken as yet another resistance to uptake of the solute by the target cells. This latter resistance term is a function of the reciprocal of the product of the volume of blood flow per unit of time to the target tissue (V) and the concentration of the solute in the bulk phase of the blood—i.e., $1/VC_1$[5] (17).

Thus, in many anatomically intact tissues, the rate of cellular uptake is profoundly influenced by the magnitude of the total resistance to molecular movement imposed by such complex diffusion barriers. This total resistance, it should be emphasized, is made up of such factors as the rate of blood flow to a particular organ, the solubility of the solute in blood, and the rate of diffusion of the molecule across a series of unstirred water layers and cell membranes.

Relative Importance of Membrane and Diffusion Barrier Resistances in Determining Rates of Lipid Movement Across Biological Membranes

It is apparent from equations 11 and 12 that two extreme situations may be encountered in various membrane systems. First, the rate of movement of the solute molecule across the diffusion barrier may be very rapid relative to its rate of movement across the cell membrane; i.e., the term S_wD/d may be very much larger than P_d. In this case, unstirred layer resistance is negligible and the rate of molecular penetration through the cell membrane becomes totally rate limiting to cellular uptake—as described by the following equation.

$$J_d^{net} = (C_1 - C_3)P_d \tag{13}$$

Second, the rate of movement of the solute molecule might be very much faster through the cell membrane than across the unstirred water layer; i.e., if P_d is very much larger than the term S_wD/d. In this case, the value of the term dJ_d^{net}/S_wD in equation 12 essentially equals the value of C_1, and the value of C_2, therefore, approaches 0. In this case, the rate of solute movement

[5]This term is actually much more complex than stated here; see reference 17 for a fuller explanation.

across the diffusion barrier becomes wholly rate limiting to cellular uptake; it is described by the following equation.

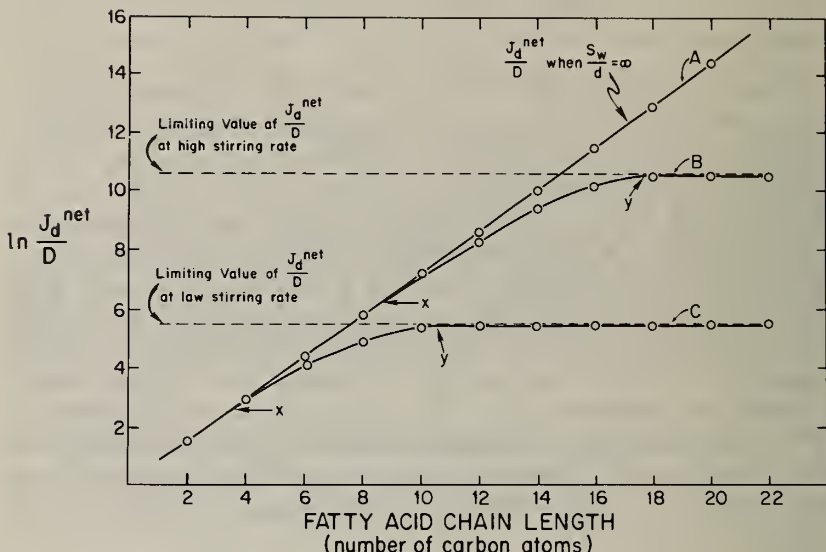
$$J_d^{net} = (C_1 - C_3)\left(\frac{S_w D}{d}\right) \qquad (14)$$

These two extreme situations, as well as the intermediate condition where both the unstirred water layer and membrane resistances influence uptake rates, are shown in figure 8. In this diagram, the logarithm of the value of J_d^{net}/D has been plotted as a function of the fatty acid chain length at several different values for unstirred layer resistance. In these examples, it is assumed that the concentration gradient between C_1 and C_3 is the same for each fatty acid. Curve A represents the extreme case (equation 13), where S_w/d is infinitely great and unstirred layer resistance is, therefore, negligible. In this situation, J_d^{net} is determined by the passive permeability coefficient for each fatty acid so that the term ln J_d^{net}/D increases as an essentially linear function of the fatty acid chain length. However, there is significant deviation from this behavior as the diffusion barrier begins to exert a finite resistance.

In the first example, curve B, fatty acids with two to eight carbon atoms have such low passive permeability coefficients that membrane permeation is still totally rate limiting, and the value of ln J_d^{net}/D still falls on the linear portion of the curve (the segment of line B to the left of point x). In contrast, the passive permeability coefficients for the longer chain length fatty acids with 18, 20, and 22 carbon atoms are so high that uptake becomes totally diffusion limited; these were described in equation 14. In this case, the term J_d^{net}/D reaches a constant and limiting value dictated by S_w/d (the portion of curve B to the right of point y). The portion of curve B between points x and y delineates those fatty acids where the unstirred water layer and membrane both contribute in determining the rate of cellular fatty acid uptake.

When an unstirred water layer of even greater resistance is introduced in front of the membrane, as shown by curve C, the diffusion barrier becomes totally rate limiting to cellular uptake for all fatty acids with more than 10 carbon atoms. The important principle illustrated by figure 8 is that the higher the passive permeability coefficient for a particular solute molecule, the more likely that diffusion barriers in the intestine, rather than those in the cell membrane, will be rate limiting to monomolecular uptake.

Figure 8. Effect of Diffusion Barriers of Varying Resistance on the Cellular Uptake of Fatty Acids of Different Chain Lengths



In this example, it is assumed that the concentration gradient across the cell membrane is the same for each fatty acid. Under these conditions, the rate of net uptake, J_d^{net}, will equal the product of the passive permeability coefficient of each fatty acid times its concentration gradient. If no diffusion barrier is present outside of the cell membrane, then the rate of uptake will essentially be a linear function of the fatty acid chain length (curve A).

Curve C represents the theoretical findings that would be observed when the bulk solution was stirred at a very low rate so that the diffusion barrier was relatively thick; curve B represents the results anticipated at a higher rate of stirring. For the latter two curves, point x illustrates the point at which the diffusion barrier begins to exert significant resistance and uptake rates begin to deviate from the linear relationship illustrated by curve A.

Point y on these curves represents the point at which diffusion of fatty acids across the unstirred water layer becomes totally rate limiting to cellular uptake so that J_d^{net} is proportional to D and the quantity J_d^{net}/D becomes constant. These theoretical curves are based on actual experimental data derived in several types of epithelial tissues and reported in references 4, 5, and 6.

Effect of Diffusion Barriers on Measurement of Activation Energies

This recognition—that either the cell membrane or the diffusion barrier outside of the cell may be rate limiting to the uptake of lipids in a particular tissue—has important implications with

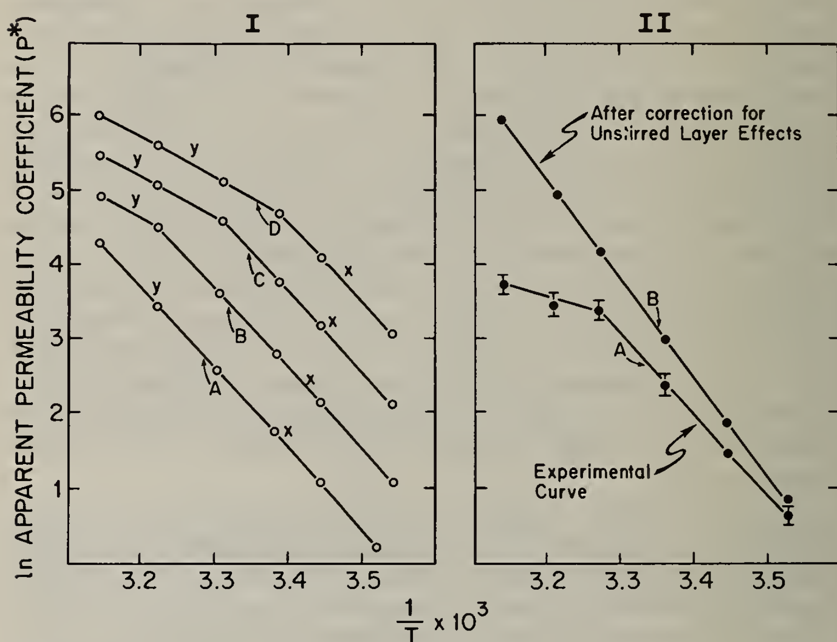
respect to the interpretation of temperature effects on the transmembrane movement of solutes. In the past, the passive monomolecular diffusion of a solute across a biological membrane has been said to have low Q_{10} values and correspondingly low activation energies. Furthermore, in some instances, an abrupt change in apparent activation energy has been found when the temperature is lowered. This effect has been attributed to a temperature-related phase change in the lipid molecules making up the structure of the cell membrane. Such behavior is illustrated by the "experimental curve" in panel II of figure 9. At the higher rate of solute uptake of approximately 1.2 for each 10°C change in temperature (an activation energy of only about 2,800 cal/mol) the following occurs: As the temperature is lowered, however, a "transition" point is apparently reached, below which the line acquires a steeper slope. It may correspond to a Q_{10} value, varying from 2.0 to 4.0, and to activation energies varying from approximately 10,700 to 21,000 cal/mol. However, when such data are corrected for unstirred layer effects (curve B), the "transition" point disappears and a single linear regression curve is produced; it has a steep slope corresponding to a high activation energy for the passive penetration of this solute across the cell membrane (18).

Data such as these suggest that, in many instances, both the low activation energies and the apparent transition points reported for passive solute uptake across biological membranes are artifacts caused by researchers' failure to recognize that the uptake of the solute is diffusion limited at physiological temperatures.

At higher temperatures, the low Q_{10} value simply reflects the low activation energy for the diffusion of the solute through the aqueous environment of the unstirred water layer. As the temperature is decreased, a point is reached at which penetration through the cell membrane, rather than through the diffusion barrier, becomes rate limiting, and the apparent activation energy abruptly increases. Thus the "transition" point shown in panel II (figure 9) actually corresponds to the point where the major resistance to molecular uptake of the solute shifts from the unstirred water layer to the cell membrane (18).

The resistance encountered by a solute in crossing the diffusion barrier is also a function of the passive permeability coefficient of that molecule (and, hence, the J_d^{net} term in equation 12), so it follows that the apparent "transition" temperature seen in a given membrane should vary inversely with the P value for a series of solute molecules. Such a situation is illustrated by the series of curves shown in panel I of figure 9. Curve A represents the

Figure 9. Effect of Diffusion Barriers on Apparent "Transition Temperatures" and on Activation Energies for Solute Molecules With Varying Permeability Coefficients



The logarithm of the apparent permeability coefficient (P^*) has been plotted against the reciprocal of the absolute temperature. Panel I shows the results that might be obtained with four different solute molecules that penetrate a biological membrane at different rates. Curve A represents a molecule having the lowest passive permeability coefficient; curve D represents a molecule with a much higher P value.

In this diagram, the segment of each curve labeled "x" has a relatively steep slope and, therefore, yields a high value for the activation energy; segments labeled "y" have lower slopes and correspondingly lower values for activation energies.

In panel II, curve A illustrates the results obtained when experimental data on uptake are plotted; curve B shows the results obtained after correction of the experimental data for unstirred layer effects. These theoretical relationships are based on actual experimental data such as those reported in reference (18).

situation encountered with a compound having such a low passive permeability coefficient that the cell membrane is rate limiting to uptake at all temperatures.

As solutes with progressively higher P values are tested, an apparent transition point is seen (the change in slope between the line segments labeled x and y). This apparent "transition" temperature is not constant, however, but occurs at a progressively lower temperature for each more permeant solute tested. In each case, correction for unstirred water layer resistance would eliminate this transition point and yield curves reflecting the true activation energies for the passive penetration of these solutes through this particular biological membrane (18).

These data illustrate two important generalizations concerning the effect of diffusion barriers on the determination of activation energies. First, it now seems likely that the passive penetration of biological membranes by various lipid molecules is very temperature dependent and manifests high activation energies. It has been reported, for example, that the activation energy for such uptake increases by 2,500 to 3,600 cal/mol for each $-CH_2-$ group added to a solute molecule (18). Low activation energies previously reported for such processes probably reflect failure to make appropriate corrections for unstirred layer effects. Second, it is also likely that some previous reports, suggesting that temperature-dependent changes in the activation energy for passive uptake reflect phase transitions in the lipid structure of the cell membrane, are also in error and result from failure to make these corrections.

Effect of Diffusion Barriers on Kinetics of Transport of Lipids by a Finite Number of Membrane Sites

Thus far in this section, the influence of diffusion barriers on the passive monomolecular uptake of solutes into cells has been examined. However, such barriers also have profound effects on the kinetic characteristics of the second type of transport, which depends on the interaction of a solute with a finite number of receptor sites on the cell membrane. In the simplest case, the kinetics of such a transport system are described by equation 2—the rate of uptake (J_d) bears a hyperbolic relationship to the concentration of the solute molecule present at the aqueous-membrane interface (C_2). The kinetics of a particular transport system are described in terms of the maximal transport rate that can be achieved (J_d^m) and the concentration of the solute molecule necessary to reach half of this limiting rate (K_m). This equation, it

should be emphasized, is written in terms of C_2, the concentration of the solute molecule "seen" by the membrane. In the presence of a diffusion barrier, however, this equation must be rewritten in terms of C_1, i.e., the concentration of the molecule present in the bulk perfusate. C_2 equals C_1 minus the term dJ_d/S_wD, so this expression can be substituted for C_2 in equation 2 to give the following expression.

$$J_d = \frac{J^m\left(C_1 - \frac{dJ_d}{S_wD}\right)}{K_m + \left(C_1 - \frac{dJ_d}{S_wD}\right)} \tag{15}$$

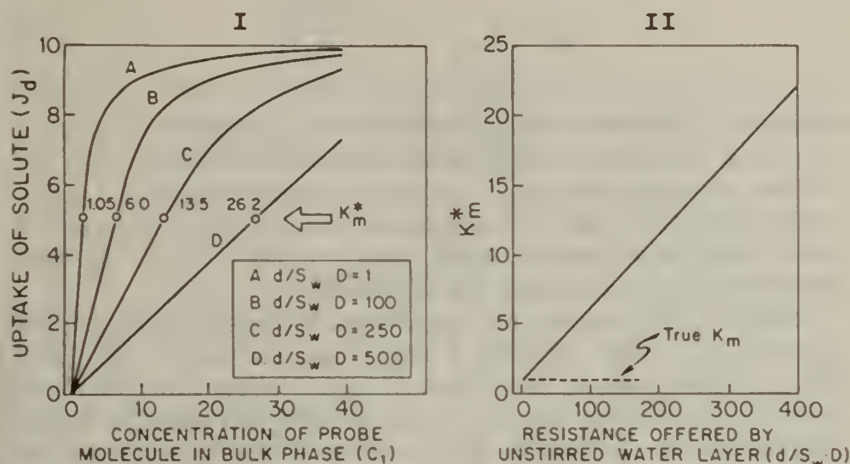
When solved for J_d, this equation yields the following quadratic expression (19).

$$J_d = (0.5)(D)\left(\frac{S_w}{d}\right)\left[C_1 + K_m + \frac{dJ_d^m}{S_wD} - \sqrt{\left(C_1 + K_m + \frac{dJ_d^m}{S_wD}\right)^2 - 4C_1\left(\frac{dJ_d^m}{S_wD}\right)}\right] \tag{16}$$

Thus, under most physiological circumstances (where the membrane transport sites are separated from the bulk perfusate by a diffusion barrier), this equation describes the relationship between the rate of uptake and the concentration of the solute (or lipoprotein) in the bulk perfusing medium. It should be emphasized, therefore, that the value of J_d is influenced by the resistance of the diffusion barrier, given by the expression dJ_d^m/S_wD, as well as by the values of the K_m and J_d^m terms.

This formulation has four important consequences. First, in the presence of a significant diffusion barrier, resistance J_d becomes essentially a linear function of C_1 and the "saturable" appearance of the kinetic curve is lost. This effect is illustrated by the series of curves shown in panel I of figure 10, which were derived from equation 16. In this example, it is apparent that when the resistance term, i.e., dJ_d^m/S_wD, is low (as in the case of curve A), the rate of uptake exhibits saturation kinetics with respect to C_1. However, when the resistance term is increased 500-fold (as in the case with curve D), J_d increases in essentially a

Figure 10. Effect of Diffusion Barriers of Varying Resistance on the Kinetics of Transport Through a Finite Number of Sites on a Biological Membrane



In panel I the rate of solute uptake is plotted in arbitrary units on the vertical axis, and the concentration of the solute molecule in the bulk perfusate (C_1) is plotted in arbitrary units on the horizontal axis. In this illustration, it is assumed that the true K_m value for the transport process equals 1.00. This figure shows the effect of increasing the resistance of the unstirred water layers overlying the transport sites 500-fold on the apparent K_m values (K_m^*).

In panel II, the apparent K_m values are plotted against the resistance of the unstirred water layers as given by the quantity d/S_w·D. These theoretical curves are based on values for the various parameters of transport likely to be encountered in biological systems and are given in detail in reference (19).

linear fashion with respect to the concentration of the solute molecule in the bulk solution. Thus, in the presence of a major diffusion barrier, such linear kinetics are to be anticipated and should not be construed as evidence against the possibility that the uptake process involves translocation by a finite number of transport sites.

Second, the presence of a significant diffusion barrier leads to gross overestimation of the true K_m value for the transport process. This effect is also shown diagrammatically in panel I of figure 10, where the true K_m value for the system is assumed to equal 1.0 concentration units. As is apparent, as the resistance of the diffusion barrier is increased over a 500-fold range, the apparent K_m value (K_m^*) increases from 1.0 to 26.2 concentration units. In fact, as shown in panel II, under these circumstances the apparent K_m value increases linearly with the resistance of

the overlying diffusion barrier, as given by the following equation (19, 20).

$$K_m^* = K_m + 0.5 \left(\frac{dJ_d^m}{S_w D} \right) \tag{17}$$

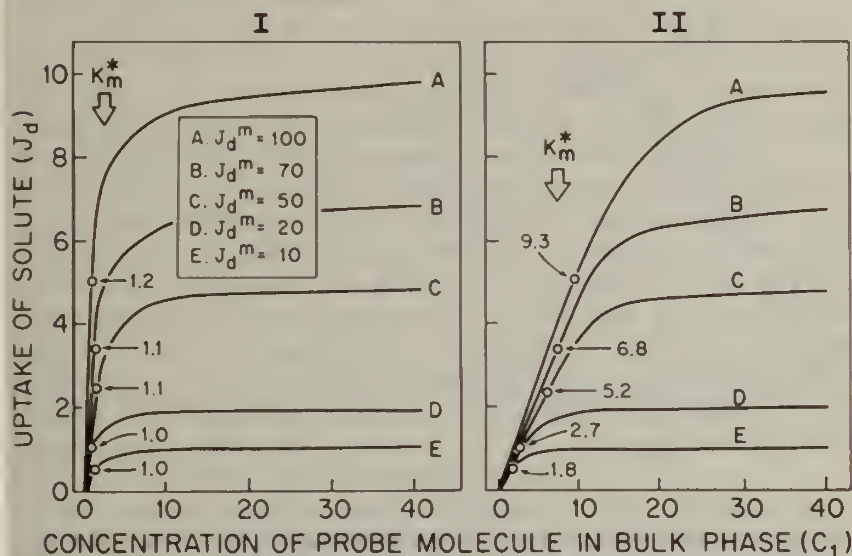
Stated in a different way, if no diffusion barrier were present, then this transport system would achieve 80 percent of the maximal transport rate (J_d^m) at a solute concentration of 4 units. In the presence of the high resistance barrier (curve D), the solute would have to be raised to 45 concentration units in the bulk perfusate to attain the same rate of transport.

Third, in the presence of a significant diffusion resistance, the apparent K_m value becomes a dependent variable of J_d^m. This effect is illustrated by the series of curves shown in panel I of figure 11, where the true K_m value is again set equal to 1 concentration unit. As seen in panel I, under circumstances where the diffusion barrier resistance is low, increasing the value of J_d^m 10-fold has only a minimal effect in increasing the apparent K_m value to 1.2 concentration units. However, a similar increase in J_d^m under circumstances where the diffusion barrier resistance is increased 50-fold (panel II) results in an increase of K_m^* to 9.3 concentration units. Thus, because the J_d^m term enters into the total resistance term in equations 16 and 17, K_m varies directly with J_d^m.

Fourth, the presence of a diffusion barrier will lead to overestimation of maximal transport rates (J_d^m) if these values are estimated from double reciprocal plots. As shown in figure 12, in the absence of a diffusion barrier, the relationship between J_d and C_1 is described by equation 2 and takes the form of curve A in panel I. When replotted in the double reciprocal form (panel II), such a curve becomes linear and has an intercept on the vertical axis that equals $1/J_d^m$. However, when a diffusion barrier is present over the transport sites, then equation 16 describes the relationship between J_d and C_1 and yields a curve such as example B in panel I.

Equation 16 does not take the form of a rectangular hyperbola, so plotting these data in the double reciprocal form does not transform curve B into a straight line: Rather, as seen in panel II, the curve turns sharply upward as it approaches the vertical axis to intercept at $1/J_d^m$. However, if, as is commonly done, the experimental points are used to construct a linear regression curve and this curve is extrapolated to the vertical axis (dashed line),

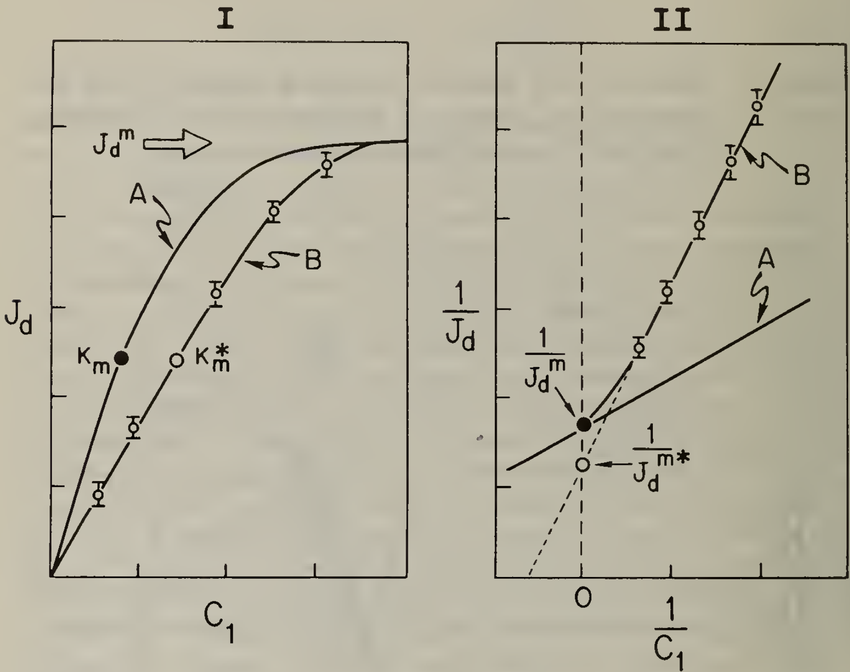
Figure 11. Effect of the Maximal Transport Rates (J_d^m) on the Apparent K_m Values in the Presence of a Diffusion Barrier of Low and High Resistance



As in figure 10, the true K_m value for the transport system is assumed to equal 1.00. As seen in panel I, under circumstances where the diffusion barrier over the transport sites exerts only a very low resistance, increasing the maximal transport rate 10-fold has only a minimal effect in increasing the apparent K_m value from 1.0 to 1.2.

However, as illustrated in panel 2, if the resistance of the diffusion barrier overlying the transport sites is increased by 50-fold, then K_m^* increases over 9-fold under circumstances where J_d^m is increased 10-fold. Thus, in the presence of a significant diffusion barrier, the apparent K_m value for the transport system has become a dependent variable of J_d^m.

Figure 12. Effect of Diffusion Barriers on Determination of Maximum Transport Velocities (J_d^m) by Use of Double Reciprocal Plots



Panel I shows two kinetic curves for the uptake of a solute molecule in the absence (A) and in the presence (B) of a significant diffusion barrier. In the presence of the diffusion barrier, the value of K_m is shifted to the right, but both curves achieve the same value of J_d^m. These same two curves are replotted in panel II as the reciprocal of these two variables. It is apparent that the linear extrapolation of the data points in curve B gives a value for J_d^m that is much higher than the true maximal transport rate, i.e., $1/J_d^{m*}$ is artefactually lower than $1/J_d^m$.

then an artefactually high value for J_d^m will be obtained. Thus, if it is experimentally difficult to directly measure the maximal transport rate for a particular transport system (because, for example, of limited solubility of the solute), then estimation of this value from double reciprocal plots will lead to an artefactually high value for J_d^m if a diffusion barrier is interposed between the bulk solution and the transport sites (20,21).

The Role of Carrier Molecules in Overcoming Diffusion Barrier Resistance

Based on these considerations, it is likely that the uptake of many lipids is limited by their diffusion across the barriers overlying the intestinal mucosa; the bile acid micelle functions to overcome this resistance. As an example of the magnitude of the effect of having the micelle present, one can calculate the rate of fatty acid absorption that takes place both in the absence and in the presence of the detergent.

In the case of stearic acid, the maximal rate of uptake that can be achieved without bile acid can be calculated from equation 14 to equal 1.35 nmol/min/100 mg tissue (assuming that the maximum solubility of the fatty acid in solution equals 4.37 μM, that S_w equals 11.7 cm^2/100 mg tissue, and that d is 137 μM (1).

In the presence of a bile acid micelle, a much higher total concentration of fatty acid can be achieved in the bulk solution, and a large mass of this solute, dissolved in the micelle, diffuses up to the aqueous-membrane interface. If this results in an aqueous concentration of the fatty acid in equilibrium with the micelle (C_2) of 4.37 μM, then the rate of uptake in this instance can be calculated from equation 1 to equal 12.8 nmol/min/100 mg tissue (using a P value for stearic acid of 2,930 nmol/min/100 mg tissue/mM). Thus, the presence of the bile acid has facilitated uptake of the fatty acid by a factor of 9.5.

Because the magnitude of the diffusion barrier resistance varies directly with the passive permeability coefficient for a particular solute (equation 12), it follows that the relative effect of a bile acid micelle in facilitating lipid absorption in the gut should increase with increasing hydrophobocity of the particular lipid under study. Such an effect has been demonstrated; for example, the presence of the detergent enhances intestinal uptake of the fatty acids with 8, 12, 16, and 20 carbon atoms by factors of 1.05, 1.48, 3.53, and 25.8, respectively, and of the still more hydrophobic cholesterol molecule by a factor of approximately 145.

Mechanisms by Which Alcohol Ingestion Could Affect Intestinal Absorption

It is apparent that the chronic ingestion of alcohol could theoretically alter the rate of uptake of a variety of different solutes through many different mechanisms. In the case of passively absorbed molecules, for example, alcohol might either

enhance or decrease the uptake of a particular solute by decreasing or increasing the unstirred layer resistance present over the intestinal villi. Such an effect could be mediated through an alteration of either intestinal or villus motility. Alternatively, ethanol conceivably could alter the permeability coefficients for a variety of solutes by altering the effective polarity of the microvillus membrane. This type of alteration would be manifest by a change in the incremental free energies associated with various substituent groups. Such a change, it should be emphasized, might be associated with enhanced absorption of some compounds and reduced absorption of others, depending on their relative polarities and, hence, their partitioning coefficients for distribution between the aqueous phase of the perfusate and the cell membrane.

Similarly, ethanol could also have profound effects on the rates of absorption of actively transported solutes. Again, these alterations could be mediated through a direct effect on the transport system so that either J_d^m or K_m are changed, or they could be the result of alterations in unstirred water layer resistance. If, for example, chronic ethanol intake decreased intestinal motility which, in turn, resulted in an increase in the effective value of d/S_w, then the uptake of a variety of solute molecules by carrier-mediated mechanisms could be significantly altered. The magnitude of these alterations, however, would be different for different molecules and would depend on the relationship between the concentration of the solute molecule in the bulk intestinal contents (C_1), the K_m value for the active transport process, and the value of the term dJ_d^m/S_wD.

Although little work of this type has been done thus far, it is now apparent that there is sufficient information available—on both the theoretical and technical aspects of this problem—to carry out appropriate experiments to precisely identify the effect of acute and chronic ethanol intake on the intestinal absorption of many different types of nutrients.

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Discussion of Paper by Dietschy

Dr. Lester: One peculiarity in your data concerning polarity of membranes was that even liposomes appeared to be less polar than one might have guessed that they would be. I can understand how membranes might be rather more polar than what one would anticipate, because of the presence of polar proteins or other things, but I notice that liposomes were down on your list. How do you explain that, since their only constituent is phospholipid, and since I assume that the thing that determined their permeability characteristics was the fatty acid that essentially hooked onto the phospholipid?

Dr. Dietschy: I think that is true, and, in general, those data support the fact that the major determinant of cell membrane permeation is, in fact, simply its cholesterol, phospholipid, and interactions with water, that whole sum total. Those data come from work by Diamond et al. Clearly, the phospholipid liposome, and therefore a model of the cell membrane, if you like, is just as polar as most cell membranes. I presume that the sum total of the polarity of the membrane is determined by the interaction of the polar head groups, as well as by the hydrocarbon chains.

Dr. Lester: This is not true.

Dr. Dietschy: Well, that may or may not be the case. This is the sum total of the net polarity of all of those groups interacting, and all I can say is that maybe water molecules get interdigitated and, in fact, behave as a more hydrated structure. Those are the results. Jerrod has gone into the thermodynamic analysis of this in great detail and essentially says what I have said—that there is a very polar region on the outside, a very nonpolar region on the inside.

Dr. Schenker: To bring us back to alcoholism, again, I am aware of two ways in which alcohol can alter transport. One is the mechanism that I think Dr. Lieber's group has shown and others including our own, which is damage to the mucosa and maybe changes in permeability. The other is the postulated effect on active transport of some amino acids. This occurs, especially at higher concentrations.

If the primary location of sodium potassium ATPase is in the basolateral membrane, and if the carrier, protein if you will, the

carrier mechanism is in the brush border, and if there has to be a coupling between the ATPase in the basolateral membrane and the carrier in the brush border, then how do you visualize the interrelationship between the carrier and the energy source? How do you view this?

Dr. Dietschy: The technical problems in measuring valid permeability coefficients and in measuring valid rate constants for active transport are only now becoming apparent. So first of all, I would have to say that if you look back at much active transport data or much "passive permeability" data, they are invalid, because corrections were never made for unstirred layer effects. I don't mean Charles Lieber specifically, I am just saying in general.

What happens to the passive permeability coefficient if you expose a membrane to alcohol? That could be now measured. But I do not know if it has been measured. The active transport kinetics are profoundly influenced by unstirred layers, and the corrections here are much harder to make mathematically. So I am not really quite sure we understand what the effects of any particular manipulation, like the administration of alcohol, are on the true kinetics of the membrane. If the alcohol affects motility, motility affects K_m and V_{max} values, through an effect on unstirred layers. It is true in enzymes. Much of the enzymology is suspect if you fail to correct for unstirred layer effects getting up to the active sites. Same thing with transport.

Now the final question: First of all, I am not convinced that ATPase and active transport have anything to do with one another. The data are very indirect if you look at them. If you get some profound effect on transport, you may measure a 20-percent change in sodium potassium-dependent ATPase; it is all indirect coupling evidence. So first of all, I am very suspicious that the two do not have anything to do with one another; at least I remain to be convinced. Second, again we get into the technical problem of where you have manipulated the system, either in vivo or in vitro, and you have an apparent change in the kinetics. Those kinetics are suspect because none of the corrections has been made. At this point, I do not know how to answer your question. I am saying I do not think there are any valid measurements yet upon which to make a decision about these things. But that is my own personal bias.

Dr. Halsted: One of the theories for the saturability of folic acid transport holds that the polarity of folic acid is altered as it goes through the unstirred layer because of pH changes in that layer. I do not recall you saying anything about the importance of pH.

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Dr. Dietschy: Well, if one has a molecule which can either be ionized or not ionized, as a weak acid or a weak base, then in the past the teaching was that the ionized form of a molecule does not penetrate membranes. Well, that is clearly wrong. It was based on the wrong assumptions to begin with and failure again to make the appropriate corrections. I think they do.

The charge does affect the degree of penetration, because a charge determines how much hydrogen bonding you have in the water. So any time you make a molecule charged to get a lower penetration rate, it has a finite passive permeability. That is quite clear from a variety of experimental work. If you get bulk phase pH changes and therefore bulk phase changes in the distribution between a non-ionized and an ionized species, that has profound influence on the rates of penetration of the two species together.

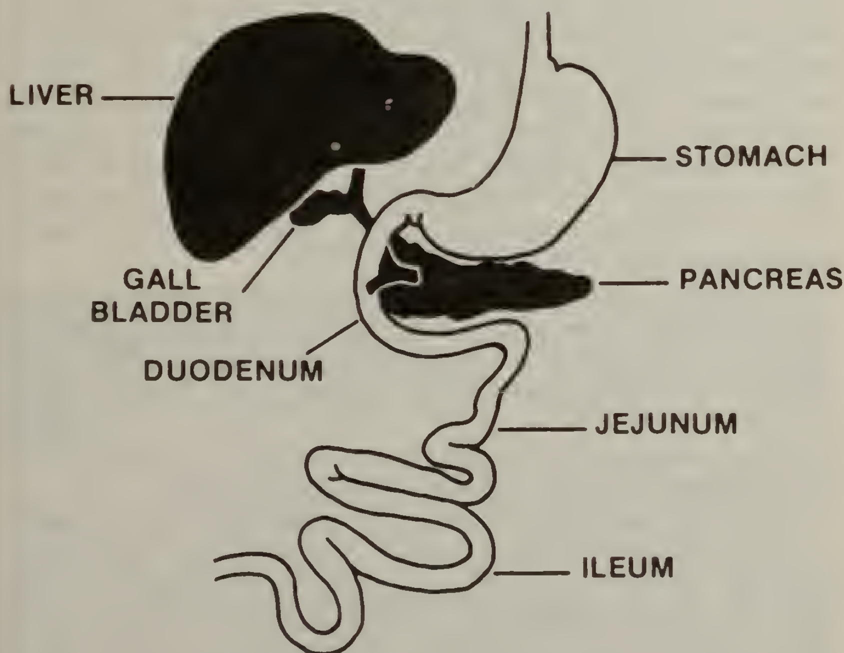
Now the question I think you are asking is, could a pH gradient develop within an unstirred layer, and that as a group of molecules move into that region, is there a shift in the distribution that is dictated by an unrecognized shift in the pH gradient? I think that clearly does occur, in a sense, but we do not quite know how to measure that pH gradient. I do not think it can be very big.

Effect of Ethanol on the Determinants of Intestinal Transport

Linda L. Shanbour

Any discussion of intestinal transport, at least from a physiological viewpoint, should take into consideration influences from other gastrointestinal tissues. Figure 1 illustrates the interplay between the stomach, liver, pancreas, and small intestine. Alterations in gastric acid secretion may influence intestinal transport by altering the pH of the environment for the intestinal enzymes involved in the breakdown of foodstuff and thus altering presentation of material for transport, as well as, possibly, by affecting the transport process itself. Indirectly, alterations in gastric acid secretion

Figure 1. Interactions of Various Tissues of the Gastrointestinal System



may influence hormonal release mechanisms, such as gastrin from the antral mucosa and secretin and glucagon from the small intestine. These hormones may then influence the pancreas and liver—secretin inducing pancreatic water and bicarbonate secretion, and glucagon stimulating gluconeogenesis in the liver. There are obviously many other actions, but these are the major focus of this paper.

Stomach

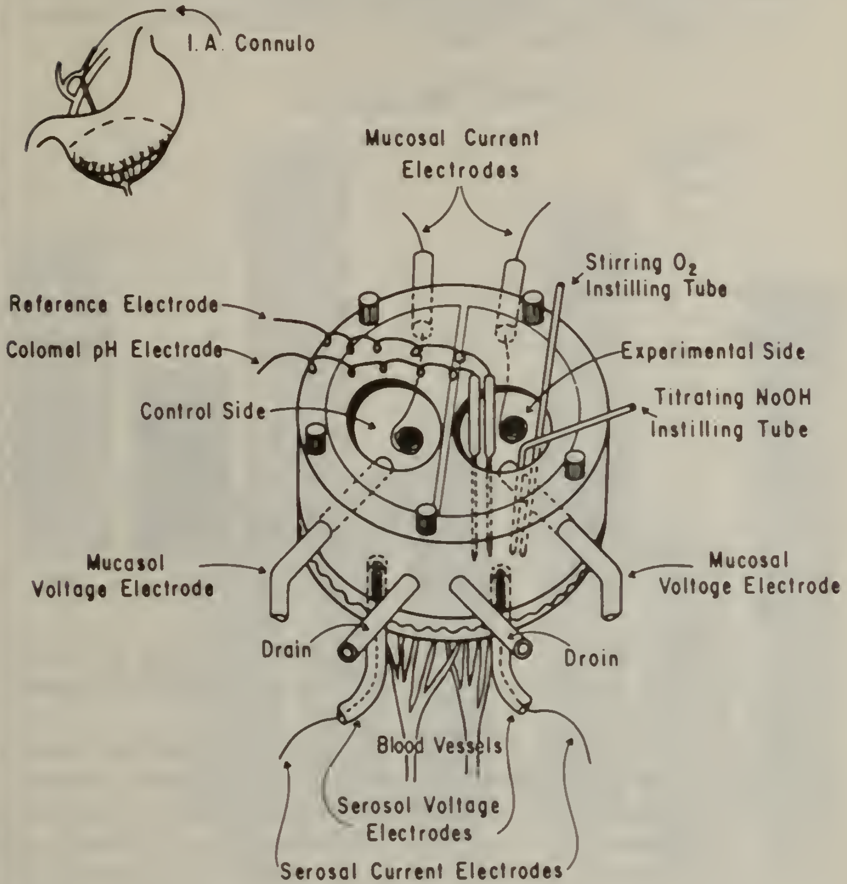
The stomach, which receives high concentrations of alcohol as compared to most other regions of the body, has received relatively little attention in terms of basic mechanisms which may be altered by ethanol. For many years, it has been assumed that ethanol stimulates gastric acid secretion. However, in most literature reports of stimulated acid secretion, it is impossible to discriminate between a direct action of ethanol on the parietal cells or an indirect effect through the possible release of gastrin from the antral mucosa.

In a preparation designed to separate the fundic or acid-secreting portion of the stomach from the antral or gastrin-releasing segment, the effects of alcohol on acid secretion in the dog were determined (figure 2). A laparotomy was performed, the stomach was antrectomized, and the fundic portion was mounted in the the double lucite chamber. Acid secretion collected at intervals cannot distinguish between possible increase in back-diffusion of H^+ or decrease in the active secretion of H^+. Therefore, the luminal solution was maintained neutral, and acid secreted was titrated continually with a pH stat technique.

A second method, maintaining the luminal solution neutral with TES buffer, was used to verify results (1). Figure 3 illustrates the effects of ethanol as compared to pre-ethanol values in the histamine-stimulated preparation. Ethanol, at a 20-percent concentration (which is equivalent to one martini on an empty stomach), decreased acid secretion to one-third of control values. The potential difference was also markedly decreased.

Some investigators have used potential difference measurements to indicate damage or increased permeability of the mucosa. However, potential difference alone cannot distinguish between increased tissue permeability and inhibition of active transport of ions. An automatic voltage-clamp system (2) was developed to permit continuous monitoring of PD and periodic determination of electrical current to calculate electrical resistance. A decrease

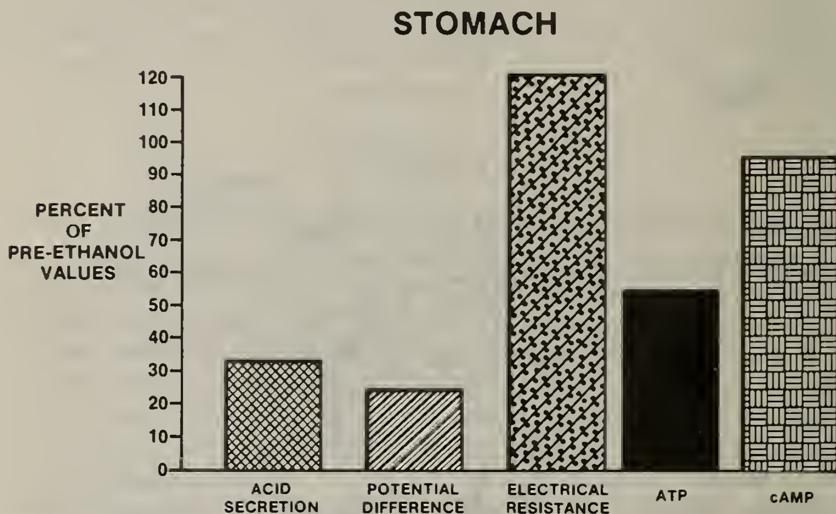
Figure 2. The Intact Fundic Chamber Preparation



in electrical resistance implies an increase in tissue permeability, and increased resistance suggests inhibition of active transport. Ethanol increased electrical resistance, thus suggesting a prime effect on the inhibition of active ion transport in the gastric mucosa.

Other studies on the isolated gastric mucosa, in which unidirectional and net isotopic flux determinations were made, have confirmed the inhibition of active ion transport by ethanol (3). Concomitant studies have demonstrated that ethanol does not alter the cAMP content of the gastric mucosa, but does decrease ATP content (4). The decrease in ATP content may be the mechanism by which ethanol inhibits active transport of ions in the gastric mucosa. These effects are observed only when ethanol is present on the luminal side of the gastric mucosa. Intra-arterial

Figure 3. Effects of 20-Percent Ethanol on Acid Secretion, Potential Difference, Electrical Resistance, Tissue ATP, and cAMP Contents in the Fundic Mucosa, Compared With Pre-ethanol Values



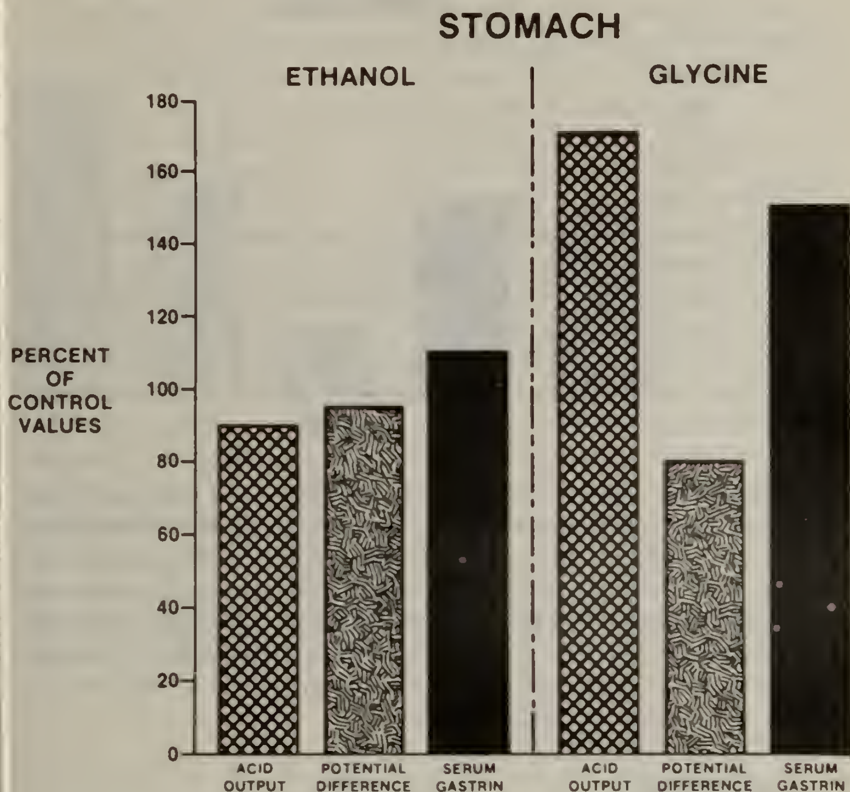
infusion of ethanol into the stomach, at concentrations as high as 30 percent, fails to produce any changes in the measured parameters (1).

To test the possibility that ethanol may stimulate acid secretion by producing the release of gastrin from the antral mucosa, the previous preparation was used—with the exception that the antrum was made into a pouch for the instillation of ethanol or other test substances (5). Ethanol in the antral pouch produced essentially no change in acid output or potential difference in the fundic chamber or in the serum gastrin level (figure 4). However, when glycine was instilled in the antral pouch, acid output from the fundic chamber increased by approximately 70 percent, with a slight decrease in the potential difference. Serum gastrin increased by 50 percent. These studies suggest that any release of gastrin from the antrum is insufficient to stimulate fundic acid secretion.

Pancreas

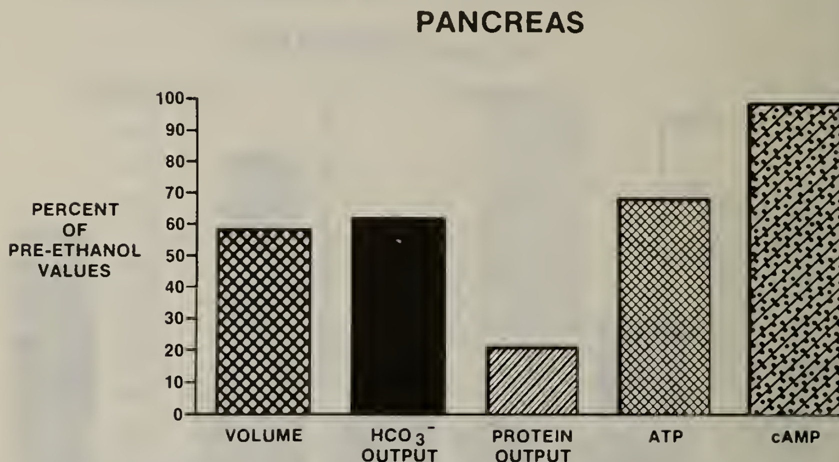
Consideration of any net effects of ethanol on intestinal absorption should include factors that may influence absorptive

Figure 4. Effects of Ethanol and Glycine in the Intact Antral Pouch on Fundic Acid Output and Potential Difference and Serum Gastrin Levels as Compared With Controls



processes, such as pancreatic exocrine secretion. The pancreas secretes digestive enzymes, water, and electrolytes into the duodenum. Until recently, it had been assumed that ethanol stimulates pancreatic secretion. However, Mott et al. (6), using human subjects, and Bayer et al. (7), using conscious dogs, have shown that ethanol inhibits secretin- and cholecystokinin-stimulated pancreatic secretion of water, bicarbonate, and protein. The preparations prevented acid from entering the duodenum, so the inhibition was probably due to a direct effect of ethanol on pancreatic secretory cells. We have tested this hypothesis by using the isolated perfused rabbit pancreas preparation of Rothman and Brooks (8) and an in vivo perfusion method (9). Figure 5 illustrates that ethanol inhibits volume and bicarbonate output by approximately 50 percent and markedly decreases protein output.

Figure 5. Effects of Intravenous Ethanol on Pancreatic Secretion and Tissue ATP and cAMP Contents, as Compared With Pre-ethanol Values



Studies have shown that ATP is necessary for pancreatic enzyme secretion (10) and that secretion of water and bicarbonate depend on oxidative phosphorylation. Cyclic AMP has been implicated as a mediator of secretin-stimulated pancreatic water and bicarbonate secretion (11). Ethanol decreases pancreatic ATP content, but has essentially no effect on tissue cyclic AMP content. The decrease in ATP may be the mechanism by which ethanol inhibits pancreatic exocrine secretion.

Liver

Oral ingestion of ethanol may produce the release of hormones from the small intestine. These hormones may then influence other tissues in the body. There is only one report in the literature, by Straus et al. (12), demonstrating an ethanol-induced increase in secretin release. In this area of alcohol studies, knowledge is severely limited. The lack is due primarily to the technical difficulties in establishing the radioimmunoassays for the gastrointestinal hormones.

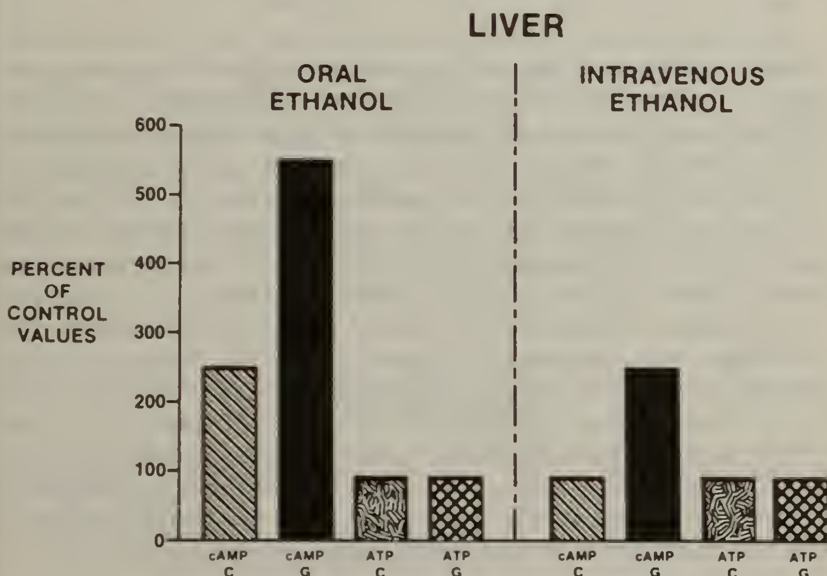
Some excellent studies have been conducted on the metabolic effects of ethanol on the liver. Unfortunately, many of these studies have been conducted using an isolated perfused liver preparation or tissue slices. To determine whether the effects of ethanol on the liver are different when ethanol is administered via a

more physiological route, i.e., orally, studies were designed to evaluate the effects of ethanol given orally versus intravenously on the glucagon-mediated increase in hepatic cyclic AMP (13). Figure 6 summarizes the results of this study.

Each group is compared with saline control values. Ethanol, infused intravenously to achieve blood alcohol levels ranging from 60 to 200 mg/%, did not alter hepatic cyclic AMP or ATP values. However, glucagon (50 μg/kg) increased hepatic cyclic AMP by approximately 2.5-fold, but did not alter hepatic ATP levels. When ethanol was administered orally as a 20-percent solution, cAMP levels more than doubled, as compared with saline controls, but ATP levels were unaltered. The glucagon response in the presence of oral pretreatment with ethanol was approximately 5.5-fold the saline controls and more than double the glucagon-alone response.

The synergistic effect of oral ethanol on the glucagon-mediated increase in hepatic cAMP was also demonstrated to be inversely correlated with the blood alcohol level. This finding suggests that, the more alcohol retained in the intestinal tract, the greater the degree of intestinal hormones released. These hormones, when released, may then activate hepatic adenyl cyclase, the hormone involved in the formation of cAMP, or change the sensitivity of the hepatic receptor to glucagon.

Figure 6. Effects of Oral versus Intravenous Ethanol Administration on Tissue cAMP and ATP Contents in the Liver



The most likely candidates for hormones released from the intestinal tract are gut glucagon and secretin. These hormones evoke hyperglycemia under normal physiological conditions. Both hormones stimulate the production of adenyl cyclase, but apparently via different receptors (14). These results emphasize the importance of conducting integrated studies under as physiological conditions as possible.

Jejunum

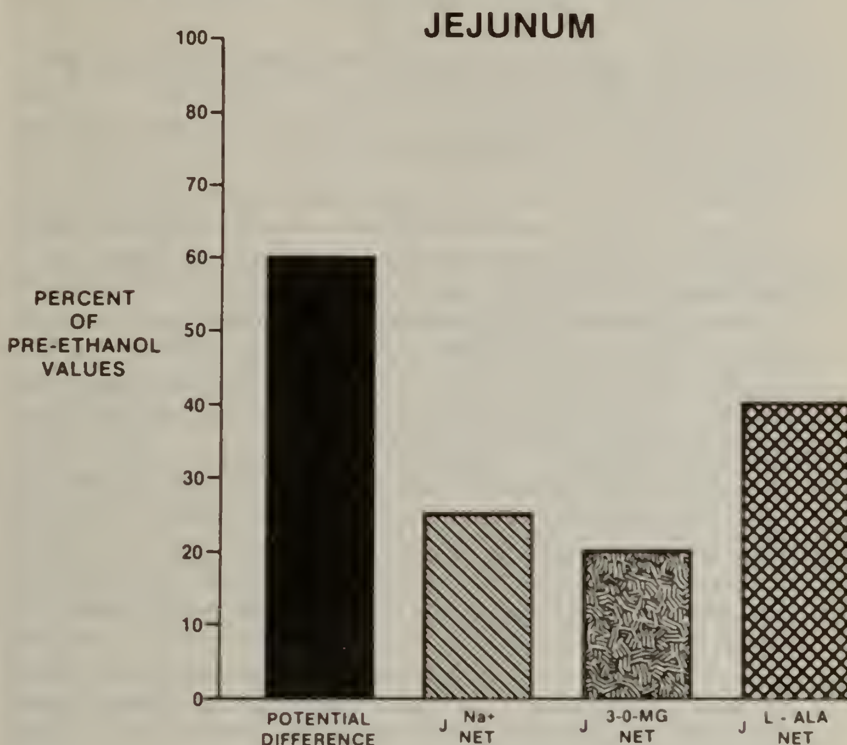
The jejunum is the major gastrointestinal region for absorption, and all enzymatic activity of pancreatic enzymes is performed in duodenal and jejunal lumen. The absorption of amino acids and small peptides is 80-percent complete in the upper 100 cm of the jejunum. The jejunum actively transports sodium, glucose, and amino acids and thus requires the expenditure of metabolic energy.

Ethanol has been demonstrated to inhibit the absorption of glucose and amino acids (15, 16, 17). The absorption of these substances is dependent, to a considerable extent, on the active transport of sodium in the intestine. Dinda et al. (18) have reported that ethanol inhibits glucose transport and mucosal to serosal Na^+ flux, but does not affect net Na^+ flux. However, in their studies, the presence of electrochemical gradients prevents definitive interpretation concerning the effect of ethanol on active transport of Na^+.

With the Ussing chamber preparation—by which electrochemical gradients across the mucosa can be maintained at zero, and passive (unidirectional) and active transport (difference between unidirectional fluxes) can be determined—studies were designed to evaluate the effects of ethanol on the active transport of sodium, 3-*o*-methylglucose, and L-alanine (19).

Figure 7 illustrates the effects of 3-percent ethanol on the jejunum. This concentration of ethanol may be found in the human upper jejunum during moderate drinking (17). The potential difference, which was determined periodically during the experiments, was decreased to approximately 60 percent of pre-ethanol values. The active transport of Na^+, 3-o-methylglucose and L-alanine were decreased to less than 50 percent of pre-ethanol values. These effects are observed when ethanol is present on only the luminal side of the jejunal mucosa or on both sides. However, when ethanol is present on only the serosal or blood side, the values are essentially unchanged from controls.

Figure 7. Effects of 3-Percent Ethanol on Electrical Potential Difference and Active Transport of Sodium, 3-*o*-Methylglucose and L-Alanine in the Jejunum



Based on their findings that long-term administration of ethanol given with balanced liquid diets still produces fatty liver, other investigators (2) have proposed that ethanol has direct toxic effects on the liver. Ethanol inhibits the active absorption of Na^+, 3-*o*-methylglucose, and L-alanine, as well as enhancing possible fluid loss through increased permeability, so an effective nutritional deficiency could still exist, despite adequate and nutritious dietary intake.

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Discussion of Paper by Shanbour

Dr. Schenker: I wanted to focus on one point: the decrease in the ATP in the pancreas and in the stomach. I have two questions. I wonder if you have any morphological data on those tissues at the same time to see what the mitochondria looked like? Do we have any idea as to whether this is an abnormality in synthesis or an increase in degradation of the ATP?

Dr. Shanbour: We have examined the pancreas. We have conducted EM studies, and the pancreas looks pretty normal. We cannot see any obvious histological damage with the ethanol.

Now, we have approached the ATP question in our studies on the gastric mucosa, and we have looked at the effects of ethanol in various doses on adenylate cyclase, phosphodiesterase, and various ATPases (e.g., magnesium-bicarbonate ATPase), and we find a general inhibition in the gastric mucosa. So it is still difficult for me to answer whether the primary effect is in the synthesis or the degradation of ATP. However, in the liver studies, where we have used essentially the same techniques and looked at the ethanol effects on adenylate cyclase, we have essentially confirmed Gorman Betinsky's work on stimulation. So in terms of the gastric mucosa, it looks like a general enzyme inhibition.

Dr. Lieber: I would like to ask two questions, but before I do, perhaps I can answer the question you raised at the very end. You raised the question about what happens in terms of absorption when a liquid diet is given with alcohol, and whether there could be a nutritional deficit. Well, one way we answer that is by measuring the overall balance, by measuring the protein output in the stools compared to the intake, and there was no overall deficit. So whereas segmental absorption may be impaired, I do not know of any good evidence that this results in overall impairment of at least amino acid absorption. With regard to your studies where you compared the effect of intravenous alcohol to oral alcohol, and you mentioned that you were careful to maintain, or achieve, the same blood level, was this peripheral alcohol, or did you mean portal blood levels of alcohol?

Dr. Shanbour: It was peripheral blood alcohol levels.

Dr. Lieber: This raises the important issue: What the liver sees are different blood levels, depending on the two modes of

administration. I think that is an important consideration when you compare the effects.

Now, another question concerns the inhibition of protein secretion by the pancreas under the influence of alcohol. Did you have an opportunity to see what happens in terms of tissue enzyme levels? Was there retention of enzymes normally synthesized, or was there decreased synthesis?

Dr. Shanbour: These are studies that we have not been able to pursue at this point. They are planned, looking more in this direction.

Dr. Lieber: The question is really, do you ever achieve, under your experimental conditions by intravenous alcohol, a level comparable to your portal blood alcohol level secondary to oral administration? It is conceivable that your intravenous system did not reach the same level, even at this high concentration.

Dr. Shanbour: That is possible.

Dr. Lumeng: There are two explanations for your finding. One is the solvent effect of ethanol. The other one could be that ethanol might be metabolized by mucosa, such as gastric mucosa contains ADH. Do you have any evidence that the acetaldehyde is formed in this chamber?

Dr. Shanbour: Some studies that we have just recently completed have demonstrated that the alcohol dehydrogenase activity in the gastric mucosa is very low. Considering the amount of tissue we are examining, and the level of alcohol, the amount that is metabolized should be a minor factor under those conditions.

Section II:
Effects of Alcohol on Mineral Metabolism

302-749 O - 79 - 7

Recent Advances in Zinc Biochemistry*

Bert L. Vallee

I feel privileged to have been asked to come here, because zinc biochemistry, the subject which I am to address, relates to alcohol—directly or indirectly. I thought that I would briefly survey for you the overall state of zinc biochemistry, a problem not usually discussed in toto. Much as the biochemistry of bulk constituents of biological matter (for example, proteins, carbohydrates, lipids, nucleic acids, and that of substances occurring in lesser quantities, i.e., vitamins and minerals such as Na^+, K^+, Ca^{2+} and Fe^{2+}) are now subjects for general discussion, zinc has not been among them.

The role of zinc in biology has been studied for more than 100 years, but its actions have been elucidated only in the last decade or two, to a point where they can now be discussed in a meaningful manner. There is an ever-increasing body of knowledge, but its integration continues to require attention. Clearly, there is insufficient time to give all relevant background, but I will try to highlight what to me seems to be of the greatest interest at the moment, while delineating what is seemingly the present frontier of knowledge.

Based on the work of McCance and Widdowson, it has been known for over a quarter of a century that the body of an adult weighing 70 kg contains about 4 g of iron and 0.2 g of copper. Surprisingly, there are about 2.5 g of zinc. This finding did not result in much increased scientific activity. It was not considered to be of biological importance, but, rather, thought to reflect the passive accumulation of an environmental contaminant.

One might wonder why there is a difference in attitude regarding the role of zinc versus that of iron or copper? The reason is obvious but not visible: Iron and iron salts are red. Hence, macromolecules or other compounds containing this element call attention to themselves, leading to ready purification and isolation (for example, hemoglobin, identified and crystallized by Pryor as

*This work was supported by Grant-in-Aid GM-15003 from the National Institutes of Health.

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early as 1866). As a result, the biochemistry and physiology of iron as well as of iron metabolism have been studied intensively for a long time and are now the foundations of a medical specialty, hematology.

Similarly, copper salts and copper proteins are generally blue and also identify themselves quite readily, largely for that reason. In fact, the first copper protein, haemocyanin, the oxygen carrier of arthropods such as crustacea and xiphosera and of gastropods and cephalopods, was first recognized in 1847 by Harless. Copper did not make it into medicine quite as early as iron, but then it is not quite as abundant.

Thus, the visible color of iron and copper proteins were decisive to their early recognition, but zinc and its proteins are colorless, and hence, do not give away their presence. They were not recognized until highly sensitive and accurate methodology became available, e.g., techniques such as emission spectrography and atomic absorption spectrometry, to detect and identify zinc and other metals in proteins and enzymes regardless of the optical properties of the complexes.

Despite its wide distribution and functional significance, zinc is not unique to a single protein that occurs in large quantities and is readily accessible as, e.g., iron is to hemoglobin. Hence, its possible biological function escaped notice. I was attracted to this "invisible" element a long time ago and I will give a brief, highly personalized, and, hence, markedly truncated history.

In 1869 Raulin found that *Aspergillus niger*, the bread mold, did not grow in the absence of zinc. The studies went completely unnoticed. The next step took place in France, in 1877, when zinc was found in plants, vertebrates, and the liver. Not until 1934, however, did both Bertrand and Elvehjem independently show that zinc deficiency results in a series of abnormalities in rodents. In 1940 Keilin and Mann found that carbonic anhydrase is a zinc enzyme, the first one to be discovered, and the first enzymatic role for zinc became known. For the next 15 years, many biological studies of zinc attempted to relate its presence to carbonic anhydrase function.

In 1954, in collaboration with Hans Neurath, I found—by means of emission spectrography—that zinc is an enzymatically essential constituent of carboxypeptidase A. The establishment of that method gave the first impetus to systematic search for and studies of zinc enzyme; the advent of atomic absorption spectroscopy 10 years later accomplished the rest. In the next few years, we found zinc in carboxypeptidase B, and, importantly, in the alcohol dehydrogenases of yeast and horse liver. The yeast enzyme

had been known since Pasteur's days, and scientists had searched for it in liver, among other sources.

Because this symposium is devoted to the question of ethanol metabolism, it is appropriate to give some background regarding our earlier studies. They were not entirely accidental. Work done about 1950, by Nathan Kaplan, Sydney Colowick, and Alvin Nason, all of them at the McCollum Pratt Institute, had initiated studies of zinc deficiency in *Neurospora crassa* and found that—in contrast to the normal—the deficient organisms did not exhibit alcohol dehydrogenase activity. Understandably, at the time, they interpreted the finding to mean that a zinc-requiring enzyme was responsible for the synthesis of alcohol dehydrogenase. For lack of suitable methods, the obvious could not be tested.

On founding of our laboratory, one of our first experiments concerned the metal analysis of yeast alcohol dehydrogenase, and we found it to contain 4 g-atoms of zinc per mole. At that time, the physical chemist, Charles Coryell of MIT, was one of the few who believed that metals might be important in biology—an article of faith at that time. I went to see him and said, "Charles, I have what seems to me a most improbable finding: yeast alcohol dehydrogenase contains zinc and the enzyme's function depends on it. Would you have expected that?" He looked at me, thought for 2 minutes, and said, "No, but now that you have found it, I will explain to you why it is there." He did, but we—and many others—are still trying.

Our earliest (nonenzymatic) work in 1956 dealt with alcohol and zinc metabolism in cirrhosis, and we found some relationships. The work revealed that the zinc content of serum and liver of such patients decreases, but its excretion in urine increases. This finding has been confirmed widely since, but decisive therapeutic results have not yet been reported.

In subsequent years, biochemistry generally, and the characterization of enzymes specifically, rose to unprecedented heights in terms of opportunities for isolation and study by methods hitherto unavailable, and we developed and applied such procedures to isolate and characterize metalloenzymes. For some reason, attributed erroneously by some to our persistence in pursuing zinc, we found a number of zinc enzymes. Actually, I must give credit again to emission spectrography, which is quite free of bias even though some of our best friends claimed that our spectrograph contained a "built-in" zinc line to be recorded whenever we analyzed any enzyme. We have a number of examples, however, that show this supposition to be much exaggerated.

Very quickly then, the questions became, what could one learn about this element? What is its function? Zinc had never engendered a great deal of enthusiasm on the part of chemists, biochemists, or biologists: Chemically, it seemed quite uninteresting. It is neither oxidized nor reduced; it is not paramagnetic; and it does not yield colored products; in fact, it is reasonably inert, much as any element can be. Why should biology bother with the extensive use of such an element? Biologically, too, it seemed quite improbable. And so, the emergence of its importance became then, a matter of the concurrent advances in chemistry, botany, biochemistry, microbiology, nutrition, teratology, and medicine. Now it seems that zinc has no end of effects on everything and in anything.

Thus there has been a tremendous explosion of knowledge and understanding, and workers from many disciplines have participated to establish a new discipline that is still developing: metallobiochemistry, encompassing that of zinc. Although there were but two zinc metalloenzymes, carbonic anhydrase and carboxypeptidase, in 1954, by 1977, there were 92. Table 1 gives some examples.

Table 1. Some Zinc Metalloenzymes

Enzyme	Source
Alcohol dehydrogenase	Yeast; horse and human liver
D-Lactate cytochrome reductase	Yeast
Glyceraldehyde-phosphate dehydrogenase	Beef and pig muscle
Phosphoglucomutase	Yeast
RNA polymerase	*E. coli*
DNA polymerase	*E. coli*
Reverse transcriptase	Avian myeloblastosis virus
Mercaptopyruvate sulfurtransferase	*E. coli*
Alkaline phosphatase	*E. coli*
Phospholipase C	*Bacillus cereus*
Leucine aminopeptidase	Pig kidney and lens
Carboxypeptidase A	Beef and human pancreas
Carboxypeptidase B	Beef and pig pancreas
Carboxypeptidase G	*Pseudomonas stutzeri*
Dipeptidase	Pig kidney
Neutral protease	*Bacillus* sp.
Alkaline protease	*Escherichia freundii*
AMP aminohydrolase	Rabbit muscle
Aldolase	Yeast; *Aspergillus niger*
Carbonic anhydrase	Erythrocytes
δ-Aminolevulinic acid dehydratase	Beef liver
Phosphomannose isomerase	Yeast
Pyruvate carboxylase	Yeast

We were responsible for establishing the criteria by which they were characterized, but we certainly did not identify all of them as zinc enzymes by any means—although we can be held accountable for a number of them. Alcohol dehydrogenase is at the top of the list alphabetically. It has been found in yeast, plants, and in the liver of vertebrates, particularly that of the horse.

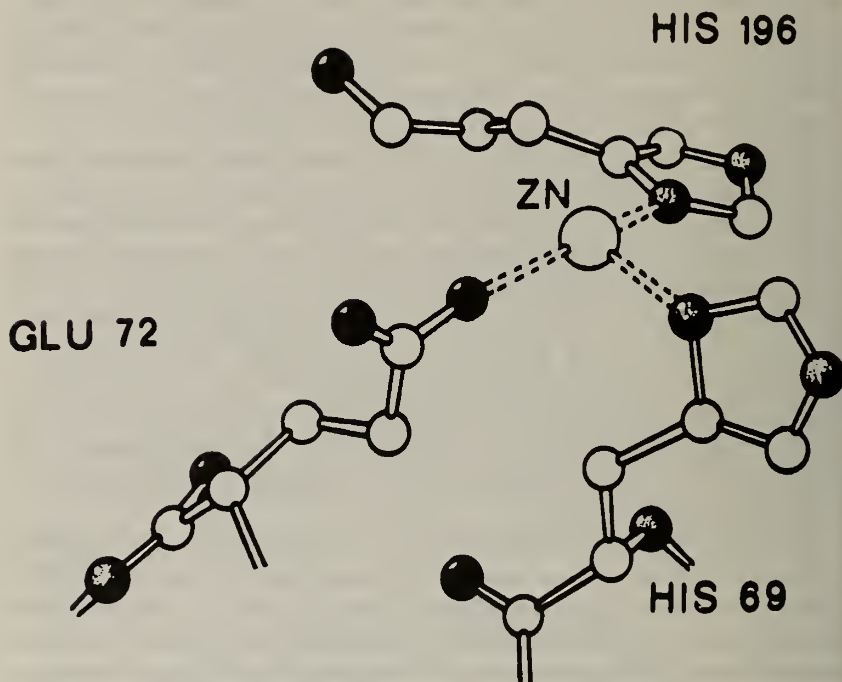
It is not possible to discuss the characteristics, properties, and implications of even a fraction of those in this list. Suffice it to say that there is at least one zinc enzyme among the six classes of the international nomenclature of enzymes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. That some of these turned out to be zinc enzymes were great surprises: thus, δ-aminolevulinic acid dehydratase, critical to the synthesis of porphyrins, was an unexpected addition to the list. Similarly, many others are involved in the synthesis of nucleic acids, i.e., DNA- and RNA-dependent DNA and RNA polymerases, respectively, a finding which came as another great sensation to almost everyone.

At this point I would like to say a few words regarding the manner in which zinc interacts with enzymes and how this might differ for various zinc metalloenzymes, and also to discuss a few details about human alcohol dehydrogenase, which we have isolated and purified recently after trying for about 15 years. I will touch then on some of the consequences of zinc deficiency, both in lower and higher organisms, particularly humans, but will leave the relevant details of metabolic studies to Drs. Falchuk, Li, and Bosron and, to Dr. Hurley, for the teratological consequences which were not anticipated. At the end, I will say a few words about how we actually came into the field, because that relates to the discovery of zinc in the reverse transcriptase from oncogenic C viruses.

If one were to play a "parlor game" in inorganic chemistry regarding metals and their ligands, and one were to say "zinc," the reply would be "sulfur," much as the reply to "calcium" would be "oxygen." So knowledge of inorganic chemistry would lead one to expect sulfur to be the predominant zinc ligand in enzymes. But this is not so, not in carbonic anhydrase. Both the primary sequence and three-dimensional structure have been established long since and show that the zinc atom is bound to three histidines. Nor, for that matter, in carboxypeptidase, an enzyme on which I have spent 23 years.

In this case, there are two histidines, residues #196 and #69, and one glutamic acid residue #72; hence zinc is bound to two nitrogens and one oxygen, much as is the case in thermolysin

Figure 1. Zinc Ligands at the Active Center of Carboxypeptidase



(figure 1). The primary sequence and three-dimensional structure of all three of these enzymes have also been studied, and, in all of these instances, the metal is bound in a most unusual manner as compared with model systems. I will not discuss the details of the implications, but we have elaborated the entatic state hypothesis, which states that the geometry of enzyme·metal coordination is highly irregular and has low symmetry compared with that seen in conventional models, this geometry relating in some manner to the mechanism of action. In any event, all of the X-ray structures that have been performed on enzymes containing zinc or other metals are consistent with this prediction. Apparently, biochemical systems have found characteristic ways to bind and handle metals such as to result in enzymatic activity. Thereby they simultaneously augment the tremendous diversity of biological specificity required, which can only be achieved by innumerable, different arrangements of the various components of biological macromolecules.

The active site of alcohol dehydrogenase, of course, is organized differently again. There, sulfur donor atoms are actually involved; in fact, two cysteines and one histidine, i.e., two sulfurs and one

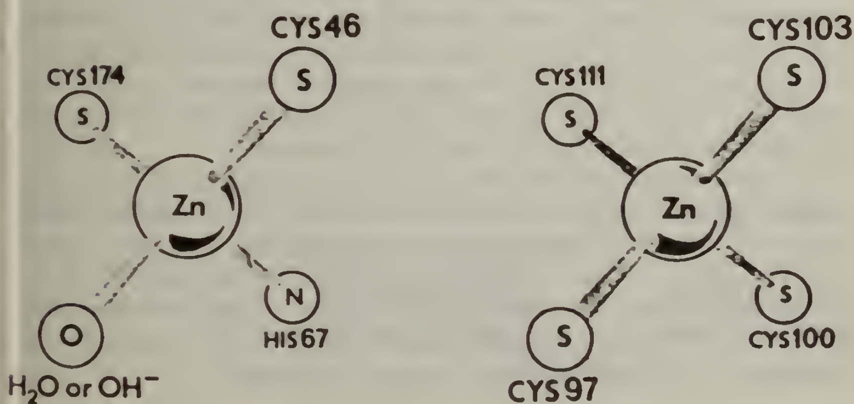
nitrogen bind the active-site zinc atoms, while the fourth coordination site is open to water or substrate. The horse and human liver enzymes contain two zinc atoms per each of the two subunits, hence a total of four zinc atoms.

A long time ago, we found that only two of the four zinc atoms of the holoenzyme are active. They bind two NADH's, two moles of substrate, and two moles of inhibitors per each of the subunits. The second pair of two zinc atoms is not active; it is bound to four cysteines, and hence four sulfur donor atoms fill the coordination sphere; no further site is open for any interaction of water or substrates (figure 2). These zinc atoms are bound very firmly. They have no known role other than perhaps structure stabilization. A similar second set of "structural" atoms exists in the human enzyme but is not present in the yeast enzyme.

I won't dwell on that further except to say that X-ray structure analysis, as determined by Brandén and coworkers, has found the coordination at the active site zinc to be "entatic." Zinc is indispensable for the activity of these enzymes, even though, of course, we have succeeded in substituting zinc with cobalt or cadmium and both these derivatives are catalytically active. The chemical properties of these elements are advantageous to probe the active center environment. So much of our work on this and other zinc enzymes has dealt with exchanging it for other elements. But these aspects of the work are not really pertinent here.

For this audience, I must spend a moment on human alcohol dehydrogenase. Certainly liver alcohol dehydrogenase is the enzyme primarily responsible for oxidizing the bulk of the ethanol consumed. Dr. Lieber, no doubt, will have comments about

Figure 2. Ligands of the Catalytic (left) and Structural (right) Zinc Atoms in Horse Liver Alcohol Dehydrogenase



other oxidative pathways. In any event, pyrazole was thought to inhibit all liver alcohol dehydrogenases. This compound, and many of its derivatives, were thought to bind to zinc, although this was mostly implied and never proved. It is now difficult to see how that could be true, as will be apparent.

Dr. Bethune and I first tried, with Dr. Von Wartburg, when he was a research fellow in my laboratory years ago, to isolate the human enzyme, but the isolation of enzymes from a complex tissue like the human liver proved difficult, to say the least. Nevertheless, we were able to show that it is a zinc enzyme, but we did not succeed in purifying it to homogeneity. More recently, Dr. Lange obtained it in purified form while working on his thesis. With assistance from members of the laboratory staff, particularly Dr. Riordan, he designed an affinity chromatographic procedure by linking it to 4-methylpyrazole through an arm 14–16 Å long. This was then attached to an insoluble carrier, resulting in an affinity-chromatographic column that selectively binds alcohol dehydrogenases from any source. The inhibition constant, K_I, for alcohol dehydrogenase is particularly favorable. Pyrazole is an excellent and selective inhibitor of the enzyme. Now the Theorell-Chance mechanism postulates that, for catalysis to occur, the sequence of coenzyme followed by substrate binding is obligatory. When substituting an inhibitor like pyrazole for substrate, an abortive complex forms, which, in the present instance, causes alcohol dehydrogenase to bind to the pyrazole column, while all other proteins pass through. However, the substrate, ethanol, when in excess, will then compete with the enzyme for 4-methylpyrazole and displace it, thereby eluting the enzyme, followed by subsequent catalysis resulting in NADH and the aldehyde.

The double ternary complex affinity chromatography used here was based on the validity of the Theorell-Chance mechanism proposed; the successful isolation of the enzyme, indeed, confirms it. Elution with ethanol dissociates the enzyme from the pyrazole column, resulting in an active enzyme homogeneous by physicochemical criteria and a preceding peak of varying height, the nature of which I shall mention later on. Drs. Li and Bosron will elaborate on this problem in their presentations.

The procedure requires about 8 hours to isolate human liver alcohol dehydrogenase in contrast to our earlier efforts—2-1/2 years with Dr. Von Wartburg and then, later on, another 2 years with Dr. Blair—without achieving purification anywhere close to that obtained now. The substrate specificity of this pure material turns out to be quite broad. Ethylene glycol will do nicely as a substrate as will methanol.

Two diseases are now curable when treated in time, based on the relative affinities of the enzyme toward methanol and ethylene glycol as compared with ethanol. Poisoning with methanol and ethylene glycol can be handled by administering ethanol, which the enzyme oxidizes preferentially, competing with the potential poisons and preventing the formation of toxic products that cause the resultant disorders.

The characteristics of horse and human enzymes are amazingly similar (figure 3), although there are some differences in terms of the K_m and K_I. The mystery remains why the horse's liver contains so much of an enzyme of which it presumably needs so little; providence seems to have prepared the horse more than adequately for a problem which that species does not have. Humans, instead, seem to have been shortchanged, but I hope to say a word about that in a minute.

A large amount of work has been done on the enzyme, of course, including its genetics and catalytic properties, but all of it on impure material; in fact, much of it has been done on crude tissue extracts. These were examined on agar slabs and gels, and the genetic propositions that have been advanced are based on what one might consider somewhat uncertain biochemical foundations. However, although there are clearly many isoenzymes, the

Figure 3. Properties of Horse and Human Liver Alcohol Dehydrogenases

	HORSE	HUMAN
M.W.	83,000	85,000
ISOELECTRIC POINT	6.8	9.1
METAL CONTENT	4 g-ATOM Zn PER DIMER	4 g-ATOM Zn PER DIMER
OP pK_I	3.6	4.7
SUBSTRATES	PRIMARY ALCOHOLS (EXCEPT METHANOL), ALDEHYDES, SOME SECONDARY ALCOHOLS, KETONES HYDROXY STEROIDS	AS FOR HORSE + METHANOL AND ETHYLENE GLYCOL

genetics are not known definitively. We have isolated the enzymes from the rabbit, the rat, the horse, and the human by means of 4-methylpyrazole column chromatography, and we are now addressing the isolation of isoenzymes from the human material to undertake genetic and other studies with pure material.

Dr. Li has pursued the problem in Indianapolis and has obtained the α, and β, and γ bands, identified previously by Harris and coworkers. In addition to these cathodic bands, he has also observed an anodic species which he has called "the anodic band." Apparently this band had not been observed by other investigators; certainly it had not been reported. This lack is probably because the enzyme responsible for its occurrence disintegrates rapidly unless the liver specimen is fresh—and even then it does not seem to be detectable in all human livers studied so far and to the same degree. Yet, working in collaboration with them, we have now isolated and purified this material using variations of the new double ternary complex affinity chromatograph method.

I am not going to preempt Drs. Li and Bosron's "thunder"; they are going to report on this work later on. Suffice it to say that our collaboration with them resulted in the isolation and purification, in homogeneous form, of a new alcohol dehydrogenase that has a high K_m and a very high K_I toward 4-methylpyrazole—the latter so high, in fact, that the enzyme remains virtually uninhibited, except by extraordinarily high concentrations. Otherwise its characteristics are the same as those of the other bands, and this form constitutes about 16 percent of the total liver enzyme. We have named it Π-alcohol dehydrogenase; its characteristics have been described in a manuscript that has just appeared (*Proc. Nat. Acad. Sci.*, 74, 4378, 1977). Its detection may well require a reappraisal of present views of the genetic basis of formation of this enzyme.

I will insert a word for Dr. Noble's sake because he considers it important that the public be aware of the biochemical problems underlying alcohol oxidation abuse. While Dr. Li and I were writing this manuscript, we were reminded that the daily newspapers have become accustomed to reporting on the "carcinogen of the week." The country has been placed into a state of chronic "carcinophobia," to coin a word. As best as one can tell, if we do what is implied by all the well-meaning advice given, we should progressively cease to eat or drink most anything that comes our way because virtually all food or beverages might contain a carcinogen, and, hence, be lethal. Our data do not bear on that phobia and such concern may be justified, but much of the discussion regarding those public health problems remains

hypothetical, at least when compared with those regarding the ethanol problem.

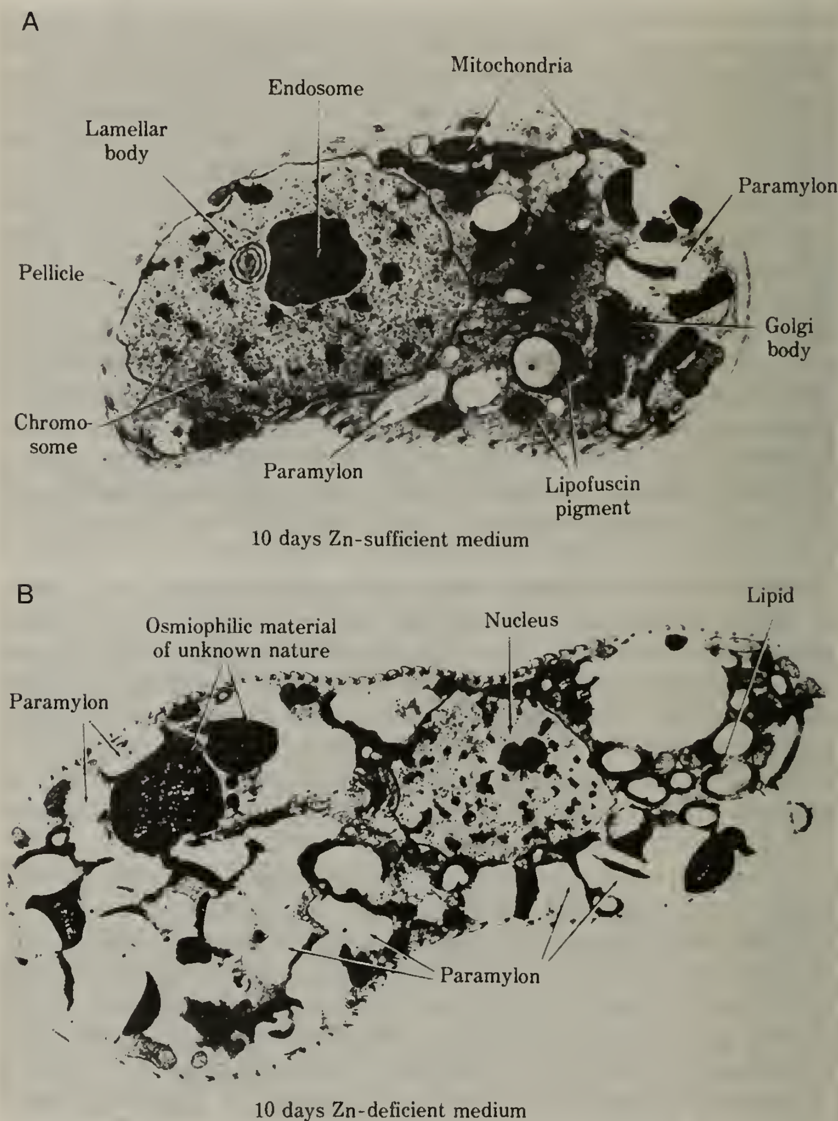
This is hardly hypothetical: It is real. There is no question that ethanol consumption can be both toxic and lethal, and there is complete certainty that morbidity and mortality are direct results of its consumption. However, the obvious chemical basis of these effects is investigated with such reluctance that one might wonder whether or not the known chemical origins of the resulting diseases are either ignored or rationalized away. Some 10 to 15 million affected individuals in this country are afflicted, with some 140,000 a year dying, I am told. Hence in this instance, no guess is required, and I am amazed at the lack of support for intensive biochemical studies. One is compelled to wonder whether or not these circumstances denote unwarranted indifference. Compared with "carcinophobia," there is very little public discussion, and considering the known effects of ethanol as compared with those postulated for infinite numbers of other agents, the expenditures for either are even more discrepant than might be reasonable. I think the ethanol problem is one of the most widespread, pressing, and intriguing problems yet to be dealt with in terms of toxicology and medicine.

Well, little time remains to say much that should be included. In rapid order now, I should say that a large number of organisms can be rendered zinc deficient, the flagellate *Euglena gracilis* (figure 4), which Dr. Falchuk will discuss, being one of these. His studies of its cellular metabolism are of general import regarding the function of zinc in nucleic acid, protein synthesis, and cell division. As a consequence, the teratological consequences of zinc deficiency to be discussed by Dr. Hurley can be appreciated better in biochemical terms.

A number of diseases in both animals and humans are zinc related. It has been known since 1955 that swine fed a calcium-fortified diet develop parakeratosis, a conditioned zinc deficiency. The zinc content of the diet is normal, but added calcium renders the amount of zinc present insufficient for normal growth. Cessation of growth, weight loss, and ultimately death are accompanied by collagen infiltration of the skin and esophagus. Dr. Prasad's (and other investigators') work on dwarfism in Egypt and Iran, where zinc deficiency results in arrest of growth and sexual development of humans, has been discussed widely, and I will not dwell on it further.

Three years ago, a genetic disease, first described in 1942, *acrodermatitis enteropathica*, was shown unequivocally to be a zinc deficiency. It manifests with dermatitis, diarrhea, and alopecia in

Figure 4. Electron Micrographs Comparing the Ultrastructure of *E. gracilis* grown 10 Days With or Without Zinc, Respectively

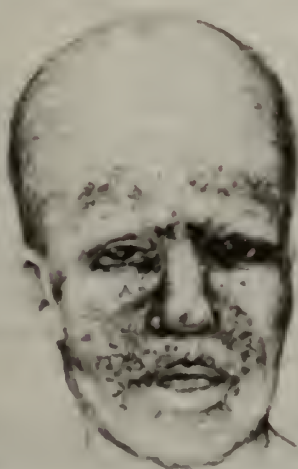


The zinc-deficient organisms are significantly larger; their cytoplasm contains an abundance of paramylon, and large masses of dense osmiophilic material presumably rich in lipid. The size difference is actually greater than is apparent, because the micrograph of the zinc-deficient *E. gracilis* is shown at somewhat lower magnification so that the whole organism can be included, i.e., X 8500 and 6500, respectively.

childhood and is accompanied by mental retardation, generally first seen in infancy. Those afflicted have been known to survive into and beyond puberty, but the disease has been fatal. However, zinc is a specific and completely curative agent (figure 5). The malady apparently represents the first instance of a completely zinc-dependent human disease. Neither the mechanism nor the basis of the pathophysiology is known as yet, but, finally, now there is a proved instance of a one-to-one relationship between zinc and a human disease process; presumably, it will be possible to study it in a decisive manner.

Interestingly the impact of this discovery has already become apparent in surgical patients. A group of workers in New Zealand has reported a zinc-deficiency syndrome due to intravenous alimentation postenterectomy. The patients in question were fed exclusively by vein subsequent to massive resection of various parts of their intestinal tracts because the remainder of the tracts were insufficient to allow adequate absorption of nutrients. All patients developed a syndrome that resembled the signs and symptoms described in *acrodermatitis enteropathica* in virtually all

Figure 5. Artist's Rendition of the Consequences of Zinc Therapy in *A. Enteropathica*, Before (left) and After (right) Therapy



details. Addition of zinc to the intravenous feeding promptly reversed all manifestations. Thus, zinc may be expected to join calcium, magnesium, sodium, and potassium in the care of surgical patients.

I thought I would close on a rather personal note retrospective to my entry into this field many years ago; to me, it reflects the field's development and growth. Time precludes the presentation of details, but zinc has turned out to be involved in virtually all phases of metabolism, a great surprise to me, but one that is not exactly unpleasant.

In a way, the manner in which I got involved is somewhat "offbeat," as is often the case. I was working on blood preservation for the Office of Scientific Research and Development in a joint Harvard-MIT project at the end of World War II and decided to investigate the preservation of normal and abnormal leukocytes. Hence, I separated them from erythrocytes by flotation on human serum albumin, a novel procedure then. I examined the leukocytes by emission spectrography, and surprisingly, normal leukocytes contained a relatively large amount of zinc while that of leukemic leukocytes was markedly diminished. In a paper submitted in 1947, we said, "It is possible that there is a zinc enzyme concerned with myelopoiesis, and that there is some disturbance of this enzyme in leukemia." I had ruled out that it was carbonic anhydrase, the only zinc enzyme then known.

The reverse transcriptases from oncogenic type C viruses (viruses that cause leukemia in a number of species) participate in the incorporation of viral into cellular DNA. In view of the presence of zinc in DNA and RNA polymerases and its essentiality to their action, it seemed reasonable to suspect that this element was also present in the reverse transcriptases.

Unfortunately these enzymes were available in amounts too small to perform metal analyses with equipment then available. However, inhibition studies with chelating agents indicated the presence of a functional metal—but not its identity, of course. We therefore developed a new instrument with detection limits sufficient to detect transition and IIB elements in picogram quantities of enzyme, of which—in turn—we could only obtain microgram amounts. The result, a microwave excitation emission spectrometer, can identify and quantitate from 10^{-11} to 10^{-5} g-atom of metal in 5 μl containing about 1 microgram of protein with 5-percent precision.

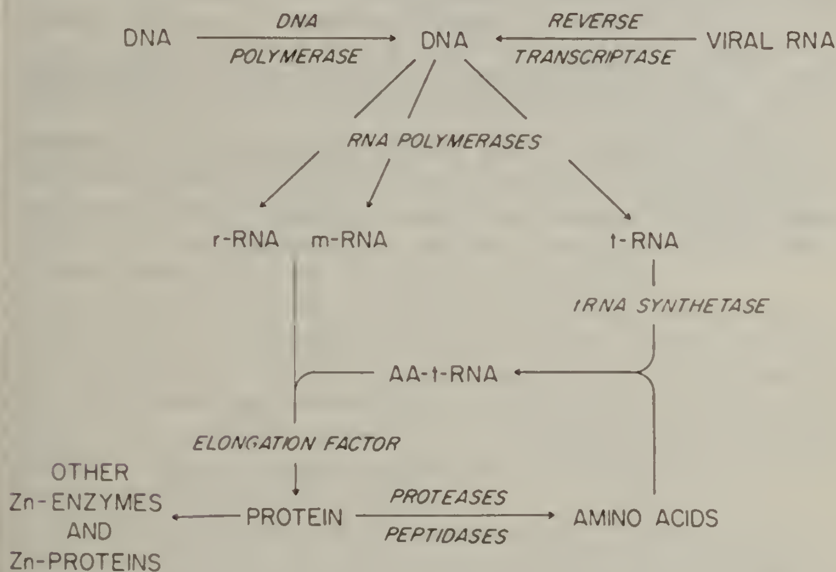
Dr. Auld devised the micromethod, using one single drop of a sample from a Sephadex microcolumn used to purify the enzyme from avian myoblastosis virus. Zinc, protein content, and activity

were determined in all Sephadex fractions, resulting in a peak coincident for all three parameters. Two g-atoms of zinc per mole of enzyme were found in the most active material.

Since then, we have analyzed the analogous enzymes from mouse, monkey, gibbon, ape, and baboon viruses with analogous results: All of them are zinc enzymes. Zinc is clearly critical to cellular division, replication of DNA, formation of RNA, and protein synthesis. A metabolic chart now shows where zinc is involved in translation, transcription, and formation of zinc proteins and enzymes (figure 6).

Clearly, zinc biochemistry is not wanting for opportunities to perform imaginative work to define its role, including that in alcohol metabolism.

Figure 6. Zinc in Nucleic Acid and Protein Metabolism



Italics indicate steps dependent on zinc enzymes or zinc proteins.

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Effects of Zinc on Cellular DNA and RNA Metabolism*

Kenneth H. Falchuk

It has been more than 100 years since zinc was found to be indispensable to the growth of *Aspergillus niger* (1), and almost that long since its presence was established in plants and animals (2,3).

It is now evident that zinc is essential to normal growth and development of all living matter (4). Zinc deficiency results in major abnormalities of composition and function, although the manifestations are complex and can vary depending on the particular species studied (4,5,6,7,8). An increasing number of diseases are proving to be related to zinc deficiency, both in animals and humans (9,10,11,12). Zinc deficiency during pregnancy results in congenital malformations of the embryo, particularly by affecting growing or proliferating tissues (13). The consequences of more subtle metabolic interactions, as in alcoholic cirrhosis (14) and other diseases (15), and the basis of genetic or teratological defects (16) have not been examined widely.

In spite of the major advances establishing both the participation of zinc in enzymatic catalysis and many aspects of presumable mechanisms, knowledge concerning the roles of this element in cell division and the associated metabolic events is surprisingly sparse. The reactions in which zinc is essential and in which its failure to occur becomes limiting to cell growth have not been recognized, defined, or integrated.

We initiated a series of experiments with *E. gracilis*, strain Z, a eukaryotic organism which we found suitable for studying the biochemical basis for the requirement of zinc for growth, and particularly, for defining the metabolic consequences secondary to its deficiency (17). The organism is obtained in homogeneous form; its growth is sensitive and responsive to the zinc content of the medium; and it can be disrupted readily to allow definitive

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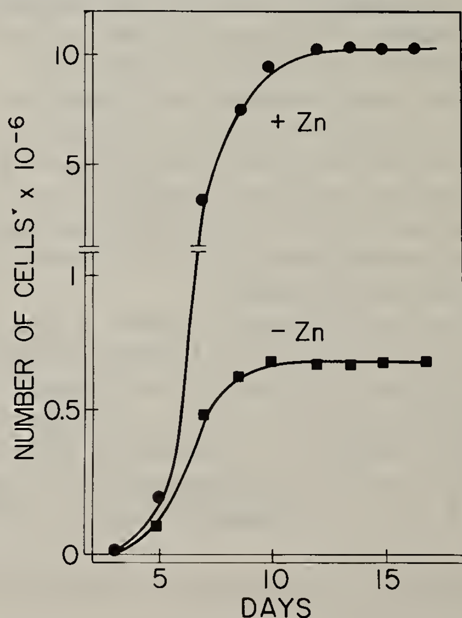
measurements on subcellular organelles as well as such metabolically critical components as nucleic acids, proteins, carbohydrates, lipids, phosphates, metals, etc. Decrease of the zinc concentration of the culture medium from 10^{-5} M to less than 10^{-7} M arrests its growth (figure 1). Concurrently, its zinc content (table 1) decreases to less than 10 percent of that of zinc-sufficient cells. Raising the zinc content of the medium to 10^{-5} M completely restores normal growth within 36 to 48 hours (17,18,19).

A number of striking chemical changes accompany the proliferative arrest induced by zinc deficiency. Cellular DNA content doubles; cell volume increases; the protein content and ^{3}H-uridine incorporation into RNA both decrease; and peptides, amino acids, nucleotides, polyphosphates, and unusual proteins accumulate. Further, the intracellular content of Mn, Mg, Ca, Fe, Ni, and Cr increases from 3- to 35-fold (18,19), and we recently found that the Cu content of the organism increases as well (table 1).

As part of our aim to determine the basis for the proliferative arrest, we undertook to detail, delineate, and define those steps of the cell cycle of *E. gracilis* affected by zinc deprivation. Toward

Figure 1. Growth of Zinc-Sufficient (+Zn) and Zinc-Deficient (–Zn) *E. gracilis* Grown in the Dark



Zinc-sufficient medium contains 1 × 10^{-5} M Zn^{2+}; zinc-deficient medium, 1 × 10^{-7} M Zn^{2+}.

Table 1. Metal Content* of (+Zn) and (-Zn) *E. gracilis*

Metals	(+Zn)	(-Zn)
Zn	5.80	0.80
Mn	0.01	0.35
Mg	3.00	15.00
Ca	0.20	4.00
Fe	1.60	12.00
Ni	0.09	0.24
Cr	0.12	0.36

*$\mu g \times 10^7$/cell

this end, we have examined the DNA content of zinc-sufficient and -deficient cells by means of laser excitation cytofluorometry (20).

Aliquots of cells were collected for analysis of DNA content by flow cytofluorometry. The cells were stained with propidium diiodide solution and analyzed in a cytofluorograph (Model 4801, Bio/physics System, Inc., Mahopac, N.Y.). The flow system of this instrument allows passage of one cell at a time through a 100-micron orifice (Restrictor valve) into a flow chamber where laminar flow is induced by a sheath of water. The cell traverses through exciting, monochromatic radiation from an Argon ion laser beam tuned to emit at 488 nm. The resultant fluorescence of propidium diiodide—DNA complexes of the cell nucleus—is converted to an electrical signal by a photomultiplier; the output is displayed on the horizontal axis of a cathode ray tube. The signal also enters a multichannel pulse height distribution analyzer (Model 2100, Bio/physics Systems, Inc., Mahopac, N.Y.) where the frequency distribution of the pulses as a function of the magnitude of the signal is stored in a memory unit and subsequently displayed as a histogram. A total of 100 channels are used, and the abscissa of the histogram reflects increasing linear values of the fluorescence signal. The numbers of cells recorded in each channel are registered simultaneously on a printout tape system, allowing quantitation of the number of cells fluorescing at a characteristic intensity.

Incubation of mammalian cells with RNAse, prior to staining, has been shown to obviate interference by RNA in DNA analysis (21,22), as was shown to be true also for *E. gracilis* (20).

The histograms of DNA content of cells in each phase of the cell cycle have been identified by using synchronized cell population

(20). Log phase cultures contain organisms in all stages of the cell cycle. A histogram from a log phase culture of *E. gracilis* stained with propidium diiodide and analyzed in the cytofluorograph is shown in figure 2. The major fractions of cells are in G_1, with an unreplicated genome; the remainder are in S or in G_2 or M phase of the cell cycle.

The DNA content of early stationary, nondividing cells was examined next. Figure 3 compares the pattern of early stationary phase zinc-sufficient cells with that of zinc-deficient cells, obtained when cell division ceases. The histogram of zinc-sufficient cells demonstrates that the majority, although not all, of the cells in stationary phase are in G_1, with a smaller number in S. In contrast, the pattern of nondividing zinc-deficient cells is typical of that of S/G_2. The latter cells have previously been shown to cease dividing on depletion of zinc in the growth media (19).

The resulting histogram of DNA content suggests that as the nonsynchronously growing cells in the zinc-deficient media are deprived of zinc, those cells that are in S do not continue to G_2, and those that reach G_2 do not proceed to mitosis. Moreover, a small fraction in G_1 is also present. To further detail the effect of zinc deprivation on the G_1 to S transition, early stationary phase zinc-sufficient cells, known to be mostly in G_1 (figure 3), were incubated in media deficient in zinc. Figure 4 illustrates that, following incubation in this medium, there is a 25-percent increase

Figure 2. Schematic Diagram of Flow Cytofluorometer

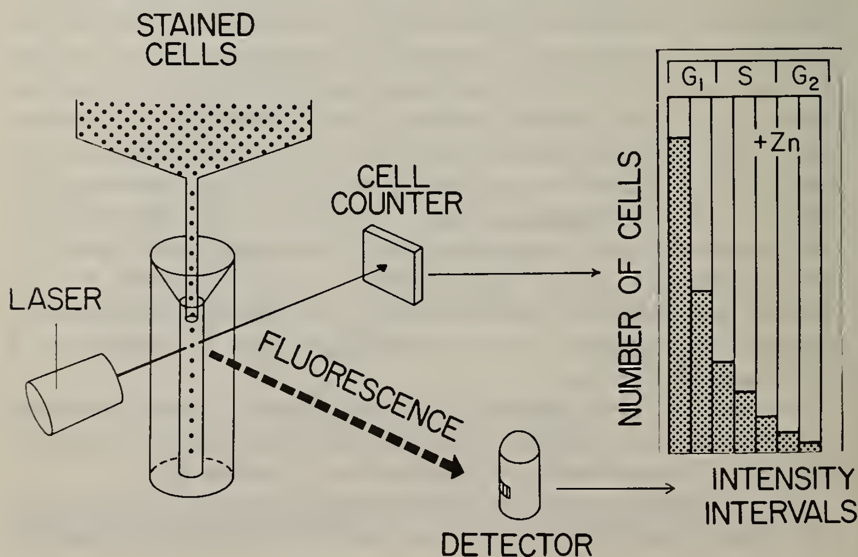
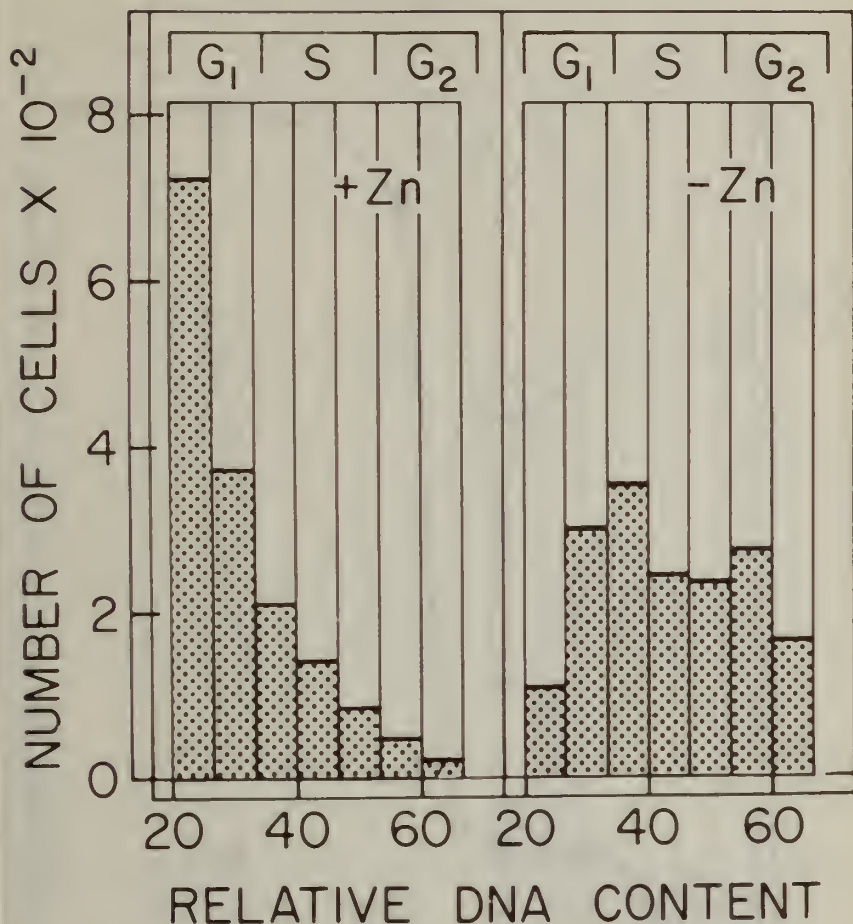


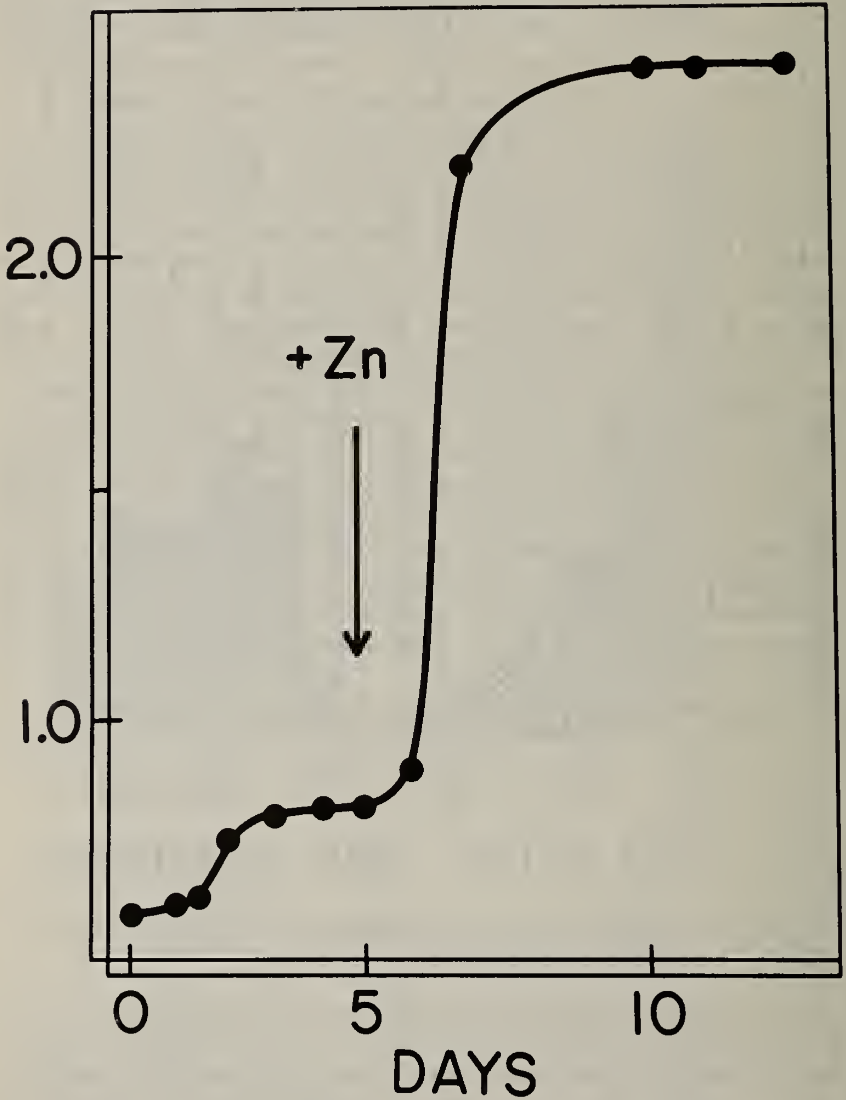
Figure 3. Comparison of DNA Histograms of (+Zn) and (-Zn) *E. gracilis*



The majority of (+Zn) cells are in G_1 with a small fraction in S. In contrast, nondividing (-Zn) cells are mostly in S or G_2, with a small fraction in G_1.

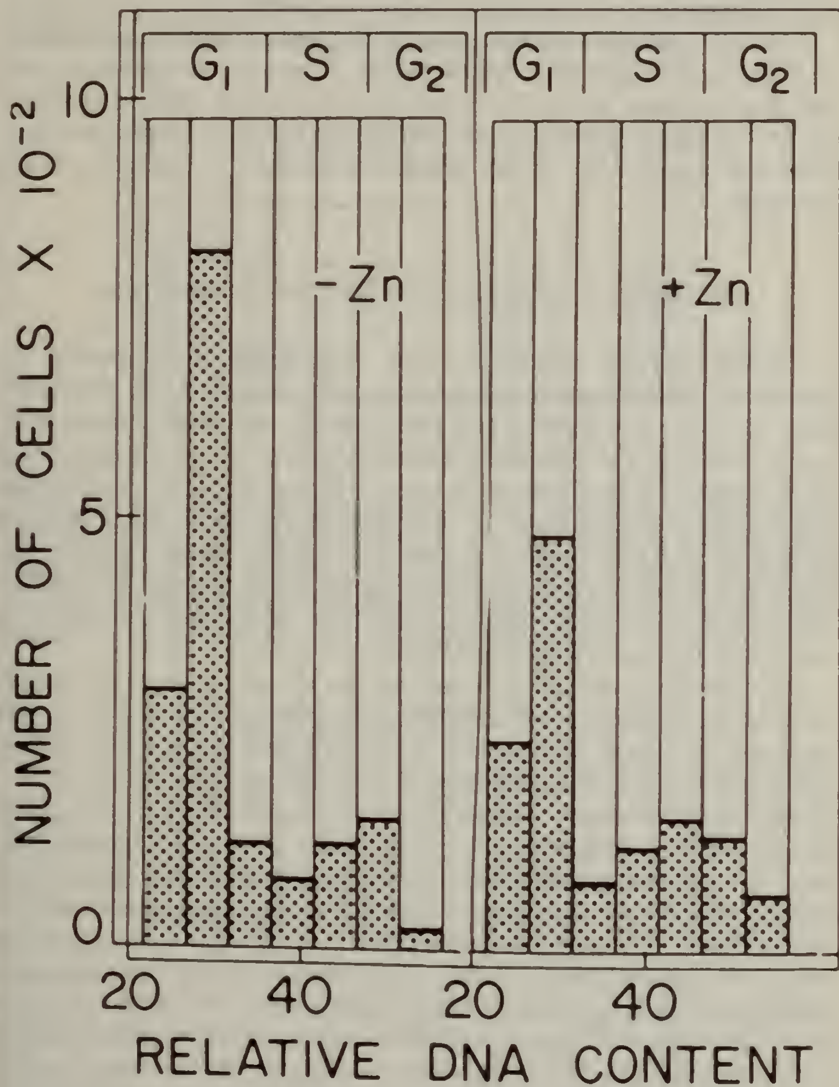
in cell number, followed by cessation of growth. Addition of zinc to the medium confirms that the absence of zinc is responsible for the inhibition of cell growth. Within 24 to 36 hours of the addition of zinc, the cell numbers increase by 200 percent, reaching those expected for a zinc-sufficient culture. The cytofluorometric analysis of these cells (figure 5) demonstrates that, prior to the addition of zinc, when cell division has ceased, almost all cells incubated in zinc-deficient media are in G_1 phase. Hence, zinc deprivation of cells in G_1 blocks their progression into S. Addition of zinc to these cells reverses the block of their cell cycle, restoring

Figure 4. Growth Characteristics of Early Stationary Phase (+Zn) Cells Incubated in (-Zn) Media



Following incubation there is a small increase in cell density, followed by a cessation of cell division. On raising the zinc content to 1×10^{-5} M, there is a striking increase in cell division, the final density reaching that expected of a (+Zn) culture.

Figure 5. Comparison of the Histogram of DNA Content of *E. gracilis* Incubated in (-Zn) Medium Prior to and Following Addition of Zinc



Following cessation of cell division, the majority of the (-Zn) cells are in G_1, with a small fraction in S. On addition of zinc, the number of cells blocked in G_1 decreases and a histogram typical of log phase cells results.

the normal pattern of division of cells, which becomes identical to that shown in figure 2. Clearly, the biochemical processes essential for cells to pass from G_1 into S, from S to G_2, and from G_2 to mitosis depend on the presence of zinc, and its deficiency can block all three phases of the growth cycle of *E. gracilis.*

Indirect evidence suggests that zinc is essential for the function of DNA polymerase of *E. gracilis* (23). However, the results of our cell cycle studies led to the conclusion that the limiting steps leading to the abnormalities of the cell cycle and the consequent proliferative arrest cannot be restricted solely to impaired DNA synthesis.

Studies of *E. Gracilis* RNA Metabolism

Relative to zinc-sufficient cells, zinc-deficient *E. gracilis* incorporate ^{3}H-uridine into RNA at a reduced rate (19). However, they accumulate peptides and amino acids and their protein content is reduced (18). These observations focused on derangements at the level of translation in zinc-deficient cells. Such derangements could potentially be responsible for both the observed blocks of the cell cycle and the proliferative arrest because ongoing RNA and protein synthesis are required for G_1, S, and G_2. Alterations in their synthesis could then block the cell cycle at each of these stages (24,25,26,27).

Accordingly, we next focused on the details of the role of zinc in RNA metabolism of *E. gracilis* as another possible basis for the observed chemical lesions. We first examined the RNA polymerases I and II from zinc-sufficient *E. gracilis* (28,29).

The enzymes were isolated from cells harvested in the log phase of growth. A cellular homogenate was precipitated with ammonium sulfate and the pellet was dissolved in 0.15 M ammonium sulfate buffer. At this stage, the RNA polymerases are bound to DNA. The DNA was precipitated with protamine sulfate, leaving the RNA polymerases in the supernatant. The preparation was then chromatographed on DEAE-Sephadex A-25. The enzymes, now free of DNA, were purified further by either affinity chromatography on a DNA cellulose column or by chromatography on phosphocellulose. Polymerases I and II have been purified to homogeneity.

As shown in table 2, both polymerases are entirely dependent on an exogenous DNA template for activity. The product of their enzymatic reaction is RNA, as evidenced by an absolute substrate requirement for ribonucleotide triphosphates and by digestion of

the product by ribonuclease. As with other polymerases, the *E. gracilis* enzymes are inactive in the absence of Mg^{+2} or Mn^{+2}. Both these DNA-dependent RNA polymerases are homogenous on polyacrylamide gels, and their estimated molecular weights, determined on SDS gels, are between 650,000 and 700,000 for both polymerases. They are composed of multiple subunits.

Table 2. Properties of *E. Gracilis* RNA Polymerases I and II

Property	I	II
Template dependence	DNA	DNA
Product	RNA	RNA
Activating metals	Mg, Mn	Mg, Mn
M. W. (SDS-PAGE)	650,000	700,000

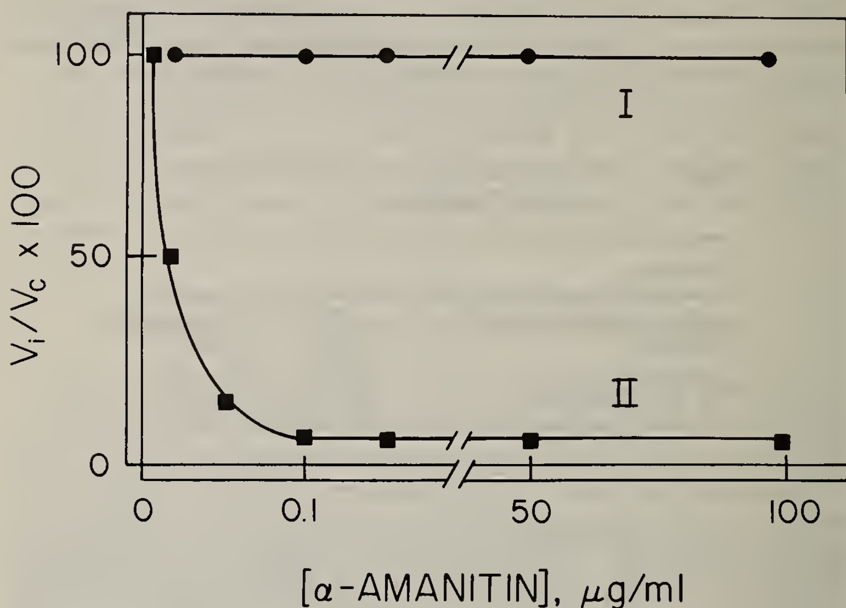
α-Amanitin differentiates type II from type I RNA polymerases. The activity of the polymerase I is not inhibited by α-amanitin at concentrations up to 200 μg/ml. In contrast, increasing concentrations of α-amanitin progressively decrease and at 0.1 μg/ml nearly abolish activity of RNA polymerase II (figure 6).

Inhibition by 1,10-Phenanthroline

The answer to the question of metal dependence clearly has to be approached by undertaking studies of inhibition with metal binding agents which, while conserving material, give first a valuable indication of the involvement of a metal in activity.

The chelating agent, 1,10-phenanthroline (OP) has proved exceptionally suitable to study the inhibition of zinc metalloenzymes (31). To determine its effect on *E. gracilis* RNA polymerases I and II, the effect of OP concentration on enzyme activity was studied. A stock solution of OP, 10^{-2} M, pH 7.5, was diluted variously to range from 10^{-2} to 10^{-7} M. The concentrations of template, nucleotide, and other components were standard in all assays. Throughout, Mg^{2+} was the only activating cation. The effects of the nonchelating isomers. 1,7-, or 4,7-phenanthroline in the concentration range from 1 to 3×10^{-4} M was determined also.

Figure 6. The Effect of α-Amanitin on the Activities of RNA Polymerases I and II



Inhibition Studies With Other Chelating Compounds

The effect of other chelating agents on activity was studied as a function of their concentrations. Stock solutions of 8-hydroxyquinoline-5-sulfonic acid, EDTA, α-α′-bipiridyl or 8-hydroxyquinoline, all 5×10^{-2} M, were diluted with metal-free water to prepare dilutions ranging from 10^{-6} to 10^{-2} M, adjusted to pH 8. Assays were performed with 10 μg enzyme and under standard conditions. Magnesium was the activating metal in all cases.

Both polymerase I and II are inhibited by saturating amounts of chelating agents (table 3). 1,10-phenanthroline and EDTA inhibit both their activities completely. Other chelating agents such as 8-hydroxyquinoline 5-sulfonic acid, EDTA, and α-bipyridine, also at saturating concentrations, reduce the RNA polymerase II activity from 70 to 50 percent. At saturating amounts, the nonchelating analogs of 1,10-phenanthroline, 1,7- or 4,7-phenanthroline, do not inhibit either polymerase. Hence, the inhibition by the 1,10-isomer must be due to chelation of a functional metal atom.

The relative sensitivities of polymerase I and II to 1,10-phenanthroline were studied in detail over a range of inhibitor

Table 3. Inhibition of RNA Polymerases I and II by Chelating Agents

Agent	$V_1/V_c \times 100$	
	I	II
Chelating		
1,10-Phenanthroline	0	0
EDTA	0	0
8-Hydrozyquinoline	—	0
8-Hydrozyquinoline	—	—
5-Sulfonate	—	70
2,2′ Bipyridine	—	55
Nonchelating Analogs		
1,7-Phenanthroline	100	100
4,7-Phenanthroline	100	100

concentrations (figure 7). Both RNA polymerase I and II are inhibited by this agent but with different pK_I's, 5.2 and 3.4, respectively, further differentiating them (28,30). Collectively, these results were almost diagnostic of the presence and functional essentiality of zinc rather than of any other metal (31) although, of themselves, such studies cannot be decisive.

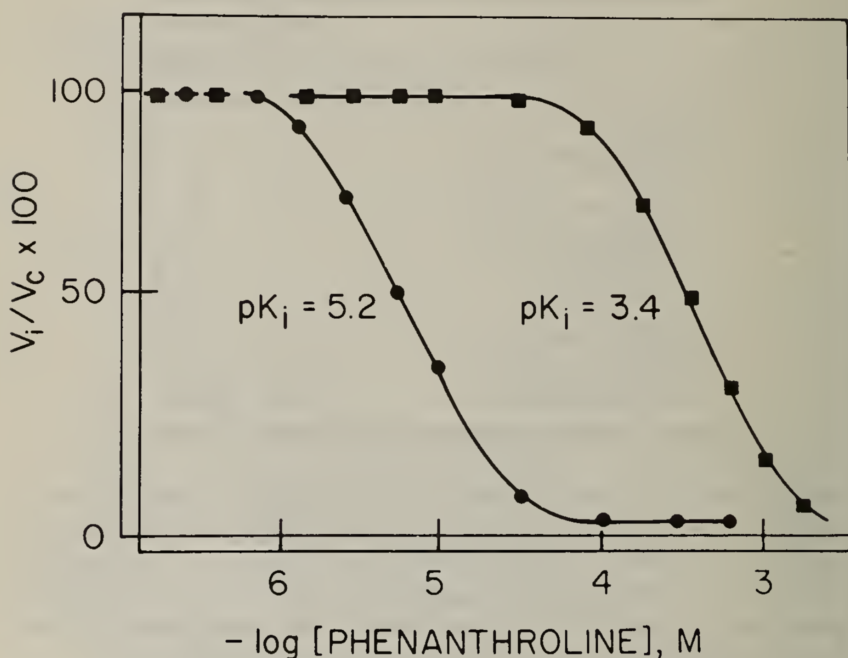
Studies of Metal Content of *E. Gracilis* RNA Polymerases

The presence of stoichiometric quantities of metal is, of course, essential to verify that chelating agents exert their effect by binding to a functional and/or structurally essential metal.

The presence of Zn, Cu, or Fe could account for the observed inhibition of the *E. gracilis* RNA polymerases. These elements and Mn were determined by microwave excitation emission spectrometry after removal of metal-quenching agents and low molecular weight protein contaminants by gel exclusion chromatography or dialysis against metal-free buffers. The elements and protein were measured quantitatively with high precision in fractions containing maximal activity when absolute amounts of metal varied from 10^{-11} to 10^{-14} g-atom and utilizing ~0.6 μg of enzyme (!) for analysis, a determination that would have been technically impossible only 3 years ago.

The *E. gracilis* RNA polymerase I and II contain 0.2 μg of Zn/mg of protein. Keeping in mind the as yet provisional nature of

Figure 7. The Effect on Enzyme Activity of Incubating RNA Polymerases I and II With 1,10-Phenanthroline



Both polymerases I and II are inhibited by this chelating agent, with pK_i's of 5.2 and 3.4, respectively.

the molecular weights of these polymerases, which form the basis of the metal/protein ratio, the stoichiometry of both is essentially the same; i.e., 2 g-atom of zinc per mole (table 4) (28,30).

Table 4. Metal Content of RNA Polymerases I and II as Measured by Microwave Excitation Spectroscopy

RNA Polymerase	Protein mg/ml	ZN μg/mg Prot.	g-atom/mole
I	0.12	0.19	2.0
II	0.10	0.21	2.2

Fe, Cu, Mn < 0.2 g-atom/mole
Metal content is expressed as g-atom per mole of 650,000 and 700,000 for the RNA polymerases I and II, respectively.

The sum of Cu, Fe, and Mn is less than 0.2 g-atom/mole. Thus, both RNA polymerase I and RNA polymerase II from zinc-sufficient *E. gracilis* are zinc metalloenzymes. Concurrently, in our laboratory, the eukaryotic DNA-dependent RNA polymerase I of yeast was also shown to be a zinc enzyme, containing 2.4 g-atom of zinc per molecular weight 650,000 (32).

Studies With Zinc-Deficient (-Zn) *E. Gracilis*

Our demonstration that RNA polymerase I and II from (+Zn) *E. gracilis* were both zinc enzymes (15,28) suggested that the metabolism of these enzymes might be affected in (-Zn) cells and prompted us to study RNA polymerase function in these organisms. We have found that (-Zn) *E. gracilis* contain a single, unusual RNA polymerase that is also a zinc metalloenzyme (33). This difference in the content of RNA polymerase in (-Zn) cells confirmed that deficiency of this metal indeed altered the metabolism of RNA polymerases and, further, highlighted the need to investigate its RNA products. Consequently, we compared the amounts of RNAs synthesized by both (+Zn) and (-Zn) cells. The total RNA from (+Zn) and (-Zn) cells was isolated by a standard phenol ethanol extraction.

The various RNA classes in the total RNA's were separated by using a series of affinity columns. Cytoplasmic mRNA contains a poly A segment, which is absent in other RNA's. Oligo-(dT)-cellulose columns will bind only the poly A containing mRNA and, thus, afford a rapid purification method (34). The RNA's that did not bind to the Oligo-(dT)-cellulose column were chromatographed on a dihydroxyborylamino ethyl (DBAE) cellulose affinity column. This column binds transfer RNA and separates it from the bulk of ribosomal RNA and the minor amino-acylated transfer RNA fractions (35). By sequential use of these columns, the three major classes of RNA were obtained. Each fraction was hydrolyzed in preparation for analyzing their base composition using high pressure liquid chromatography.

(+Zn) *E. gracilis* contain 20 μg of RNA/10^6 cells. This value is virtually unaltered by zinc deficiency (table 5). In both cases, the total RNA content is consistent with values obtained for *E. gracilis* grown to early stationary phase (36).

The total RNA is resolved into three fractions by sequential chromatography on Oligo-(dT) and DBAE celluloses. The mRNA fraction binds to Oligo-(dT)-cellulose, and approximately 90 percent or more of the RNA does not bind. This larger RNA fraction

Table 5. RNA Content* of Zinc-Sufficient, (+Zn), and -Deficient, (-Zn), *E. Gracilis*

	Total RNA μg × 10^6/cell	Ribosomal %	Transfer %	Messenger %
(+Zn)	20 ± 5	79 ± 5	15 ± 3	6 ± 2
(-Zn)	19 ± 5	74 ± 4	15 ± 3	11 ± 3

*Values are the mean ± 1 S.D. of six preparations

is then applied to a DBAE cellulose column that separates the tRNA from the ribosomal fraction. The amounts of ribosomal, transfer, and messenger RNA obtained by these methods are similar to those reported by others for eukaryotic organisms, including *E. gracilis* (36). In (-Zn) cells, the fraction of the total RNA present as rRNA is slightly less, while that present as tRNA is essentially the same as in (+Zn) cells (table 5). In contrast, the mRNA content of (-Zn) cells, 11 percent of the total RNA, is almost twice that of (+Zn) cells (table 5).

The analysis of base composition of the various RNA fractions, determined by high-pressure liquid chromatography, is highly reproducible, varying by about 1 to 2 percent in multiple experiments (37).

The base compositions for ribosomal, transfer, and messenger RNA from (+Zn) cells are in agreement with the values obtained for *E. gracilis* using other methods (36). For rRNA (table 6), the purine and pyrimidine contents are identical for (+Zn) and (-Zn) cells. For tRNA we analyzed only for the four major bases and found that the guanine content decreases in (-Zn) cells from 34 to 24 percent, while the cytosine content increases from 27 to 38 percent. The adenine and uracil contents are identical (table 6).

The base composition of mRNA from (-Zn) cells differs strikingly. Figure 8 compares the chromatogram of an mRNA from (+Zn) cells with that of an mRNA sample from (-Zn) cells eluted from the cation exchange gel of the high-pressure liquid chromatogram (HPLC). In the former, only four bases are found. They are identified as uracil (U), guanine (G), cytosine (C), and adenine (A), respectively, based on their elution volumes from the (HPLC) system and the UV spectra of each fraction. In contrast, the mRNA from (-Zn) cells (figure 8) contains seven major peaks and several minor ones. The uracil, quanine, cytosine, and adenine fractions in this chromatogram also have been identified by their

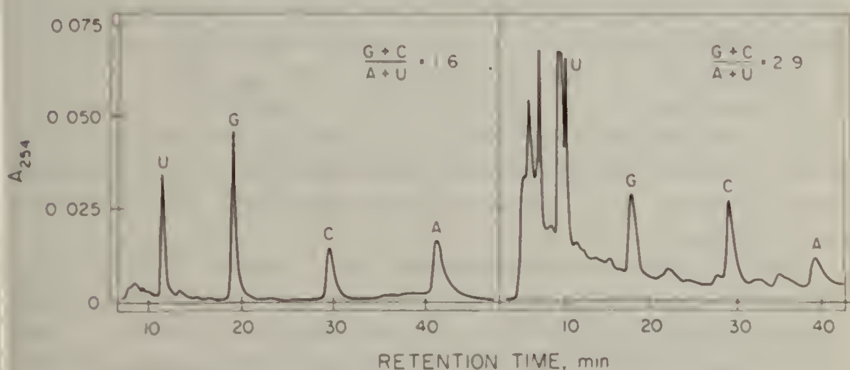
Table 6. Base Composition of *E. Gracilis* RNA

Base	+Zn			-Zn		
	Ribosomal	Transfer	Messenger	Ribosomal	Transfer	Messenger
Guanine (G)	36	34	35	36	24	25
Cytosine (C)	26	27	26	26	38	49
Adenine (A)	21	19	21	22	20	10
Uracil (U)	17	20	18	16	18	16

The data are expressed as μg of each base/μg G + C + A + U × 100. The mRNA from (-Zn) cells contain a number of additional bases (figure 8) that are not included in this calculation. Each value is the mean of three analyses.

characteristic UV spectra. The remaining fractions represent bases whose identities are presently unknown but which are not found in mRNA from (+Zn) cells. The calculated G + C/A + U ratios of the mRNA samples, 1.6 for (+Zn) and 2.9 for (-Zn) cells, differ strikingly. Thus, mRNA from (-Zn) contains additional bases, and the ratio of the known major purines and pyrimidines is nearly double, as compared to the mRNA from (+Zn) cells.

Figure 8. High-Pressure Liquid Chromatographic Analysis of Base Composition of mRNA from (+Zn) and (-Zn) *E. gracilis*



mRNA from (+Zn) (left) and (-Zn) (right) cells were acid-hydrolyzed prior to analysis by high-pressure liquid chromatography. The G + C/A + U ratio of the mRNA's from both (+Zn) and (-Zn) cells differ, being 1.6 and 2.9, respectively. In addition, a number of bases are present in the mRNA from (-Zn) cells which are absent in the control (+Zn) cells.

There are various mechanisms through which zinc deficiency could affect RNA and DNA metabolism (19,28). In particular, we have emphasized recently the essentiality of zinc for the function of both DNA and RNA polymerases. We have also noted the importance of Mn(II) [or Mg(II)] for the activity of these enzymes. However, the possible synergism and/or antagonism of these and other metals in nucleic acid polymerase action in general, and particularly, on the base composition of the resultant RNA product, has not been examined critically. This is of interest because one consequence of zinc deficiency in *E. gracilis* is a 35-fold increase in intracellular Mn and a 5-fold increase in Mg content (18,20). This, together with earlier experiments using micrococcal DNA polymerase (38), *E. coli* RNA polymerase (39), and viral reverse transcriptases (40) demonstrating that the base composition of the nucleic acid product synthesized in vitro varied according to the particular activating cation employed, prompted us to examine the role of these metals in determining the composition of the products. Thus, we have determined the effects of varying Mn(II) concentrations on the incorporation of bases into RNA produced by RNA polymerases I and II from (+Zn) cells and the single, unusual RNA polymerase from (-Zn) *E. gracilis*. These experiments were carried out in cell-free systems, and the incorporation of CMP, UMP, or AMP served as the criterion of the base composition of the resultant RNA.

The effects of various Mn concentrations on the relative incorporation of UMP and CMP into RNA by the different *E. gracilis* RNA polymerases are shown in table 7. Increasing the Mn concentration from 1 to 10 mM in assays with RNA polymerase I or II from (+Zn) cells decreases the ratio of UMP to GMP incorporated from 1.7 to 1.0 and 2.1 to 0.8, respectively. Similarly, in assays of the single enzyme from (-Zn) cells, this ratio decreases from 3.5 to 0.4. Thus, the base composition of RNA synthesized by polymerases from either cell type varies as a function of Mn concentration.

These studies show that the total RNA content per cell is not altered by zinc deficiency (table 6). Moreover, each of the RNA classes—ribosomal, transfer, and messenger—is present in these cells. Thus, changes in the amount of each RNA class synthesized would not appear to be responsible for the biological effects of zinc deficiency in *E. gracilis*. A remarkable difference does exist, however, between the composition of mRNA from (-Zn) and (+Zn) cells. The mRNA from (-Zn) cells has an unusual base composition as demonstrated by the twofold increase of its G + C/A + U base ratio and the presence of significant amounts of bases

other than uracil, guanine, cytosine, and adenine (figure 8). Although the sequence of bases in mRNA coding for different proteins does vary, such differences in mRNA base composition are most unusual because the ratio of G + C/A + U bases has been found to be consistently uniform for nearly all mRNA's.

The changes in mRNA composition of the degree observed between (+Zn) and (-Zn) cells have not been reported previously in cells deprived of essential nutrients or as a function of growth or cell cycle stage. Therefore, the changes in composition of mRNA from (-Zn) cells reveal critical differences in their mRNA metabolism. These must be the consequence of either an alteration in the processes that normally regulate the incorporation of bases into mRNA or the accumulation of mRNA molecules coding for large amounts of a specific protein(s). We have obtained evidence suggesting that the increases in the content of other metals such as Mn in (-Zn) cells could play a role in the production of such mRNA's (table 7). These results, together with the finding of a single RNA polymerase in (-Zn) organisms, represents the first major metabolic difference between these and (+Zn) organisms whose manifestations would provide a basis for the arrest of cell division in this organism. Thus, mRNA plays a central role in the translation of information from the genome into proteins, which in turn, determine the phenotype. The mechanism by which genetic information is faithfully translated into proteins is dependent on the base composition and sequence of mRNA molecules. These two variables are involved in binding mRNA to ribosomes and determine the amino acid composition of the proteins synthesized. Our demonstration of the unusual composition of

Table 7. The Effect of [Mn(II)] on the Relative UMP/CMP Incorporation by *E. Gracilis* RNA Polymerases*

Mn(II) mM	RNA Polymerase		
	I	II	Single Enzyme From (-Zn) Cells
1	1.7	2.1	3.5
5	1.4	1.7	1.5
10	1.0	0.8	0.4

*Analogous results are obtained on comparing the relative incorporations of UMP/AMP.

3[illegible]-[illegible]49 [illegible] - [illegible]3 - 11

mRNA from (-Zn) cells suggests that, in these organisms, translational processes may be altered, leading to the formation of products of translation with unusual amino acid composition and/or to changes in the rate of synthesis of specific proteins that may be either essential for or inhibitory to cellular function. Indeed, abnormalities of protein metabolism in (-Zn) *E. gracilis* (18) and plants (41) have been documented, albeit only in terms of total protein and changes in amino acid contents. The effects of such alterations in protein metabolism would be decisive and result in the arrest of cell division in (-Zn) organisms because ongoing protein synthesis is required for this process (24,26,27,42).

Similarly, if extended to other systems, derangements in the metabolism of proteins involved in the formation of tissues and organs could lead to the developmental abnormalities characteristic of (-Zn) mammals (13).

These studies are part of an ongoing investigation aimed at identifying the biochemical basis for the role of zinc in cell division. They have led us to explore details of both DNA and RNA metabolism in both (+Zn) and (-Zn) cells, whose importance remains to be further elucidated. In general, however, the present systematic studies are relevant to understanding the essential role of zinc in cell division and development and also direct attention to a novel mode of regulation of the metabolism of nucleic acids and proteins, with profound implications for their mechanism.

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Discussion of Papers by Vallee and Falchuk

Dr. Russell: Dr. Vallee, in this last year we have had experience with six zinc-deficient alcoholic cirrhotics who were also night-blind. Night blindness persisted about 1 month after cessation of alcohol abuse. Two of these patients had abnormally low serum vitamin A levels, and they were treated with vitamin A and did not respond. They also had low serum zinc levels, and the dark adaptation responded within a week after replacing the zinc. The other patients had normal serum vitamin A values but abnormally low zinc levels. Within a few days of replacing zinc, in the form of zinc sulphate, their dark adaptation curves came back to normal. So my comment is that this may be a new thing that we ought to look for more often in our alcoholic subjects who are zinc deficient. My question is whether there are good, quantitative data in humans on how ethanol metabolism is actually affected in a zinc-deficient individual?

Dr. Vallee: To the best of my knowledge, no good data exist. We certainly have never done this, and in fact our efforts to relate zinc metabolism to human disease in terms of cirrhosis date back 20 years. I really have not done any more. But there is a point I am terribly interested in—your comment on night blindness. As a matter of fact, if you were to go back and look at the papers in the *New England Journal of Medicine*, you would find that we commented on this. At the time, I was particularly interested, but I think it is sometimes good to point out on these occasions that the literature does contain information not one's own, but other people's. There is a gentleman named Bliss who worked at Simmons College in Boston. He worked on alcohol dehydrogenase as a retinal reductase in eye tissue and claimed that it was the same enzyme we saw, as alcohol dehydrogenase. And indeed the alcohol dehydrogenase seems to attack that particular alcohol-aldehyde pair.

I then talked to some people in Boston at the City Hospital who were knowledgeable about cirrhosis and was guided to a paper by Patek, I think, in 1936, who had reviewed absolutely everything he had ever seen about cirrhosis and then some, and he talked about night blindness. Well, I knew Bliss, who has long since died, and I made a comment in our papers simply on this matter of

vitamin A and night blindness. Since that time, I had in my laboratory an expert on vision, Dr. Franz Damon, from Holland, and I asked him about this problem. He wrote a review later on it in *Biochim. Biophys. Acta*, and he was absolutely certain that the enzyme in the retina was not alcohol dehydrogenase. He said it had an alcohol dehydrogenase-like activity, but was not the same enzyme. But since then he has written that up, it must be about 2 or 3 years ago, in a *BBA* review. I think that the observations are extremely interesting, and I also really do believe (in fact, I am kind of incredulous) that this has set for 20 years and nobody has ever taken a recent real look at it. I think I would have, probably, if I hadn't had other things to do.

Dr. Schenker: It has been said and written that in people with alcoholism and liver disease, serum zinc level falls. Is this due to decreased intake, impaired transport of zinc via the gut, decreased storage of zinc, increased urinary elimination of the cation, or some combination of these? What do we know about these sort of things?

Dr. Vallee: The answer is, nothing is known. Absolutely nothing. That isn't to say that there isn't a lot written. But nothing is known. I don't believe that the matter of the relationship of zinc metabolism to alcohol metabolism has really ever been studied seriously in the manner in which you've asked. It is astounding. I would just simply like to add to that one item.

There is one particular protein, metallothionine, which I am sure you have learned of, which we found about 20 years ago. This protein has been accused of being everything, from a detoxifier to a storage agent; its role has never been known. The truth is, nobody knows if there is a storage protein for zinc, and it isn't even known how it is transported in serum. Nor is it known how it is absorbed. It is conjectured upon, but how all of that might relate to alcoholism, I haven't the faintest idea.

Dr. Noble: I found both presentations, Drs. Vallee and Falchuk, extremely intriguing. They raised a question in my mind about some of the studies we have been doing on the effect of alcohol on brain protein and RNA metabolism. We have actually found a decrease in RNA synthesis as well as in DNA synthesis, and I think Dr. Schenker has been working on that area, too. We have been searching for a mechanism to explain why we get these decreases in the brain. In a couple of the places we have looked at, one is the RNA polymerases and the other is the tRNA synthetases, we found a decrease in the activity of both those enzymes. Now, this raises the association of what role zinc plays in the findings we have obtained here.

Dr. Falchuk: The interrelationships of all of these systems, whether it be the DNA polymerases or the RNA polymerases, and all the way down, are so complex. There are so many factors involved, even just in terms of tRNA synthetase function, what it does beyond that. How tRNA binds, i.e., the binding factors, the elongation factors, and the releasing factors, many of which are very poorly characterized, particularly in mammalian systems, and certainly in brain. I think it would be dangerous to make any conclusions based on the decreased activity unless you knew all of the factors involved. What zinc would be doing in that condition, I think, is totally unknown, and I think that is something that really needs to be explored.

Dr. Vallee: I think that having findings such as you do, it would really be worthwhile to see whether or not this is a metalloenzyme. It is no longer that difficult. It is now about as obvious a thing to look for as one used to look whether or not an enzyme had NAD dependence or not. And so I think there is certainly a reason to look. The caveat that Dr. Falchuk brought up is surely correct. Those data that Dr. Falchuk showed, I think as far as I am concerned, are very important. There are major fundamental changes in the genetic material, environmentally conditioned in a manner that you simply could not possibly guess at. Not only is the coding obviously influenced intrinsically, but environmental changes occur.

Dr. Schenker: In connection with Dr. Noble's question, I wonder if I could ask Dr. Lieber a question. In the animal system that Dr. Noble and Dr. Tawari have used to show the changes in RNA in the brain of animals drinking alcohol for a prolonged period of time, I wonder, Dr. Lieber, if anyone has ever assayed the tissues of the animals that have been on the liquid alcohol diet to actually determine what the zinc concentration would be in various tissues of these animals—to see whether prolonged alcohol ingestion of the type that Dr. Noble has discussed has, in fact, induced measurable changes in zinc level in these tissues. I was unaware of any such data.

Dr. Lieber: I don't believe there are such data, and the reason we have not looked at those, although we have thought about it, is we keep our rats in galvanized cages, and so we felt that there was not much point in doing that until we shifted the way we keep our animals. Then we will probably do that one of these days.

Dr. Hurley: I have a comment on Dr. Lieber's answer. If alcohol ingestion produces a metabolic zinc deficiency, then it wouldn't matter if the rats have galvanized cages or not, although

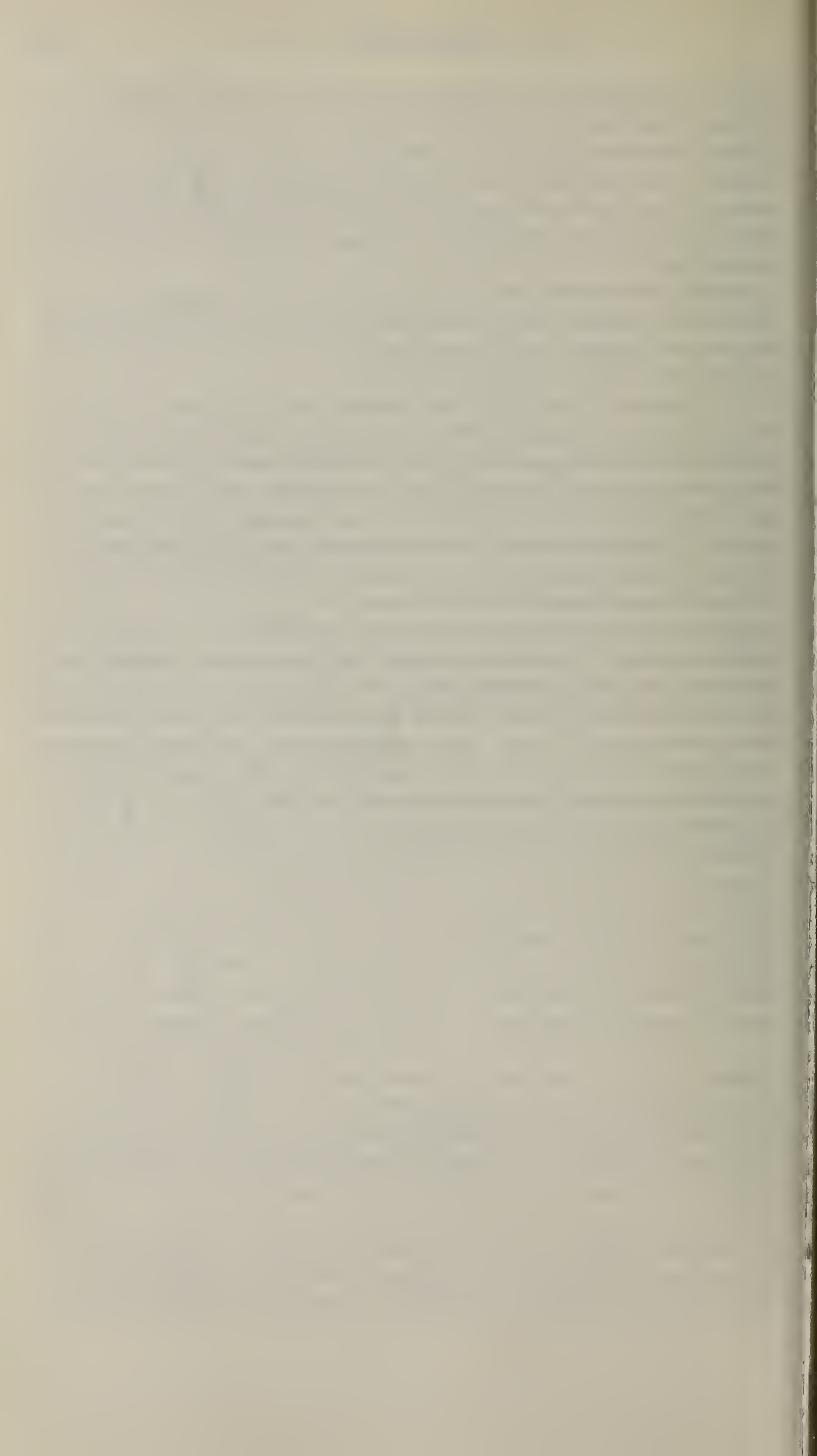
there should still be an effect. It is not, perhaps, as strict as it would be the other way.

Dr. Lieber: It is my understanding that whatever zinc deficiency has produced can be easily corrected with zinc administration, even in the presence of alcohol. I believe that we will have to get rid of our galvanized cages to get the effect of zinc deficiency.

Dr. Hurley: Dr. Falchuk, in the case of the change in the enzyme with manganese and magnesium, do you think that these metals are actually substituting for the zinc in the enzyme and thereby producing a different effect?

Dr. Falchuk: First of all, the answer to your question has two parts. No one knows to date what the role either of zinc or of manganese and magnesium or any of the other metals is in the actual function of the RNA and DNA polymerases. There are many hypotheses that have to do with binding of template and binding of nucleotide, but that isn't really known specifically. So to begin with, there is no way we can actually answer that question from a functional point of view.

I think it is clear that both enzymes, or all of the enzymes we isolate, contain 2 g-atoms of zinc. And you cannot dialyze that zinc out, either by routine dialysis or dialysis against a chelating agent. The effect of the addition of manganese and magnesium is phenomenological. Now, until we know the specifics of what these metals do, there is no way that your question can be answered, but I do not think at this time that they are going in and replacing zinc as a metalloenzyme.



Effect of Ethanol on Zinc Metabolism

Ananda S. Prasad

Zinc Deficiency in Human Subjects

In the fall of 1958, Dr. James A. Halsted brought to my attention a 21-year-old patient at Saadi Hospital, Shiraz, Iran, who looked like a 10-year-old boy and had severe anemia. In addition to dwarfism and anemia, he had hypogonadism, hepatosplenomegaly, rough and dry skin, mental lethargy, and geophagia. The patient ate only bread made of wheat flour, and the intake of animal protein was negligible. He consumed nearly 1 pound of clay daily, and on further investigation it was clear that the anemia was related to iron deficiency. There was no evidence for blood loss. Shortly thereafter, 10 additional cases were studied in Shiraz, Iran. In our initial report concerning this syndrome, it was speculated that a deficiency of zinc was probably a complicating factor accounting for growth retardation and hypgonadism (1). We were convinced that the growth and gonadal effects were not due to iron deficiency, because under experimental conditions iron deficiency does not affect growth or gonadal functions.

Subsequently, similar cases were investigated in Cairo, Egypt. We were able to study such patients extensively and concluded that, indeed, several of their clinical manifestations were related to a deficiency of zinc (2,3). This conclusion was based on the following information. The zinc concentrations in plasma, red cells, and hair were decreased. Radioactive zinc-65 studies revealed that the plasma zinc turnover rate was greater, the 24 h exchangeable pool was smaller, and the excretion of zinc-65 in stool and urine was less in the patients than in the control subjects. Finally, a controlled trial with zinc supplementation resulted in reversal of the clinical features, thus supporting the hypothesis that zinc deficiency existed in human subjects. Poor availability of zinc from the village diet, excessive loss of zinc due to sweating, and blood loss due to hookworm infestation were considered to be the major factors accounting for zinc deficiency in Egypt.

Many etiological factors responsible for zinc deficiency in humans have been recognized in the past decade (4). Besides

nutritional factors, liver disease, malabsorption, certain renal diseases, burns, acrodermatitis enteropathica, sickle cell anemia, and total parenteral nutrition are some of the conditions in which zinc deficiency may be a complicating feature.

The clinical manifestations of human zinc deficiency include growth retardation, hypogonadism, rough skin, abnormal oral glucose tolerance curve, general lethargy, and poor appetite. Poor wound healing in marginally zinc-deficient patients has been reported by several investigators. Other manifestations, such as susceptibility to infections and lymphopenia, have been reported in experimental zinc-deficient animals. Fetal abnormalities and behavioral changes, as a result of zinc deficiency during pregnancy, have also been reported in animal models.

Severe zinc deficiency may be seen in patients with acrodermatitis enteropathica, patients on total parenteral nutrition, and patients on penicillamine therapy. In such cases, skin changes seem to predominate and consist of parakeratosis or moist eczematoid dermatitis, most severe in the peri-oral, -anal and -orbital areas, and alopecia. In acrodermatitis enteropathica, dermatological manifestations also include progressive bullous-pustular dermatitis of the extremities and the oral, anal, and genital areas; paronychia; and alopecia. Diarrhea, malabsorption, steatorrhea, lactose intolerance, mental apathy, retardation of growth, and hypogonadism have been also reported in acrodermatitis enteropathica.

Laboratory findings of human zinc deficiency include a decreased level of zinc in plasma, red cells, hair, and urine. Zinc deficiency may be associated with hyperzincuria in liver disease, sickle cell anemia, certain renal diseases, after injury and surgical trauma, burns, acute starvation, and as a result of total parenteral nutrition.

Zinc-65 studies show an increased plasma zinc turnover rate and a decrease in 24-hour exchangeable pool. Zinc balance study reveals a positive retention of zinc. The activity of ribonuclease in the plasma may be increased and, following supplementation with zinc, the activity of alkaline phosphatase in plasma increases.

Biochemical Effects of Zinc

Many enzymes need zinc for their function (5). During the past 15 years, at least 30 enzymes that require zinc for their activity have been identified. If related enzymes from different species are included, then more than 70 zinc metalloenzymes would be on record. Zinc is present in several dehydrogenases, aldolases,

peptidases, and phosphatases. Alcohol dehydrogenase, carboxypeptidase, carbonic anhydrase, and alkaline phosphatase are some examples of zinc metalloenzymes.

Zinc plays a significant role in RNA and DNA synthesis and catabolism of RNA. RNA and DNA polymerases, and RNA-dependent DNA polymerase in the reverse transcriptase of avian myeloblastosis and other viruses have been shown to be zinc metalloenzymes. Recent studies suggest that thymidine kinase is a zinc-dependent enzyme and is very sensitive to a lack of zinc (6). The activity of RNase is inhibited by zinc, thus the catabolic rate of RNA is zinc dependent. Growth retardation, so commonly the result of zinc deficiency, is most likely due to its effect on nucleic acid metabolism and decreased protein synthesis.

Zinc content of testes and bone decrease within 3 weeks of institution of zinc-restricted diet in rats. A decrease in the zinc content of rat kidneys and esophagus becomes apparent at the end of 4 to 5 weeks of dietary zinc restriction. Carboxypeptidase, thymidine kinase, and alkaline phosphatase appear to be very sensitive to zinc restriction, inasmuch as their activities are affected adversely within 1 week of dietary manipulation in rats. Recent studies indicate that there is a specific effect of zinc on testes and that gonadal function in the zinc-deficient state is affected through some alteration of testicular steroidogenesis and spermatogenesis.

Effect of Alcohol on Zinc Metabolism

Metabolic studies suggest that chronic alcoholism alters myocardial metabolism, even in patients without objective signs of heart or liver disease (7). The liberation of the zinc-dependent enzyme malic dehydrogenase (MDH), and the magnesium-dependent enzyme isocitric dehydrogenase (ICDH) suggests alterations of citric acid metabolism and altered membrane permeability as a consequence of chronic alcoholism. In the cardiomyopathic state, the cytoplasmic enzymes (including the zinc-dependent enzyme lactic-dehydrogenase (LDH) and aldolase) were liberated by the myocardium, both at rest and after exercise, indicating a diffuse cellular derangement. The studies suggest that repeated ingestion of alcohol may result in permanent alteration in the cellular metabolism and, in some patients, an irreversible alcoholic cardiomyopathy.

In acute studies following alcohol ingestion, zinc was liberated by the myocardium. Zinc was found in higher concentration in

the coronary vein than in arterial blood. Following alcohol ingestion, the activity of alcohol dehydrogenase (zinc metalloenzyme) increased in the peripheral venous blood as plasma zinc level decreased. Similar observations with respect to plasma zinc and other zinc metalloenzymes LDH and MDH have been made in myocardial infarction.

In another study, normal volunteers, after fasting for 10 hours, were given 6 ounces of chilled vodka by mouth (8). More than 3 hours after ingestion of alcohol, peripheral venous blood samples were obtained to determine zinc and copper levels. Urine samples were obtained as follows: fasting early morning specimen before alcohol ingestion and complete urine collection during first 3 hours and second 3-hour periods following alcohol ingestion. The urinary data were expressed in μg/g of urinary creatinine. A significant decrease of serum zinc was observed in patients 1 to 3 hours after intake of 6 ounces of vodka, parallel to the decreased serum levels of zinc. There was a significant increase in the urinary excretion in these patients. The increased excretion of zinc may have been due to loss of available binding sites for zinc on the proteins, thus rendering zinc more diffusable. Increased cellular permeability as a result of alcohol ingestion may also account for release of cellular zinc and its excretion by the kidneys. The increase in serum levels of copper could also be related to increased cell membrane permeability and a release of copper-containing proteins into the serum. Copper is, however, bound more tightly to proteins than is zinc, and may thus compete favorably with zinc for available binding sites. The extent of acetylation of proteins, amino acids, and amines due to alcohol has not been studied extensively, and awaits further examination.

Vallee et al. (9) have shown that patients with alcoholic cirrhosis of the liver excrete abnormally large amounts of zinc in the urine, even though their serum zinc levels are low. These observations have been confirmed by many other investigators. Zinc content of the cirrhotic liver was also found to be decreased. These findings have particular significance inasmuch as alcohol dehydrogenase is a zinc enzyme responsible for the initial step in the metabolism of alcohol. Previous observations have indicated that a certain percentage of alcoholic subjects also excreted increased amounts of zinc even though clinical or laboratory evidence of chronic liver disease was absent (10). The serum zinc levels of the alcoholic subjects tend to be lower in comparison to the controls. An absolute increase in renal clearance of zinc in 33 percent of the alcoholics demonstrable at both normal level and low serum zinc concentrations has been observed (11). Thus the measurement

of renal clearance of zinc may be clinically utilized for etiological classification of chronic liver disease due to alcohol in different cases. Excessive ingestion of alcohol may lead to severe deficiency of zinc. In one case, acquired zinc deficiency due to alcohol was characterized by mental disturbances, widespread eczema craquele, hair loss, steatorrhea, and dysprotenemia with edema (12). Therapy with zinc reversed these manifestations. A similar clinical syndrome has been seen among Ugandan blacks addicted to banana gin.

Wang and Pierson (13) have reported that the zinc content of alcoholic rat liver declines promptly within 2 weeks of alcohol ingestion. A more gradual decline of zinc content in muscle was seen in their experiments. Alcohol-treated rats have significantly less zinc in their mitochondria as compared to the controls. Although liver and muscle mitochondria were exposed to similar plasma alcohol levels, liver mitochondria were considerably more affected in regard to zinc depletion as compared to the muscle. Absence of alcohol-metabolizing pathways in the muscle indicates that other zinc-dependent enzymes are also inhibited by alcohol. These observations indicate that the effects of alcohol on zinc depletion are not specific for liver.

Recent studies by Yunice and Lindeman (14) indicate that both ascorbic acid and zinc exert protective effects in ethanol-intoxicated rodents. Abdulla et al. (15) have reported that ethanol inhibits the activity of delta-aminolevulinic acid dehydratase in rats, and this effect of ethanol is reversed by administration of zinc in vitro in experimental animals.

In summary, the effects of zinc deficiency in humans and experimental animals have been reviewed. The pertinent data with respect to the effects of alcohol on zinc metabolism have been discussed briefly. The circumstantial evidence implicates an adverse effect of alcohol on zinc metabolism. Definitive studies must be carried out to fully understand the implications of these findings in human alcoholic subjects.

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Discussion of Paper by Prasad

Dr. Rudman: I have two questions, Dr. Prasad. First of all, as far as the endocrine characterization of the hypogonadism in the zinc-deficient children, if this is really an end organ effect of zinc deficiency, then they should have high levels of LH and FSH in their plasma, that is, a state of hypergonadotropic hypogonadism. I wonder if LH and FSH have been measured. Second question is, dealing in the interpretation of the serum zinc level, this is an extensively protein-bound cation, and a lot of it is bound to albumin, isn't that true?

Dr. Prasad: Quite a bit is bound to albumin.

Dr. Rudman: So that hypoalbuminemia will lead to low zinc levels. I am thinking of the interpretation of the serum calcium level, for example, in people with hypoalbuminemia. If we plotted serum calcium in the patients with liver disease, we would see an apparent hypocalcemia because of the hypoalbuminemia; on the other hand, if we plotted the free or nonprotein-bound calcium, that value might not be reduced. So I am just wondering how you take into account the hypoalbuminemia in interpreting the serum zinc level.

Dr. Prasad: With respect to your first question, we do not have very good data on FSH and LH from the Egyptian dwarfs. I was there almost 14 years ago, and at that time, techniques for FSH and LH assays were not available. However, I think that sickle cell disease patients with growth retardation were also an example of zinc deficiency, and we have data on those patients. They do have high FSH and LH. With respect to the binding of zinc to albumin I would say approximately 50 to 55 percent is carried by albumin, but the binding is not very tight. It is also carried by ceruloplasmin, transferin, haptoglobin—almost every protein will tie it up. And if I am not mistaken, I think somebody has tried to correlate the serum zinc and the albumin level in the liver disease patients, and they have not come to any correlation to the same extent as you see with calcium and hypoalbuminemia. So it is important. I would say that it must have some effect, because so much of zinc is carried, but I do not know to what extent it is important.

Dr. Beard: Does anyone really know the total amount of zinc that is bound in plasma?

Dr. Prasad: Yes, I think there are very good data on that. We reported this in an extensive study in 1970 in the *Journal of Laboratory and Clinical Medicine*. There is no more than 3 to 5 percent, that is ultrafilterable, and this 3 to 5 percent is bound to the amino acids in the ultrafiltrate, and the rest is bound to the proteins. The binding to albumin is not very tight. Binding to ceruloplasmin and alpha-2 macroglobulin is very tight. In other words, if you do competition experiments with histidine and some other binding agents, the protein hangs on to it.

Dr. Beard: I was curious because we had been using filtration cones, 50 microns, and we were getting around 98 percent that was bound, and I thought it was extremely high. Do you know whether or not alcohol per se can change the binding, acutely and chronically?

Dr. Prasad: Well, I think this is something which really should be looked at, because I think some investigators have tried to correlate the hyperzincuria with amino acids in the urine, and that has not panned out. So it is possible that we are altering the protein binding site, particularly in view of the fact that the acute effects that I have seen in 3 hours seem to indicate that it must do something right at the level of protein binding. It would be a good experiment. I have not done it.

Dr. Li: Dr. Prasad, I wonder, in the experiments where you show the decrease in alkaline phosphatase activity, could you restore the activity by addition of zinc?

Dr. Prasad: Not in vitro. But you do if the induction is done in vivo. As a matter of fact, none of the zinc enzymes that I know of—this is quite different from lysyl-oxidase where you can, with respect to copper enzyme, but not with respect to zinc.

Dr. Dietschy: I am interested in the geophagia exhibited by the zinc-deficient human. Rats will also show this, and I was wondering if you feel that it is an attempt at supplementation or whether it actually aggravates the zinc deficiency?

Dr. Prasad: Well, my first impression was that geophagia made it worse. This was just because of the bulk, and we felt that they were eating a pound of clay a day in Iran, that must have some adverse effect. But I think Dr. Smith is here, maybe he can tell his experience in the rat. He thinks that perhaps, at least for the rats, when they eat clay, this has a protective effect. But I know of one different study in human subjects, and that is from St. Louis. They reported on iron metabolism and showed that geophagia does inhibit iron absorption.

Effect of Ethanol on Magnesium Metabolism

James D. Beard

In 1954, hypomagnesemia was described in alcoholic subjects with delirium tremens (10). Because the symptoms of these patients were similar to symptoms of magnesium-deficient rats (14), it was suggested that delirium tremens might be the result of magnesium deficiency. These observations stimulated considerable research in the area of alcoholism as well as in the area of magnesium metabolism in general. Most of the alcohol investigations were carried out before 1969. With the onset of atomic absorption spectrophotometry in the mid-1960's, improved, precise, and rapid methodology became available; this technology also contributed to increased research concerning magnesium metabolism in human disease. However, since 1969 there has been an almost complete absence of research on the effect of ethanol on magnesium metabolism. Indeed, the majority of the research conducted concerning magnesium metabolism has been performed on individuals following abstinence from alcohol. Furthermore, throughout the available literature, few studies have been conducted on animals.

Because one of the major purposes of this NIAAA-sponsored workshop is to discuss recent gains in knowledge concerning the effects of ethanol on nutrient balance, I am chagrined to state that the current state of the art on the effect of ethanol on magnesium metabolism is the same as it was in 1969.

This presentation will focus on the following: (1) magnesium metabolism following abstinence from alcohol, (2) effect of ethanol on magnesium metabolism, (3) current advances in magnesium metabolism, and (4) recommendations for future research.

Magnesium Metabolism Following Abstinence From Alcohol

The majority of the research effort in examining alcoholics following abstinence from alcohol is included in articles and books published in 1969 and 1971 (8,9,11,17,22,23). In these studies,

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a number of investigators reported similar findings. The studies revealed that a majority of the withdrawing alcoholic patients showed the following: (1) hypomagnesemia, (2) reduced magnesium content in muscle, (3) reduced exchangeable magnesium, (4) retention of significant amounts of intravenously administered magnesium, and (5) positive total magnesium balance. In general, evidence indicated a correlation between reduced serum magnesium concentrations and the appearance of withdrawal signs and symptoms. The more severe withdrawal states (i.e., delirium tremens) were frequently associated with the greatest reduction in serum magnesium concentrations. However, hypomagnesemia frequently occurs in alcoholics in the absence of delirium tremens, and, indeed, normal concentrations of magnesium can be found in patients with delirium tremens. Thus, the time the sample is obtained following cessation of drinking should be determined.

Many alcoholics may present with normal or slightly reduced serum magnesium levels. There can be, however, a sudden fall in serum magnesium concentration between 14 and 24 hours after the last alcoholic drink. This decrease in magnesium is correlated with the onset of signs and symptoms and a reduced threshold for photomyoclonus. If magnesium is administered, the seizure threshold is increased. The rapid decrease in serum magnesium is associated with a concomitant increase in arterial pH and reduced P_{CO_2}. Both hypomagnesemia and alkalosis are known to be associated with hyperexcitability of the central nervous system. However, the precise relationship between hypomagnesemia and alkalosis needs to be elucidated. Furthermore, the mechanism that might cause such a sudden shift in magnesium level is unknown.

More recently, it has been reported that patients with alcohol withdrawal seizures had significantly lower arterial and cerebrospinal fluid (CSF) concentrations of magnesium than did controls or individuals who had delirium without antecedent alcohol withdrawal seizures (5). However, the CSF samples were obtained 5 to 12 hours after the seizure; thus, it is difficult to ascertain whether the reduction in CSF magnesium could have resulted from the seizure, or even whether the values were lower at the time of the seizure. Excess susceptibility to seizures observed in magnesium-depleted rats was prevented when cerebrospinal fluid magnesium concentrations were replenished, although increased plasma magnesium concentrations alone were ineffective (6).

Effect of Ethanol on Magnesium Metabolism

No long-term balance study has been conducted using enough subjects to determine if the chronic ingestion of ethyl alcohol can produce a negative magnesium balance and thus lead to magnesium deficiency. Because of frequently observed hypomagnesemia and "magnesium deficiency" in chronic alcoholics, investigators turned their attention to the kidney for a possible explanation (12,16). It was found that ethanol given normal and alcoholic subjects resulted in an acute increase in magnesium excretion. The onset of the magnesium diuresis was very rapid and usually occurred during the first 3 hours following ethanol administration. The increased magnesium excretion was not accompanied by alterations in renal blood flow or in glomerular filtration rate. In addition, the magnesium diuresis was independent of the water diuresis. Thus, it was suggested that increased urinary magnesium loss could contribute to the magnesium depletion in alcoholics.

In a subject depleted of magnesium for 49 days, it was found that ethanol administered during the last 8 days did not increase the urinary excretion of magnesium during this time, nor was there any change in stool magnesium (7). This article is cited quite often to indicate that, on a chronic basis, alcohol may not contribute to increased magnesium loss. However, it is usually not stated that this study was conducted on only one subject.

Several other studies have been conducted in which alcohol has been consumed over varying periods of time, ranging from 15 to 30 days (17). However, it is difficult to analyze the data because each subject is presented individually, and no actual data are summarized. However, it is obvious from inspection that during the period of alcohol consumption there are only minor fluctuations in the serum magnesium concentration. It would appear that there is an enhancement of urine magnesium; however, this is difficult to ascertain. It is obvious, however, that urinary magnesium excretion falls rapidly following cessation of ethanol intake (17,22,23). These data are difficult to interpret because these were not balance studies. Thus the question remains as to whether alcohol significantly increases magnesium excretion during chronic intake.

In rats that received 36 percent of their total calories as ethanol for 4 weeks, it was found that there was no significant difference in muscle magnesium as compared to that of pair-fed controls (20). Because the muscle magnesium was normal, it was assumed that there was no magnesium deficiency in these animals. However, this was not a balance study, and, indeed,

considerable magnesium deficiency can occur with normal muscle concentrations of magnesium.

More recently, the acute effect of ethanol on serum magnesium has been determined. Following administration of ethanol to rats, it was found that there was a gradual increase in serum magnesium that reached significance following 4 hours (18). This alteration in serum magnesium was also found in dogs (19); in this study, a significant increase in plasma magnesium was found to occur for 10 hours following the administration of ethanol (3 g/kg body wt). In addition, the 24-hour urinary magnesium excretion level was determined, and it was found that during the first 6 hours there was a significant increase in magnesium excretion in the ethanol-treated animals. Thereafter, no significant difference was observed; indeed, very little difference in the 24-hour urinary excretion was found between controls and ethanol-treated animals. It is of interest that the increase in serum magnesium was associated with increased P_{CO_2} and reduced pH, a finding opposite to that observed following cessation of alcohol intake (22,23).

We have just completed a dose-response study performed over 24 hours. Eight rats were used for each dose of ethanol; the same animals received, at different times, the same volume of water as the ethanol solution. It was found that with the lowest dose (0.75 g/kg as a 25-percent solution), there were no alterations in the plasma magnesium concentration. An initial increase in urine magnesium was found, but on a 24-hour basis, no significant difference was observed. Initially, with the higher doses of 1.5 g/kg and 2.25 g/kg, we found an early increase in plasma magnesium concentration and greater urinary outputs of magnesium; but, again, the 24-hour urinary magnesium in the ethanol-treated animals was almost identical to that of the animals that received water. Thus, in using different doses of ethanol, there appears to be no marked difference—at least on an acute basis—in the 24-hour urinary excretion of magnesium.

From this brief review, it is clear that there is little information available on the effects of ethanol on magnesium metabolism.

Current Advances in Magnesium Metabolism Studies

Some excellent reviews have appeared recently dealing with the current status of magnesium metabolism in health and disease (1,2,15). Based on these articles, it is difficult to envision that dietary restriction alone could result in magnesium deficiency—magnesium is found in almost all foods. Green vegetables, meat,

and seafood are particularly high in magnesium. The average diet for individuals in the United States contains approximately 300 to 350 mg of magnesium per day. On such a diet, approximately 30 to 40 percent of the ingested magnesium is absorbed in the gastrointestinal tract. Fractional magnesium absorption varies with intake. Thus, if the intake is increased, absorption is reduced; when dietary magnesium is reduced, absorption is increased. Once magnesium is absorbed, it is primarily handled by the kidney. Thus, the status of body magnesium balance is largely regulated by the renal excretion of this cation.

The body of an average adult human contains approximately 2,000 mEq of magnesium. Over 60 percent of the total body magnesium is found in bone. Thirty percent of the bone magnesium is freely exchangeable. Muscle magnesium represents approximately 20 percent of the body's stores, and it appears that approximately 20 to 30 percent of muscle magnesium is in an exchangeable pool. Thus, less than 20 percent of the body magnesium is found extracellularly. In plasma, only 20 percent of the magnesium is protein bound; thus, 80 percent of the plasma magnesium is freely filterable at the glomerulus.

Muscle magnesium is found to be decreased during magnesium depletion in some, although not all, animal studies, and this finding has prompted many investigators to use muscle magnesium concentration as an index of total body magnesium stores. Recently, it has been demonstrated that there is a direct correlation between muscle magnesium and muscle potassium concentration (2). When muscle magnesium has been found to be reduced, muscle potassium has also been reduced. Furthermore, during potassium depletion (without an alteration of magnesium balance), the concentration of both of these cations is reduced in muscle. Muscle magnesium may be low and associated with low, normal, or increased total body magnesium, further invalidating the use of muscle magnesium as an index of total body magnesium.

An excellent correlation between total body magnesium and bone magnesium concentration has been found. The fact that serum magnesium was found to correlate closely with bone magnesium stores during both hyper- and hypomagnesemia suggests that, for clinical purposes, serum magnesium is a reliable indicator of total body magnesium. Thus, it appears that bone and extracellular magnesium are magnesium pools available to replenish soft tissue magnesium deficits during magnesium depletion.

Recent advances in the renal handling of magnesium have been made (15). Over 180 mEq of magnesium is filtered at the glomerulus every 24 hours, but only 3 to 5 percent of the filtered

magnesium is excreted in the urine. Thus, there appears to be an effective process of tubular reabsorption of magnesium. Magnesium reabsorption by the kidney is largely controlled by a T_m (melting time) mechanism. The T_m for magnesium reabsorption is set very close to the filtered load of magnesium present at normal plasma concentrations of magnesium. Thus, increased magnesium can be observed by increasing the filtered load of magnesium. This could occur by either an increased glomerular filtration rate (GFR) or increased plasma concentration of magnesium, or both. Thus, hypermagnesemia is usually associated with magnesuria.

A variety of factors reduce the tubular reabsorption of magnesium. These factors include the following: extracellular fluid volume expansion, renal vasodilation, osmotic diuresis, diuretic agents, cardiac glycosides, hypercalcemia, and high sodium intake. The reduced magnesium reabsorption following extracellular fluid volume expansion is of particular interest in relation to alcoholism.

Results from our laboratory have demonstrated that dogs receiving chronically administered ethanol, as well as well-nourished alcoholic patients, showed an increase in the extracellular fluid volume (ECV) (3,4,13). Magnesuria of ECV can occur despite a significant reduction in the filtered load. The expansion of the extracellular fluid volume was primarily isosmotic with normal sodium concentrations and was therefore similar to the expansion observed following ECV by the infusion of saline.

Summary: Recommendations for Future Research

We know very little about the effects of ethanol on magnesium metabolism.

First, we should determine if ethanol can produce magnesium deficiency based on a negative magnesium balance. These studies should be conducted with both normal and restricted diets. If a negative balance for magnesium is found, is it the result of dietary deficiency, reduced absorption in the gut, increased urinary excretion, or a combination of any of these?

Second, what is the mechanism of the acute increase in serum magnesium and increased urinary excretion following the ingestion of ethanol? Possibly more important, what are the mechanisms involved in the sudden reduction in plasma magnesium following abstinence from alcohol? Studies should be designed to investigate the possible mechanisms. Are these rapid changes caused by shifts in the distribution of magnesium?

Third, the lack of research in other areas of magnesium metabolism should be recognized. This cation is essential for activating a variety of enzyme systems involved in cellular metabolism (21); what would be the effect of given deficits or shifts in the distribution of magnesium if found, for example, on oxidative phosphorylation of mitochondria? Thus, a variety of biochemical experiments should be conducted in conjunction with ethanol.

Improved techniques are available, so it is hoped that with properly controlled studies, new information will be forthcoming to explain the effects of ethanol on magnesium metabolism.

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Pathogenesis and Effect of Phosphate Depletion in the Alcoholic

James P. Knochel

Abstract

Phosphorus is critically important in every known pathway of energy metabolism. Its compounds are found in every cell and supporting structure of the body. Although the biochemical, physical, and physiological roles of phosphorus have been studied extensively, the pathophysiology of phosphorus deficiency has not.

We have observed that chronic alcoholics may be seriously deficient in phosphorus. The mechanism of the deficiency is unknown. Evidence is reviewed that either phosphorus deficiency or hypophosphatemia, or perhaps both, may be implicated in a variety of serious disorders that commonly complicate chronic alcoholism.

Hypophosphatemia commonly occurs in patients with severe chronic alcoholism. The type of alcoholic patient most likely to become hypophosphatemic is one who drinks heavily and eats poorly. Such hypophosphatemia may not occur until a patient is admitted to a hospital for complications of alcoholism. A normal serum phosphorus concentration at the time of admission may be rapidly replaced by profound hypophosphatemia within 1 to 4 days, in association with administration of nutrients lacking adequate phosphorus supplementation. To detect this abnormality, it is necessary to measure serum phosphorus concentration at the time of admission and at least daily for the first 5 days of hospitalization.

In contrast, some patients demonstrate hypophosphatemia of modest degree at the time of admission to a hospital. Such patients are usually in active alcohol withdrawal, and it is likely in these cases that the hypophosphatemia is the result of respiratory alkalosis. Severe phosphorus deficiency, indicated by a marked depression of total muscle phosphorus content, may exist in the

face of a normal serum phosphorus concentration if active necrosis of tissue coexists. Presumably, necrotic tissue releases its phosphorus content into the circulation (1,2,3).

Evidence for Phosphorus Deficiency in the Chronic Alcoholic

Hypophosphatemia occurs in approximately one-half of patients hospitalized for treatment of severe alcoholism. We observed that as serum phosphorus fell to low values (less than 1 mg/dL) and remained at that level for 1 or 2 days, a sharp rise of creatine phosphokinase activity (CPK) in serum commonly occurred (1). Because of these findings, we measured phosphorus content, electrolyte content, and, in some patients, transmembrane electrical potential of muscle cells. The latter is a sensitive index of cellular permeability to monovalent ions and/or sodium transport. Altogether, studies have been conducted on 21 such patients. Their serum CPK activity was elevated, ranging between 200 and 82,000 International units/ml. The value for total muscle phosphorus content in normal controls is 29 mmols/100 g fat-free dry solids (FFDS). The average value in the patients with chronic alcoholism we studied is 20 mmols/dg FFDS. The lowest was 12 mmols/dg FFDS, which indicates a severe phosphorus deficit. In norms, the mass of skeletal muscle in a 70 kg man averages 30 kg. The fat-free dry solid fraction of skeletal muscle is about 25 percent. Thus, in norms, muscle content of phosphorus is about 2,175 mmols. If we allow for a shrinkage of muscle mass to 20 kg in a chronic alcoholic, normal total muscle phosphorus content should be 1,450 mmols. The average total muscle phosphorus content in chronic alcoholics with elevated CPK activity of 20 mmols/100 g FFDS suggested that their total muscle phosphorus content was 1,000 mmols. Therefore, their phosphorus deficit was approximately 450 mmols. This figure, of course, does not include possible deficits in other tissues.

Mechanisms of Phosphorus Deficiency in Chronic Alcoholism

Factors that could cause phosphorus deficiency in alcoholism are shown in table 1. Many chronic alcoholics develop capricious appetites; therefore an inadequate dietary intake of phosphorus must play at least a partial role in some people. In normal controls,

Table 1. Possible Factors Responsible for Phosphorus Deficiency in the Alcoholic

1. Inadequate Dietary Intake
2. Vomiting and Diarrhea
3. Magnesium Deficiency
4. Deranged Vitamin D Metabolism
5. Calcitonin
6. Acidosis

dietary phosphorus deficiency is exceptionally rare because all natural foods have a high phosphorus content. However, a diet lacking phosphorus, especially in conjunction with phosphate-binding antacids, can, in time, lead to serious phosphorus deficiency.

Many chronic alcoholics have episodic vomiting and diarrhea. However, the phosphorus content of gastric contents is low, and even voluminous diarrhea is not responsible for significant phosphorus losses in stools (5). Vomiting and diarrhea per se probably do not account for major losses of phosphorus.

Experimental magnesium deficiency in humans (6) induces a state of functional hypoparathyroidism. In association with this condition, serum phosphorus tends to become slightly elevated, and phosphaturia occurs. Phosphaturia could result from a slightly increased filtered load of phosphorus. Alcoholics often become hypomagnesemic and magnesium deficient, so such factors could play a role in phosphorus deficiency.

In unreported studies by Matter and his coworkers on human volunteers who ingested large amounts of bourbon whiskey daily for many weeks, phosphaturia appeared, but not until serum magnesium concentration fell to abnormally low levels (7). In our most recent studies of skeletal muscle composition in patients with alcoholic myopathy, we found that magnesium content, normally 8 mmols/100 g fat-free dry solids, was significantly lower than normal ($p < 0.01$), averaging 5.7 mmols in the alcoholics (4). Thus, we do have evidence that, in these patients, magnesium deficiency coexists with phosphate deficiency.

It was recently reported that plasma levels of 25-hydroxy-vitamin D_3 are abnormally low in chronic alcoholics without liver disease (8). It seems possible that this state could account for decreased formation of 1,25-hydroxy-vitamin D_3, which could in

turn decrease calcium absorption from the small intestine, cause hypocalcemia, and lead to overproduction of parathormone. Such a mechanism could occur independently of magnesium deficiency and thus could account for phosphaturia. When the severe alcoholics we studied were admitted to the hospital, there was often abundant phosphorus in the urine—before they developed severe hypophosphatemia. Some of these patients excreted 0.9 g of phosphorus in their first 24-hour urine despite a poor preceding dietary intake and despite respiratory alkalosis. Both of the latter influences would ordinarily cause hypophosphaturia. However, the phosphaturia disappeared rapidly with the advent of hypophosphatemia. Whether the phosphaturia at the time of admission was related to overproduction of parathormone was not studied.

Recent studies have shown that ethanol is a potent factor in stimulating release of calcitonin (9). Normally, calcitonin induces a slight decline of serum calcium concentration. It has been reported that it decreases intestinal absorption of phosphorus (10). Calcitonin may also induce phosphaturia and magnesuria (11). Whether such effects play a role in phosphorus deficiency in alcoholics has not been examined.

Acidosis leads to decomposition of intracellular organic phosphate compounds. The phosphorus so liberated readily diffuses into the serum and is excreted into the urine. The mechanism is of great importance in the phosphorus deficiency seen in patients with diabetic ketoacidosis. Alcoholics often develop ketoacidosis while fasting during hangovers. They may also undergo transient periods of CO_2 retention and respiratory acidosis as a result of acute severe alcoholic intoxication. It seems conceivable that either mechanism could cause intermittent phosphorus wasting.

Mechanisms of Acute Hypophosphatemia

As pointed out earlier, some severe alcoholics have abundant inorganic phosphate in the urine at the time of admission to the hospital. Usually this condition disappears rapidly, so that by the second or third hospital day, the urine becomes virtually free of phosphorus as hypophosphatemia appears. The possible mechanisms of acute hypophosphatemia are shown in table 2. It must be assumed that serum phosphorus is being incorporated into cells. It could occur by an anabolic stimulus provided by administration of nutrients or as result of acute hyperventilation with respiratory alkalosis. Because of the rapid diffusibility of CO_2, acute hyperventilation is associated with a sharp rise in intracellular pH. This

Table 2. Possible Mechanisms of Acute Hypophosphatemia in the Alcoholic

1. Administration of Nutrients
2. Acute Respiratory Alkalosis
3. Fructose Administration
4. Hyperinsulinism

situation would activate phosphofructokinase, thereby increasing phosphorylation.

The source of phosphorus to form organic phosphates in the cells is cytoplasmic inorganic phosphate. Cytoplasmic inorganic phosphate is in diffusion equilibrium with serum phosphorus. Therefore, rapid phosphorylation during an anabolic state induced by administration of nutrients or incident to respiratory alkalosis could account for the severe hypophosphatemia seen in patients during treatment for chronic alcoholism.

Administration of fructose, still recommended by some physicians caring for patients with acute alcoholic withdrawal, may also lead to acute hypophosphatemia. The hypophosphatemia associated with fructose can be more severe than that associated with administration of glucose. The mechanism of this response is related to the unregulated uptake of fructose by liver cells (12). Specific kinases in the liver catalyze phosphorylation of glucose (glucokinase) to glucose-6-phosphate and fructose (fructokinase) to fructose-1-phosphate. Increasing concentrations of glucose-6-phosphate inhibit the activity of glucokinase, thereby regulating the uptake of both glucose and phosphorus. In contrast, increasing concentrations of fructose-1-phosphate do not inhibit fructokinase. Thus, fructose phosphorylation is unregulated and, as a result, hypophosphatemia is more pronounced after administration of fructose than glucose.

An additional problem related to intracellular phosphorus trapping mediated by fructose is that it may be associated with acute hepatocellular damage (13). Thus, when inorganic phosphorus concentration inside the cytoplasm of liver cells falls sufficiently, there is an activation of AMP-deaminase (12). Adenylic compounds within the cell are irreversibly decomposed and eventually lead to a decline of cellular ATP content (13). The adenylic compounds are converted to inosine. Inosine impairs glycolysis by inhibiting aldolase (12). Inhibition of glycolysis commonly results in acute lactic acidosis. When the cellular

content of ATP falls to a critical level, certain enzymes are released from the cell, reflecting acute cellular injury (14). For such reasons, fructose should not be administered intravenously to any patient, especially one already likely to have liver damage caused by alcoholism (15).

The final mechanism that could be implicated in the pathogenesis of acute hypophosphatemia in the withdrawing alcoholic is overproduction of insulin. It has been known for many years that patients with liver disease may develop a greater depression of serum phosphorus concentration after administration of glucose than do normal persons (16). Recent studies (17) have shown that experimental phosphorus deficiency exaggerates insulin release in response to hyperglycemia. This characteristic has not been examined in the withdrawing alcoholic, but it certainly seems worthy of study.

Consequences of Phosphorus Deficiency

There are three proved, gross structural effects of phosphorus deficiency (as opposed to hypophosphatemia)—osteomalacia, a proximal myopathy, and growth retardation. Undoubtedly, other consequences will become apparent with further study.

Pure phosphorus deficiency induced by dietary deprivation and by administration of phosphate-binding antacids is associated with muscular weakness, paresthesias, bone pain, a decline of serum phosphorus concentration, and increased calcium excretion into the urine (18). Recent studies (18,19) have shown that in males with experimental phosphorus deficiency, the increased calcium excretion into the urine is matched by increased calcium absorption by the gut. Therefore, no net change of calcium balance occurs. However, this is not the case in females; phosphorus-deficient women tend to excrete more calcium into the urine than they absorb from the gut.

The response to hypophosphatemia is mobilization of phosphorus from the skeleton in an apparent attempt to correct hypophosphatemia and the associated decline of cytoplasmic phosphorus (18). As bone is mobilized, calcium is also liberated, thus producing hypercalcinuria. In adult humans and dogs, serum calcium almost always remains normal. However in rats, puppies, and possibly in children, pure phosphorus deficiency may cause hypercalcemia (21). Mobilization of bone apparently occurs by the action of 1,25-$(OH)_2$-vitamin D_3 and requires the presence of parathyroid hormone. Synthesis of 1,25-$(OH)_2$-vitamin D_3

normally increases in phosphorus deficiency (22). The recent report that 25-OH-vitamin D_3 may be low in plasma (8) does not of itself imply defective synthesis of 1,25-$(OH)_2$-vitamin D_3. However, the finding does indicate that this potentially important problem of disordered vitamin D metabolism in alcoholics should be examined in more detail.

It seems likely that many patients with chronic alcoholism could develop osteomalacia if their phosphorus deficiency were sufficiently prolonged or severe. The serum electrolyte profile displayed by severe alcoholics suggests this occurrence. These patients display hypocalcemia out of proportion to depression of their serum albumin concentration, hypophosphatemia, and hypomagnesemia. These findings are classical for chronic steatorrhea, which may be complicated by osteomalacia. However, these findings occur independently of steatorrhea in the alcoholic. If osteomalacia occurs in these patients, it must be subclinical because it is rarely recognized. Conversely, it seems possible that overt osteomalacia is seldom identified in the alcoholic despite phosphorus deficiency because the associated hypomagnesemia and magnesium depletion may sufficiently depress parathormone release so that sufficient bone is not mobilized to result in osteomalacia. Unfortunately, this area has not been examined in patients with chronic alcoholism.

In nonalcoholics, phosphorus deficiency per se leads to a proximal myopathy (23). Such a myopathy stands in sharp contrast to rhabdomyolysis, which appears with acute severe hypophosphatemia. In chronic phosphorus deficiency, patients complain of profound proximal muscular weakness and therefore often have difficulty walking. Osteomalacia is usually present and may be associated with frank bone pain and a tendency toward fractures. In such patients, creatine phosphokinase and aldolase activity are most often within normal limits. However, patients display elevations of alkaline phosphatase activity, which reflects their osteomalacia.

In contrast to rhabdomyolysis with acute severe hypophosphatemia, hypophosphatemia in patients with chronic phosphate deficiency is usually modest and ranges between 1.5 and 2.5 mg/dL. Whether the proximal myopathy of chronic phosphorus deficiency is related to chronic myopathy with proximal wasting in alcoholics is unclear. Recent evidence suggests that 25-OH-vitamin D_3 may be important in skeletal muscle protein synthesis (24) and ion fluxes in sarcoplasmic reticulum (25). Consequently, it is probably important in muscle contraction. Whether the allegedly low 25-OH-vitamin D_3 levels in chronic alcoholics bear a relationship to their weakness and proximal myopathy is unknown.

As one would anticipate, should phosphorus deficiency occur in a young person or in a growing animal, growth retardation occurs because phosphorus is an essential component of all protoplasm. In experimental animals, growth is rapidly restored by providing adequate amounts of phosphorus in the diet.

Many functional disturbances have been associated with phosphorus deficiency. In our studies of phosphorus-deficient dogs, we observed that muscle magnesium content fell slightly, but significantly. Others have observed inappropriate losses of magnesium into the urine in experimental phosphorus deficiency (26). We also observed that skeletal muscle potassium content fell moderately in dogs with experimental phosphorus deficiency (27).

It has also been well documented that experimental phosphorus deficiency may impair reabsorption of bicarbonate (28) and glucose (29) in the proximal tubule of the kidney. Thus, phosphorus deficiency can establish a situation similar to the Fanconi syndrome. It is notable that, in many of the clinical reports of somatic cell dysfunction resulting from phosphorus deficiency (30, 31,32), renal glycosuria, renal aminoaciduria, and renal tubular acidosis have often coexisted. Such findings may also occur in clinical rickets, which in many instances disappear rapidly following administration of vitamin D. There have been occasional reports of severe, generalized proximal tubular dysfunction in patients with serious alcoholic intoxication. Whether or not these dysfunctions were related to phosphorus deficiency or simultaneous ingestion of a nephrotoxin has not been clear (2).

Severe experimental phosphorus deficiency may diminish the reabsorptive capacity for bicarbonate in the proximal nephron (28). Phosphorus deficiency also diminishes renal ammonia production (20) and apparently causes intracellular alkalosis (28). Although one would anticipate that a proximal bicarbonate leak, diminished ammonia production, and the virtual elimination of buffer phosphate from the urine would all result in severe metabolic acidosis, such is not the case. Studies by Emmett and his associates (20) on phosphorus-deficient rats showed that sufficient bicarbonate is mobilized from bone, *pari passu* with mobilization of calcium and phosphorus, to forestall metabolic acidosis. Experimentally, if colchicine is given as a pharmacologic tool to block mobilization of bone bicarbonate, metabolic acidosis promptly follows. Hypercalcinuria also occurs in humans with experimental phosphorus deficiency (18,19), so it would seem quite likely that the foregoing mechanism must prevail.

Finally, depressed glucose utilization occurs with experimental phosphorus deficiency (33). It is notable that many years

ago Friedlander and associates (34) showed that untreated diabetics could use glucose more efficiently if it were given in conjunction with inorganic phosphate. This effect occurred without insulin therapy. Whether or not phosphorus deficiency plays a role in the glucose intolerance often observed in patients with alcoholism and liver disease has not been examined.

Consequences of Acute Severe Hypophosphatemia

The effects of acute severe hypophosphatemia, in many instances, are quite different from those of simple chronic phosphorus deficiency. Outstanding effects include impairment of red cell function, acute hemolysis, rhabdomyolysis, impaired leukocyte function, and profound disorders of the central and peripheral nervous systems (table 3). Many of these disorders appear to be related to failure to deliver adequate oxygen because of the disordered red cell metabolism induced by phosphorus deficiency. Additional possible consequences of acute hypophosphatemia are shown in table 4.

Two serious effects of acute hypophosphatemia on red cells have been well characterized (35, 36, 37). One is anatomic, characterized by a sequence of increased red cell rigidity, decreased red cell volume, and, finally, hemolysis. In most instances, hemol-

Table 3. Definite Consequences of Acute Hypophosphatemia

1. Impairment of Red Cell Function
2. Hemolysis
3. Disordered Leukocyte Function
4. Rhabdomyolysis
5. Nervous System Dysfunction

Table 4. Possible Consequences of Acute Hypophosphatemia

1. Myocardial Insufficiency
2. Platelet Dysfunction
3. Hepatocellular Dysfunction

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ysis in association with hypophosphatemia has occurred when phosphorus deficiency is profound and when complicated by metabolic acidosis. The clinical situations in which hemolysis has occurred have been with chronic alcoholics during withdrawal and patients with uremia who have been overtreated with phosphate-binding antacids. It has been shown that when red cell ATP content falls to a value of 15 percent or less of normal, acute hemolysis is likely to occur. This finding explains why hemolysis is generally seen only in the presence of acidosis; the latter depresses red cell glycolysis sufficiently to prevent ATP formation.

Functional disturbances of the red cell, induced by phosphorus deficiency, are much more common. In the presence of hypophosphatemia, 2,3-diphosphoglycerate (2,3-DPG) synthesis falls to low levels; the affinity of hemoglobin for oxygen increases markedly so that the oxyhemoglobin dissociation curve shifts to the left (37). Thus, a low 2,3-DPG impairs capacity to deliver oxygen to peripheral tissues. If this condition occurs in association with acidosis, the O_2-dissociation curve shifts back to the right, promotes release of oxygen from hemoglobin independently of 2,3-DPG, and thereby counterbalances the defect in oxygenation. However, alcoholics are peculiar in that they are not only phosphorus deficient and hypophosphatemic, but they almost always display acute respiratory alkalosis during withdrawal. This condition poses an exceptionally difficult and potentially disastrous problem of compromising oxygen delivery to tissue.

In the presence of a low 2,3-DPG unassociated with a disturbance in blood pH, the decreased capacity for oxygen release at peripheral tissues must lead to an increase of cardiac output. This situation occurs because the heart reacts in response to peripheral oxygen needs. It has been calculated that a decrease in red cell P^{50}* from a normal value of 26 mm Hg to 20 mm Hg, a value observed in patients with severe hypophosphatemia, would increase the demand for cardiac output from a normal value of 5 1/min to 12.5 1/min (38). If such a need were superimposed on other circulatory demands, especially in a patient who had compromised myocardial function due to malnutrition or perhaps to alcoholic cardiomyopathy, one could readily appreciate why heart failure may occur in the withdrawing alcoholic. Current unpublished experimental studies by Fuller and his associates (39) on phosphorus-deficient dogs showed a steady increase of end-diastolic pressure, a decline of cardiac output, and an impaired pressor response to angiotensin II.

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*P^{50} as used herein refers to the value for oxygen tension of whole blood when 50-percent saturated, temperature 37° C, pH 7.40.

Such events may seem paradoxic because cardiac output should increase if the capacity to deliver oxygen by red cells is decreased. However, this abnormality may not prevail in the dog because dog red cells apparently do not decrease production of 2,3-DPG when phosphorus deficiency is induced in this manner. Therefore those studies would appear to indicate that myocardial function per se is impaired by phosphorus deficiency. This important observation could have great bearing on myocardial insufficiency as observed in some patients experiencing alcoholic withdrawal. Thus, O'Connor and his associates have described congestive heart failure in patients receiving hyperalimentation without adequate phosphorus who also became phophorus deficient (40).

It has been shown that acute hypophosphatemia impairs leukocyte function. Studies by Craddock and associates (41) have shown impairment of chemotactic activity as well as of phagocytic function. Both functions can be restored to normal by correction of hypophosphatemia. It has been postulated that severe hypophosphatemia, by impairing ATP synthesis, prevents formation of adequate energy for interaction of myosin and actin in leukocytes and is responsible for the abnormality of phagocytosis. Lichtman and his associates have shown that hypophosphatemia may prevent formation of phosphoinositides, important in chemotaxis and dissolution of engulfed bacteria (42). It is tempting to speculate that such defects related to hypophosphatemia in the withdrawing chronic alcoholic could be related to their high incidence of bacterial infection.

Thrombocytopenia, decreased platelet survival, and impaired platelet function have been observed in dogs made phosphorus deficient (43). To my knowledge, thrombocytopenia or impairment of platelet function as a result of hypophosphatemia has not been documented in humans.

Acute rhabdomyolysis is common in patients with severe hypophosphatemia. In many instances, this finding is not associated with overt clinical signs of rhabdomyolysis such as muscle pain, swelling, tenderness, or paralysis. However, it may be severe and can be associated with myoglobinuria and acute renal failure. In such patients, we have demonstrated not only acute severe hypophosphatemia but also, as discussed earlier, a marked deficiency of skeletal muscle phosphorus content. Experimental studies on dogs (27) have shown that feeding them a phosphorus-deficient diet in conjunction with phosphate-binding antacids for a period of 28 days leads to an electrochemical myopathy. This symptom is characterized by a decline in resting membrane potential, a decline of muscle phosphorus content, and increases in sodium and chloride contents.

A slight decline of magnesium and potassium content also occurs. In those studies, malnutrition was prevented by gavage feeding the animals a diet that was complete except for its lack of phosphorus. Similar to some alcoholics who have not yet become acutely hypophosphatemic, there was no elevation of CPK activity, and acute rhabdomyolysis did not occur.

In further attempts to establish a model more closely resembling that seen in the patient with chronic alcoholism, 17 additional dogs were fed a phosphorus- and calorie-deficient, but otherwise balanced, diet in order to induce a 30-percent weight loss (44). At this point, the dogs were hyperalimented with a phosphorus-deficient diet providing 140 cal/kg per day. These dogs developed rhabdomolysis, preceded by acute severe hypophosphatemia. Using additional dogs prepared in a similar manner, provision of 4.6 g of elemental phosphorus each day completely prevented acute rhabdomyolysis, despite the hyperalimentation. We tentatively concluded from these studies that phosphorus deficiency per se produces an electrochemical myopathy closely resembling the subclinical myopathy observed in many patients with chronic alcoholism before acute hypophosphatemia supervenes. Thus the CPK activity in both humans and experimental animals with phosphorus deficiency (but without severe hypophosphatemia) was normal. However, with induction of acute severe hypophosphatemia in either humans or experimental animals with preceding phosphorus deficiency, acute necrosis of skeletal muscle occurs. Finally, acute necrosis of skeletal muscle can be prevented by providing sufficient phosphorus in the diet to prevent hypophosphatemia.

Disorders of the central nervous system have been well characterized in patients with severe hypophosphatemia (36). In those instances, a sequence of symptoms compatible with a metabolic encephalopathy may occur: These include irritability, apprehension, muscular weakness, numbness, paresthesias, dysarthria, confusion, obtundation, convulsive seizures, coma, and death. This clinical syndrome has been observed in patients who have not manifested other apparent causes for encephalopathy, but who have been treated with intravenous hyperalimentation. It is also seen in patients during withdrawal from chronic alcoholism. In the latter instance, the clinical picture is similar in some respects to that seen in delirium tremens. However, the distinctive hallucinations of delirium tremens have not been observed. Obviously, both conditions may coexist.

There appear to be at least two important metabolic derangements responsible for the central nervous system abnormalities

in hypophosphatemia. First, an inadequate supply of inorganic phosphorus ions would directly impair metabolism of carbohydrates and therefore limit the production of ATP to maintain cellular function. Second, the decline in red cell 2,3-DPG content would indirectly impair release of oxygen to the brain.

In a report by Travis and her coworkers (36), three of the eight patients who became hypophosphatemic during hyperalimentation developed paresthesias about the mouth and in the extremities, mental obtundation, and hyperventilation. These three patients demonstrated marked shifts of the oxyhemoglobin dissociation curve to the left because of abnormally low P^{50}. Two whose P^{50} values were 15 and 16.5 showed diffuse abnormalities of their EEG's, these disappeared after treatment.

The best evidence that hypophosphatemia can produce an encephalopathy has been the observation that it does not occur in patients receiving hyperalimentation if sufficient phosphorus is provided to prevent hypophosphatemia. An additional problem may coexist in a patient with alcoholic withdrawal. These patients characteristically hyperventilate. When hyperventilation and the ensuing respiratory alkalosis become severe, there may be a sharp decline in cerebral blood flow (45). This condition, in conjunction with a decreased release of oxygen from hemoglobin as a result of decreased 2,3-DPG content of red cells, could have devastating consequences on cerebral function.

Finally, consideration should be given to the possibility of liver damage as a result of phosphorus deficiency. Studies (46) have suggested that acute, severe hypophosphatemia may impair hepatocellular function. In patients with alcoholic liver disease, oxygen extraction was diminished in the presence of severe hypophosphatemia. Red cell 2,3-DPG content was low. Correction of hypophosphatemia was associated with increased red cell 2,3-DPG and hepatic oxygen extraction. Such studies have great potential importance and could explain the well-known clinical observation that many alcoholics tend to become worse during the first week of hospitalization. This finding could be related to the inadvertent production of hypophosphatemia.

In summary, this paper has reviewed the characteristics and consequences of phosphorus deficiency and hypophosphatemia in patients with chronic alcoholism during their management in the hospital for acute complications. It seems apparent that phosphorus deficiency may be responsible for some morbidity in these patients. The extent of the harmful effects of phosphorus deficiency and hypophosphatemia have been only superficially examined. Whether or not avoidance of phosphorus deficiency in

patients with chronic alcoholism may forestall these harmful effects is unknown.

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Discussion of Paper by Knochel

Unidentified Voice: With regard to your comments on the alcoholic and vitamin D metabolism, I think this area has not been looked into very much. We have looked now at 30 alcoholic cirrhotic patients who have stopped drinking a month prior, and in half of these patients, there is initially a low 25-hydroxy-D level. However, each one of these patients, who were low to begin with, was able to respond to vitamin D_2 replacement with elevation to normal or supernormal levels of 25-hydroxy-D, with a similar rise in serum calcium. So alcoholic cirrhotics appear to be able to respond to vitamin D as long as they are not drinking.

Now, the question is, does alcohol itself either interrupt the 25-hydroxylation in the liver? Or possibly, vitamin D is shunted into inactive metabolites for a period after stopping alcohol, much in the same way as after phenobarbitol. I think we need to look a lot more at what happens to vitamin D in the alcoholic patient.

The question I have is with the problems in phosphorus that you have shown and the low muscle levels in the alcoholics—why do you think we do not see osteomalacia very often in alcoholics? The main bone lesion in alcoholics is osteoporosis.

Dr. Knochel: Maybe for three reasons. When alcoholic patients come in, they may have hypocalcemia out of proportion to their albumin concentration. This picture is similar to any patient with osteomalacia. But at the same time, if they are phosphorus deficient, this condition might shut off their parathyroid hormone production. They may be also hypomagnesemic, which would block the effect of parathormone on bone. So for these reasons, full-blown osteomalacia may just not happen in the alcoholic.

Unidentified Voice: Right. The one other thing is that when we placed our patients on vitamin D (this has been seen in some of the renal patients now, getting replacement) half of them, as their 25-hydroxy-D levels went up, showed an abrupt rise in parathyroid hormone from formerly fairly low levels.

Dr. Knochel: This finding could be due to magnesium. The fact is, that when you give magnesium to these alcoholics, within minutes their parathormone levels become enormous.

Dr. Halsted: I wonder if you can give us some practical approaches to therapy in the alcoholic, based on what we now know about phosphate metabolism.

Dr. Knochel: Sure. Too much phosphorus given intravenously can precipitate calcium in various tissues; for that reason, our policy is that you do not give phosphorus salts intravenously until hypophosphatemia occurs. Many of these patients can have such bad muscle necrosis that they correct their serum phosphorus and may be hyperphosphatemic, especially if they have renal failure. That is why it is mandatory to measure serum phosphorus first. If the alcoholic patients become hypophosphatemic, we have been giving half of their ordinarily daily potassium supplement as potassium phosphate, and that has been adequate to maintain serum phosphorus above 1.5. You do not get in any trouble. This dose of phosphorus does not lower serum calcium. Whether or not it helps the patient in the long run I cannot say, but our staff thinks it does.

Dr. Li: What do you estimate to be the incidence of severe hypophosphatemia in the alcoholic?

Dr. Knochel: We see an average of five a week.

Dr. Li: That many. You know, a while ago, Bud Beech, at NIAAA, sent out letters to several people around the country to review records regarding its incidence. At Wishard, a county hospital, we reviewed consecutively by computer almost 5,000 patients and consecutive admissions. There were close to 6,000 determinations, and we found 10 patients with phosphorus below 1 mg percent; of these, 3 were terminal cancer patients, 2 had severe diabetic ketoacidosis, and I think 5 were alcoholics admitted because of pancreatitis and ketoacidosis, or else severe liver disease and ascites. And it could be that we are just not recognizing the population you are describing.

Dr. Knochel: Two things. I think you have to measure this condition daily, within the first 5 days, to make sure it does not happen. But the odd thing is that Jay Stein, for example, had a paper 15 years ago from Oklahoma City claiming that 50 percent of their alcoholics became hypophosphatemic. Since I have written on this, Jack Lemmon in Milwaukee tells me there is so much of it there that he does not want to see any more of it. So I think it may be timing, or it may be how aggressively these people are treated. You know, if they are just put in a ward, sort of ignored, and are not given nutrients, they are going to become hypophosphatemic.

Effect of Ethanol on Human Mineral Metabolism

Janet T. McDonald

The purpose of this research was to investigate the claim that wine has "something different" that distinguishes it from other alcoholic beverages (1). Wine has been heralded for its ability to stimulate appetite and facilitate digestion, and there have been implications that wine also has a beneficial effect on the absorption of nutrients (2). Therefore, Dr. Sheldon Margen and I designed a metabolic balance experiment to test this hypothesis. The study took place in the Human Nutritional Laboratory, otherwise known as the Penthouse, at the University of California, Berkeley.

Study Design

Six healthy male volunteers, with a mean age of 25 years, were selected for the study. All were occasional consumers of moderate, nonintoxicating amounts of alcoholic beverages. The men were housed in the Penthouse, a closed metabolic unit, for the duration of the experiment, except for occasional supervised walks. No visitors were allowed.

The study lasted 75 days and consisted of a 3-day equilibration period followed by four 18-day experimental periods. These were (1) wine, (2) dealcoholized wine, (3) ethanol, and (4) deionized water. The sequence of the four periods followed a randomized block design. Each man served as his own control.

A formula diet supplemented with crackers, Sanka, and tea was divided into four equal feedings served every 4 hours between 9 a.m. and 9 p.m. In addition, a 250 g portion of one of the test beverages was served at each meal: (1) California wine[1] with an alcohol content of 11.5 percent v/v or 9.3 percent w/v (g/100 ml), (2) dealcoholized Zinfandel wine,[2] (3) a 9.3 percent w/v pure ethanol solution in deionized water, and (4) deionized water. The

[1]Courtesy of the Department of Viticulture and Enology, University of California, Davis.

[2]Dealcoholization was achieved by distillation in a film evaporator at 45°C and 40 mm Hg. This process removed about 45 percent of the original volume, which was replaced with an equivalent volume of distilled water.

total amount of test beverage given was 1,000 g/day. During the wine and ethanol periods, the total dosage of alcohol given amounted to between 22 and 25 percent of the total calories, depending on the subject's size.

Caloric allowances were adjusted to keep body weight constant by adding to the basic formula another formula containing carbohydrate and fat, but no protein or minerals. During periods with no alcohol, an increased amount of the carbohydrate-fat formula was substituted for the calories contributed by alcohol. Furthermore, during periods when ethanol and deionized water were given, a solution containing nitrogen and minerals in the same concentrations as present in a liter of the Zinfandel wine was added to the basic formula. Additional vitamins and trace mineral supplements were administered separately to make the diet adequate in all known essential nutrients. Choline was also given (one 250-mg capsule at each meal).

Accurate 24-hour urine collections were made. Stools were collected in 3-day pools. Six-day sweat collections were made once during each metabolic period. The diet, urine, stools, and sweat were analyzed for sodium, potassium, calcium, phosphorus, magnesium, and zinc. A fasting venous blood sample was obtained at the beginning of the study and at the end of each metabolic period to measure all of the elements just mentioned except zinc (many of the samples for zinc determination were hemolyzed). Data for determining balance were compiled in 6-day pools and tested for significance by the correlated paired t test.

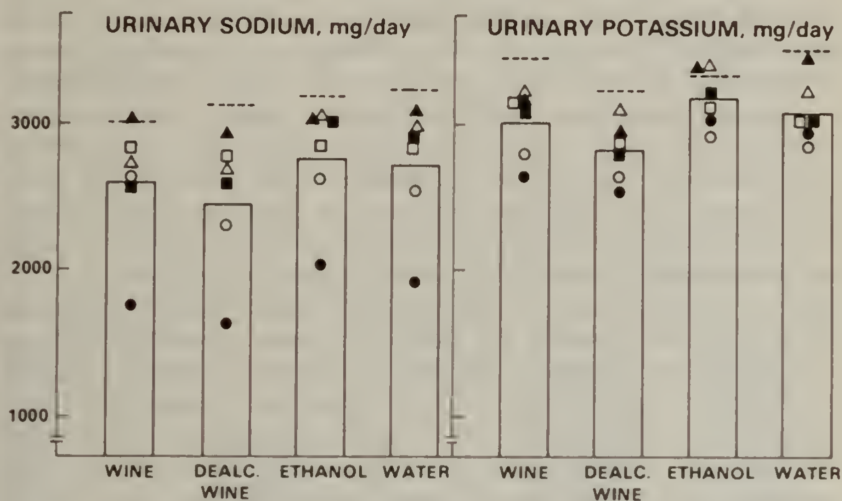
Results and Discussion

Generally speaking, the men tolerated the experiment well. There was no evidence of intoxication during alcohol periods. The blood alcohol level of the subjects was 20 mg/100 ml 1 hour after a meal; no alcohol could be detected 3 hours postprandially. No diuretic effect of alcohol was observed.

Mineral Balance Studies

There were no significant differences in fecal sodium or potassium excretion between the various test beverages. Figure 1 shows the mean daily urinary sodium and potassium during each of the four periods. Values for urinary sodium during wine and ethanol administration were not significantly different from those during water administration, as would be expected, because no diuretic

Figure 1. Mean Daily Urinary Sodium and Potassium of Six Normal Subjects During Administration of Wine, Dealcoholized Wine, Ethanol, and Deionized Water



The columns indicate average output; the horizontal dashed lines indicate average intake. Each symbol represents a separate subject:

△ = 3001		● = 3004	
▲ = 3002		■ = 3005	
○ = 3003		□ = 3006	

effect of alcohol was observed. Urinary sodium during the dealcoholized wine period, however, was significantly less ($p < 0.01$) than that during the ethanol and water periods. Consistent with this finding was a very low urine osmolality during dealcoholized wine feeding. The reason for this is unknown.

Significantly more potassium ($p < 0.01$) was excreted in the urine during ethanol administration than during feeding of the other three test beverages. This finding was consistent with the high urine osmolality observed during the ethanol period. The reason for the increased urinary potassium with ethanol (and not with wine) is difficult to explain unless the congeners in wine play some protective role. Due to the increased urinary potassium with ethanol, mean daily potassium balance was negative (−111 mg) during that period; this rate was significant at the 1 percent level.

Differences in urinary calcium between the four regimens were not significant. Fecal calcium, however, was significantly greater when subjects consumed ethanol or water than when they

consumed wine or dealcoholized wine. The mean apparent absorption[3] of calcium was 34 percent for wine and dealcoholized wine versus 16.5 percent for ethanol and deionized water. Figure 2 depicts mean calcium balance for each of the four experimental periods. Mean daily calcium balance was negative in all but the dealcoholized wine period. The differences in balance between wine or dealcoholized wine and ethanol or water were significant.

Urinary phosphorus was significantly greater during alcohol periods than during nonalcohol periods ($p < 0.01$ – difference of approximately 100 mg/d). As with calcium, significantly more phosphorus ($p < 0.01$) was excreted in the stool when subjects received ethanol or deionized water than when they received wine or dealcoholized wine. The mean apparent absorption of phosphorus was 74.5 percent for wine and dealcoholized wine versus 65 percent for ethanol and deionized water. Figure 3 portrays mean phosphorus balance for each of the test beverages. Balance was slightly negative during wine, ethanol, and water administration, but markedly positive (+113 mg/d) during dealcoholized wine feeding. The differences in balance between dealcoholized wine and the other three beverages were highly significant ($p < 0.01$). The positive balance was due to a combination of both low urinary and low fecal excretions of phosphorus.

There is some suggestion from these data that there is something in wine and dealcoholized wine (e.g., the congeners) that tends to improve absorption of phosphorus slightly. With wine, however, the increased urinary phosphorus counterbalances the increased absorption, resulting in no change in balance. We do not understand why wine had a more pronounced effect than ethanol on urinary phosphorus, but it may be because absorption was significantly greater during wine administration, and phosphate is easily cleared by the kidneys. Another possibility is that there is some synergistic effect of the alcohol and the congeners in wine on phosphorus metabolism.

The differences in urinary magnesium between periods were small. However, as with the previous two elements, the subjects excreted significantly more fecal magnesium when given ethanol or deionized water than when they were fed wine or dealcoholized wine. Mean apparent absorption of magnesium was 46 percent for wine and dealcoholized wine versus 35.5 percent for ethanol and water. Figure 4 shows mean magnesium balance for the four experimental periods. Balance was negative throughout the experiment.

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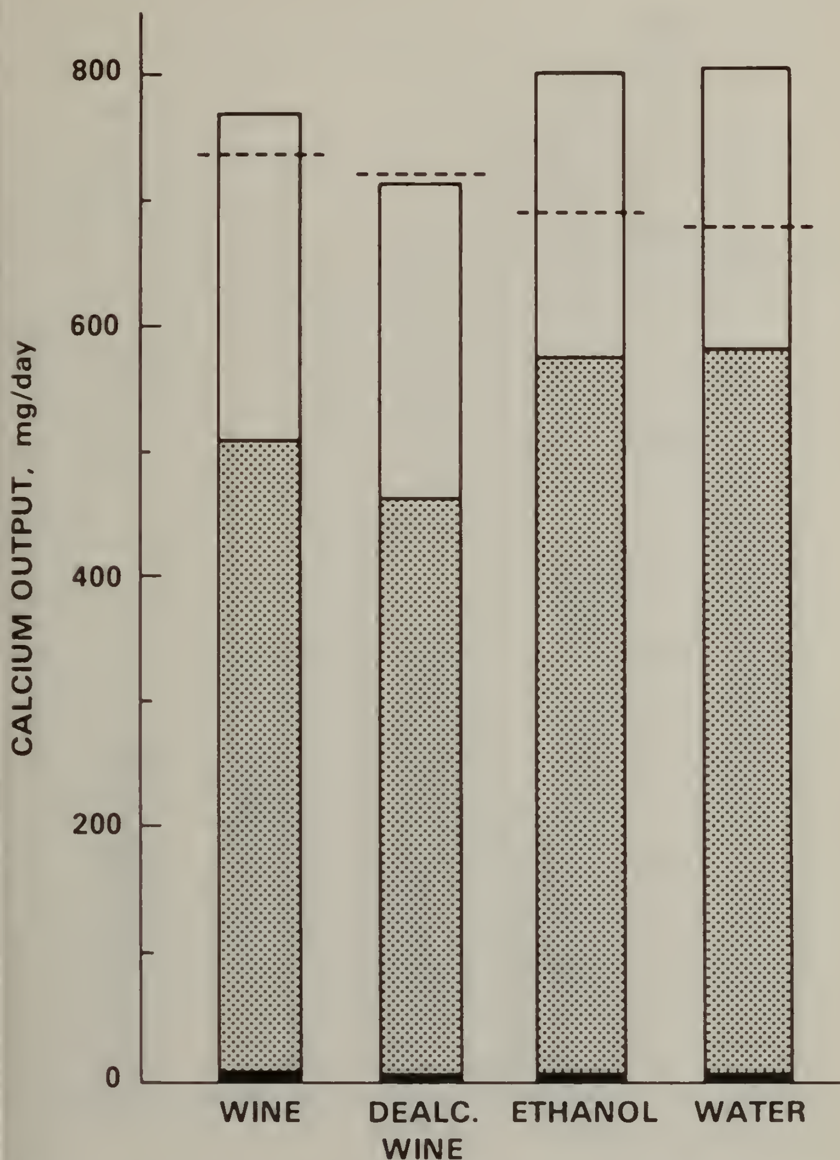
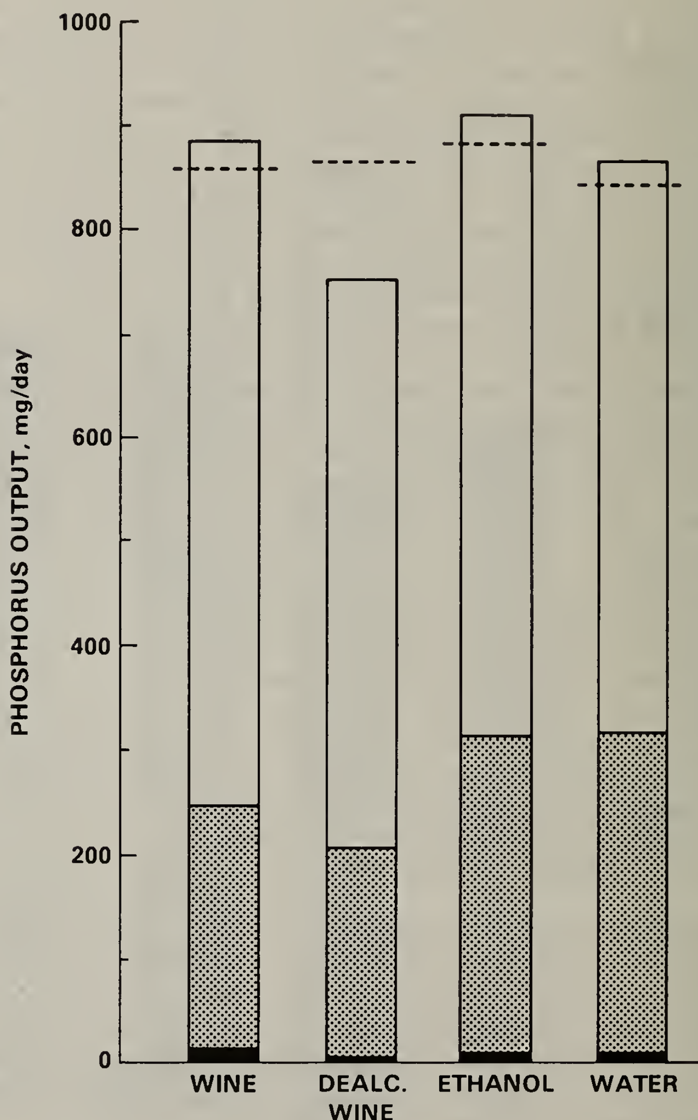
[3] $\frac{\text{Dietary intake} - \text{fecal output}}{\text{dietary intake}} \times 100 = \text{mean apparent absorption}$

Figure 2. Mean Calcium Balance of Six Normal Subjects During Administration of Wine, Dealcoholized Wine, Ethanol, and Deionized Water



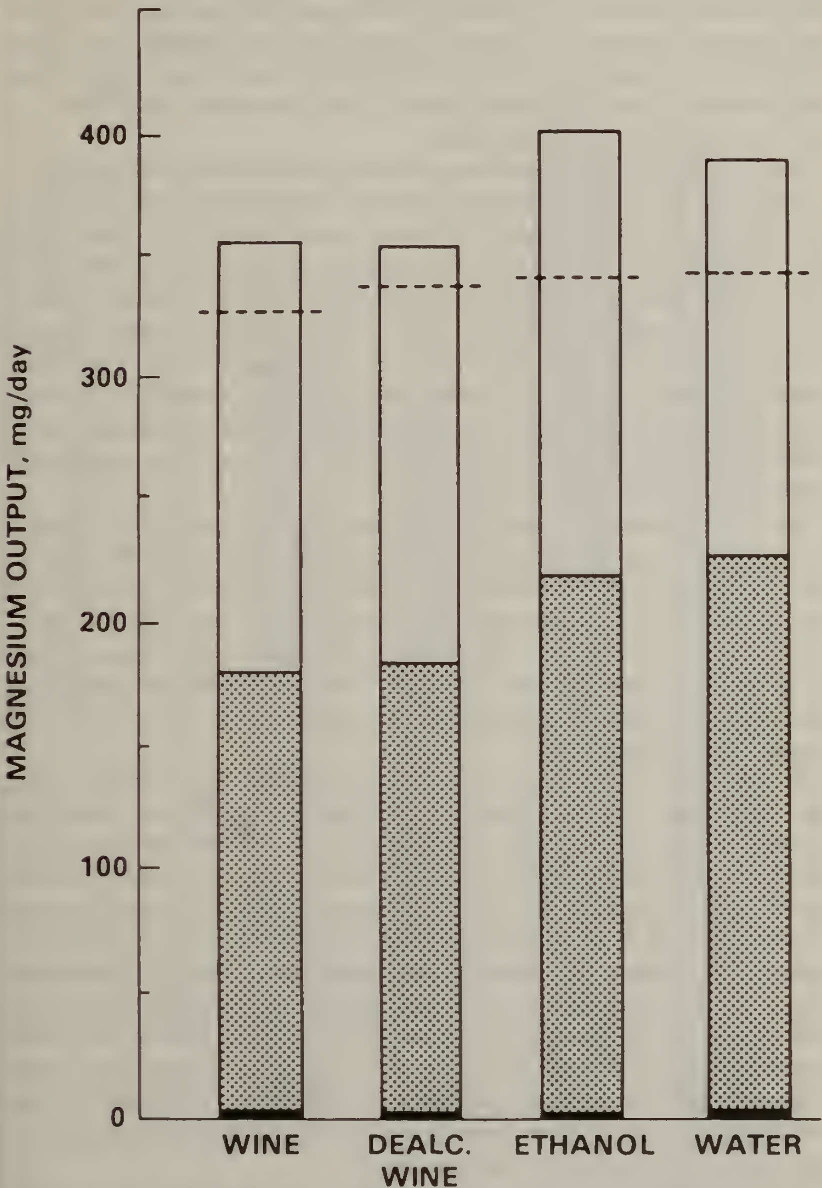
Output by way of sweat, feces, and urine is plotted cumulatively, in that order, upward from the bottom of the bars. Intake is shown by the horizontal dashed line and balance by the distance above (negative balance) or below (positive balance) the intake line.

Figure 3. Mean Phosphorus Balance of Six Normal Subjects During Administration of Wine, Dealcoholized Wine, Ethanol, and Deionized Water



Output by way of sweat, feces, and urine is plotted cumulatively, in that order, upward from the bottom of the bars. Intake is shown by the horizontal dashed line and balance by the distance above (negative balance) or below (positive balance) the intake line.

Figure 4. Mean Magnesium Balance of Six Normal Subjects During Administration of Wine, Dealcoholized Wine, Ethanol, and Deionized Water



Output by way of sweat, feces, and urine is plotted cumulatively, in that order, upward from the bottom of the bars. Intake is shown by the horizontal dashed line and balance by the distance above (negative balance) or below (positive balance) the intake line.

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This finding is difficult to explain. Intake of magnesium averaged 335 mg/d, very close to the recommended dietary allowance (RDA) of 350 mg for men in this age group. It is unlikely that the RDA for magnesium is too low. It is possible that the men who participated in this study were accustomed to higher intakes of magnesium in their prior diets, and that, with time, they would have adapted to the lower intake. This situation could be true also for calcium and phosphorus.

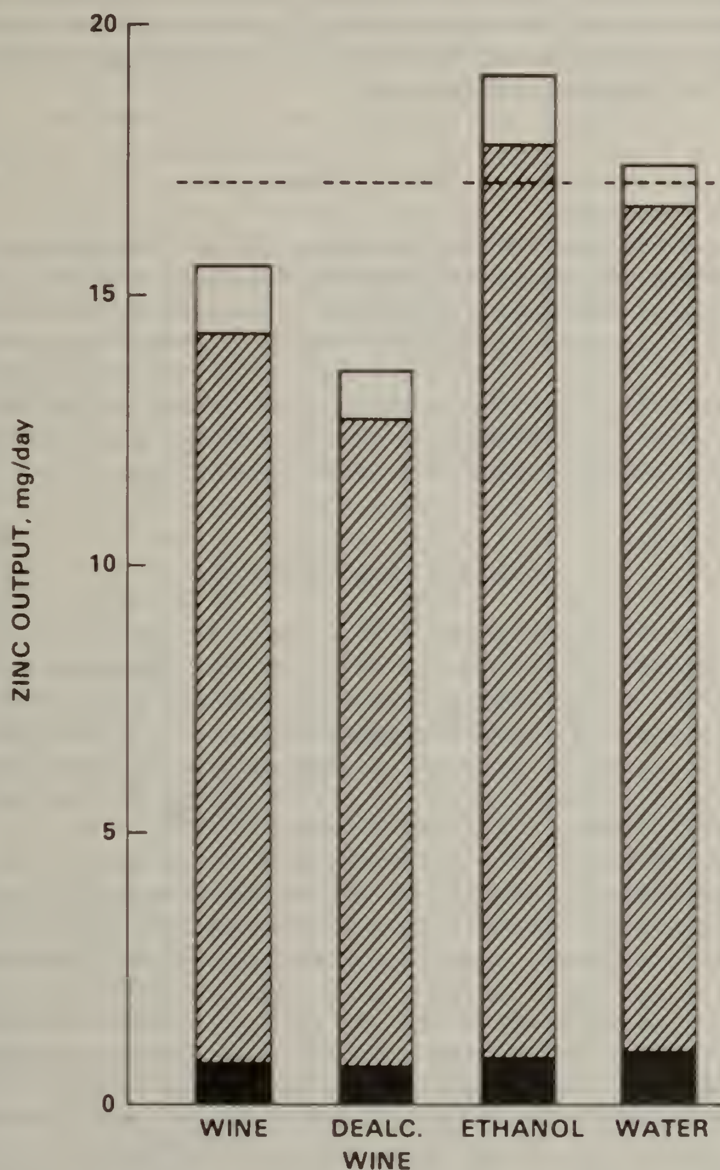
Considerably more zinc was excreted in the urine during administration of alcoholic beverages than during administration of nonalcoholic ones. The mean difference was about 46 percent and highly significant ($p < 0.01$). Our urinary zinc values tended to be higher than that generally considered normal for healthy human adults. This finding could be due to a consistent methodologic error or contamination. Nevertheless, in comparing the values between periods, evidence exists that alcohol enhances urinary excretion of zinc, even in normal subjects. It is well known that patients with alcoholic cirrhosis excrete abnormally large amounts of zinc in their urine (3,4,5,6,7).

Most of the zinc ingested was excreted in the stool. Significantly more zinc was excreted when ethanol was fed than when wine or dealcoholized wine were given ($p < 0.01$). There was a less significant difference in fecal zinc between water and dealcoholized wine ($p < 0.05$). Figure 5 illustrates the zinc balance data for the four test periods. Balance was positive for wine and dealcoholized wine and negative for ethanol and water. The differences were significant.

In addition to increased urinary potassium, phosphorus, and zinc, there was also increased urinary nitrogen with both wine and ethanol administration (8). Alcohol may affect the metabolism or renal tubular reabsorption of these substances. Muscle contains considerable amounts of all of these elements, so some muscle catabolism may have occurred during periods of alcohol administration. The urinary creatinine excretion of the subjects does not support this hypothesis, but recent reports have suggested that the correlation of creatinine excretion with lean body mass has little physiological validity (9,10). Muscle damage, as assessed by serum levels of creatinine phosphokinase following ethanol ingestion by nonalcoholic young men maintained on a nutritionally adequate diet, has been reported by others (11).

The decreased fecal output of calcium, phosphorus, magnesium, and zinc with wine and dealcoholized wine was presumably due to increased absorption but may have, in addition, reflected a decrease in the endogenous secretion of these elements. This result

Figure 5. Mean Zinc Balance of Six Normal Subjects During Administration of Wine, Dealcoholized Wine, Ethanol, and Deionized Water



Output by way of sweat, feces, and urine is plotted cumulatively, in that order, upward from the bottom of the bars. Intake is shown by the horizontal dashed line and balance by the distance above (negative balance) or below (positive balance) the intake line.

apparently was due to an effect of one or more of the many congeners present in wine and absent in a calorically equivalent amount of pure ethanol. The natural acidity of wine (average pH of 3.5 for table wines), in contrast to pure alcohol, also may have played a role in creating a more favorable intraluminal environment for absorption. Further experimentation is required to determine the underlying mechanisms.

Serum Values

Serum levels of sodium, potassium, calcium, phosphorus, and magnesium did not change appreciably during the course of the experiment, indicating that alcohol in the amounts and for the length of time given in this study had no effect on this parameter. Results of previous experimental studies in this area are conflicting. In almost all of the reported studies, however, either the subjects were alcoholics or alcohol was administered acutely in large doses; serum ion levels were measured within a few hours.

Concluding Remarks

Results of this investigation do not imply that prolonged use of large amounts of alcohol has no serious consequences on gastrointestinal or other bodily functions. Although some effects of alcohol observed in this experiment could be considered deleterious, it seems that temperate amounts are not particularly harmful, and wine, if one enjoys it, may, when taken with meals, have a beneficial effect on absorption of certain nutrients.

I would like to comment briefly on a few of the problems of alcohol research. The primary aim of this investigation was to determine the effects of wine versus pure alcohol on certain metabolic responses. One of the first effects of consuming alcohol is the recognition of its characteristic taste and smell, followed by mild epigastric sensations when the beverage reaches the stomach (12). From an experimental viewpoint, these effects are important. It is difficult to devise a dummy control drink that pleases the palate but contains no ethanol. Without a proper control beverage, distinction is almost impossible between the effects of alcohol per se, whatever the form, and the effects of drinking, with the many social and emotional factors involved. It was not feasible, however, to disguise the four test beverages, and similar efforts by previous investigators have not been successful either.

Another problem in the area of alcohol research is the lack of uniformity in the type and amount of beverage used by various investigators, making comparison of data from different laboratories extremely difficult. Wine, in particular, has a complicated composition (more than 300 components) with many variations, depending on the variety of grape used, the climatic conditions under which the grapes were grown, and the processes of fermentation and aging employed. Prior to our experiment, it was anticipated that standardization of wine research could be instituted and, it was hoped, continued. Unfortunately, the wine used in this study was of rather poor quality and disliked intensely by all subjects. The concept of using a standardized wine (i.e., from the same source and of the same type) for research, nevertheless, is an important one. For future studies, a wine of unquestionable quality (as judged by a panel of experts) should be chosen.

The data obtained in the present experiment by the balance method suggest that the nonalcoholic constituents of wine given in moderate amounts enhance the absorption of certain minerals, but do not lead to conclusions with respect to the mechanisms involved. The balance technique tells us if the subject ingested enough of an element under study to cover measured losses, but it tells us nothing about what happened to the element in the body (13). Such information is difficult to obtain without using radioactive tracers. Tracers are particularly valuable because they obviate the formidable problem of contamination. Perhaps a combination of the tracer technique and balance method should be used in future experiments.

Further research is needed to determine the minimum amount of wine that will exert a measurable effect and to determine which constituents of wine are responsible for increasing absorption. That research will involve the complex task of separating and purifying the numerous components of wine and making them available for experimental purposes. Standardization of wine, as mentioned earlier, would have to be a prerequisite. It would be of interest, also, to learn whether the nonalcoholic constituents of beer and distilled spirits exert effects on absorption similar to those of wine. The final question that will have to be answered is, "Of what clinical benefit is this information, and how can it be applied?"

Finally, most of the alcohol studies to date have either been performed with alcoholics or have involved the administration of extremely large doses of alcohol to nonalcoholic volunteers. Very few studies have tested the effect of moderate doses of alcohol, in

various forms, in normal individuals, for extended periods of time. This sort of information is clearly needed. The United States is attempting to formulate a national nutrition policy, which is long overdue. In this policy should be a statement about safe limits of alcohol consumption. How much can the average person drink without harmful effects? At this point, we don't know. Future studies on alcohol should attempt to answer this question.

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Discussion of Paper by McDonald

Dr. Li: There is some data in the old German literature that beer enhances iron absorption. Have you seen this, or do you know whether wine does that?

Dr. McDonald: We did study wine. I had not mentioned iron today because we had a problem with our fecal samples, with contamination. Fecal samples were homogenized in a colloid mill and were contaminated with iron, so I did not present these data. It is hard to say what happened. There was no difference in urinary iron, however, between any of the beverages.

Dr. Lester: I think the work is especially fascinating because your studies show differences that seem to be ascribable to wine per se as against alcohol. I have looked at this literature, and it seems to me that your studies are among the first to show an effect of the nonalcoholic part of alcoholic beverages, so I think they are very significant. One or two specific questions, if I may. Question one, although I did not see it in your summary, I thought I saw in one of your first slides that dealcoholized wine caused decreased urinary sodium excretion.

Dr. McDonald: No, that is not right.

Dr. Lester: Second question, how does one go about dealcoholizing wine? You said a word about it at the beginning, and I missed the word, and what is the residuum that is left?

Dr. McDonald: The dealcoholization was also done at University of California, Davis, by distillation in a film evaporator at 45°C and 40 mm Hg. This process removed about 45 percent of the original volume, and as I mentioned, other volatiles went off. Some of the volatile esters were lost. But the 45 percent of the original volume that was lost was replaced with an equivalent volume of distilled water. In order to use deethanolized wine, you have to come up with a synthetic mixture and replace it that way. I do not think at this state of the art anyone knows how to do that.

Dr. Beard: Dr. McDonald, I am curious as to whether the same amount of alcohol/kg was given via pure ethanol compared to the wine.

Dr. McDonald: Yes.

Dr. Beard: Then I would predict that you would have had higher blood alcohol levels from the ethanol than from the wine.

This was demonstrated many years ago. Thus, using pure ethanol as reference, about the closest you get is vodka, and then Scotch, or gin, and then bourbon, and then wines and beer. Now, if you take the same amount of ethanol/kg, you get fantastically much reduced blood levels from beer, table wine, etc., as compared to the same amount of ethanol from other forms of alcoholic beverages. And I was wondering if you found those differences, and whether they may have helped to explain some of the results.

Dr. McDonald: No, we did not find these differences. We did not draw the first blood until 60 minutes after the meal, and the levels were so low that any difference might possibly have been within the error of the method. Twenty mg percent is just quite low, and we did not see any difference. It was the same with both wine and ethanol.

Dr. Beard: The other question I had concerned your comment that you had a significant reduction in the urine osmolality, and from the data presented I would find that difficult to ascertain.

Dr. McDonald: We had a very low potassium and very low urea nitrogen with dealcoholized wine, so there was a very low urine osmolality.

Section III: Effects of Alcohol on Vitamin Metabolism

Effect of Ethanol on Folate Absorption*

Charles H. Halsted, Janice Ott, Ann M. Reisenauer, and John J. Romero**

Intestinal malabsorption may be a significant contributing factor in the poor nutrition of alcoholics. Previous clinical studies demonstrated a frequency of diarrhea in about one-third of binge-drinking alcoholics (1), which may be associated with poor absorption of dietary fat (2); of orally administered labeled thiamine (3), of folic acid (4), and of vitamin B_{12} (5); and of sodium and chloride perfused in the jejunum (6). Other experimental studies have shown that ethanol, administered in vivo or introduced to in vitro systems in concentrations similar to those obtained in the small intestine during active drinking (7), impairs intestinal transport of certain amino acids (8), stimulates intestinal mucosal adenyl cyclase (9), and may inhibit mucosal Na, K-dependent ATPase (10).

Folate deficiency is the most common cause of anemia in the alcoholic population. Contributory causes of alcoholic folate deficiency include dietary lack, inadequate hepatic storage (11), the possible development of a serum protein binder (12), and intestinal malabsorption (4,13,14). Three clinical studies have demonstrated that ^{3}H-labeled pteroylmonoglutamyl folate (^{3}H-PG-1) absorption is impaired in poorly nourished, binge-drinking alcoholics (4,13,14).

In an initial study (4), 13 alcoholics were administered ^{3}H-PG-1 orally, 1.5μg/kg, within 24 hours of admission to a hospital. Tissue folate saturation was accomplished by means of the simultaneous administration of intravenous PG-1, 30 mg, found to be adequate by measurement of its distribution, metabolism, and excretion. Compared to a group of hospitalized nonalcoholic controls and to a group of 10 stable alcoholics studied after 2 weeks of hospital care, the rise in serum radioactivity was significantly less in the group of recent drinkers.

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**We are grateful to Boris Ruebner, M.D., for assistance in interpreting histologic changes and to Elizabeth Gong for technical help.

The more direct method of jejunal perfusion was subsequently used to measure the mucosal uptake of ^{3}H-PG-1 in binge-drinking alcoholics (13). Following jejunal intubation and perfusion of an isotonic solution containing 25 mg/l of ^{3}H-PG-1 and glucose, 16.7 mM, the disappearance of the label from 30 cm of perfused jejunum was significantly less in eight poorly nourished vagrant alcoholics than in three middle-class alcoholics who had eaten normally during their binge. Repeated studies showed improved jejunal uptake of ^{3}H-PG-1 after 2 weeks of abstinence with a nutritious diet.

The administration of ethanol, 192 g per day, with a nutritious hospital diet for a 2-week period, suppressed the jejunal uptake of ^{3}H-PG-1 in only two of seven subjects. These studies suggested that the combination of poor nutrition and recent alcohol ingestion impaired the intestinal absorption of ^{3}H-PG-1. Further analysis (14) indicated that, in the initial group of 11 patients, the poor jejunal uptake of ^{3}H-PG-1, as well as of sodium, correlated with a low serum folate level on admission to the study.

The third study measured the effect of the dietary induction of folate deficiency, with and without concomitant ethanol administration, jejunal uptake of ^{3}H-PG-1, water, and sodium (14). Two alcoholic volunteers, previously stabilized in the hospital, ingested a folate-depleted diet together with ethanol, 200 g per day, until tissue evidence of folate deficiency developed. Serum folate levels became subnormal after 3 weeks, and megaloblastic erythropoiesis of the bone marrow was observed after 6 weeks. The jejunal mucosa, obtained by Crosby capsule, remained normal to light microscopy at this time. Jejunal perfusion of the glucose-saline solution containing ^{3}H-PG-1 was performed during the initial control period of abstinence, after development of megaloblastic bone marrow, and a third time, after correction of folate deficiency by 2 weeks of oral folic acid therapy, 5 mg per day. During this latter time, ethanol ingestion continued at the same daily dose. When folate depletion had been achieved at 6 weeks, perfusion of each patient showed a sharp decrease in the jejunal uptake of ^{3}H-PG-1, glucose, water, and sodium. The uptake of each substance returned to control levels after folic acid therapy despite continued ethanol ingestion.

In a third subject, folate deficiency with megaloblastic change in the bone marrow was achieved after 9 weeks of diet without ethanol. Jejunal perfusion at this time showed no change in the uptake of ^{3}H-PG-1 or of glucose, but did show a decrease in absorption to net jejunal secretion of sodium and water. The effect of ethanol alone was studied in a fourth subject in whom jejunal

uptakes were compared after a 3-week period of hospital diet supplemented by ethanol, 300 g daily, and again after a 2-week period of abstinence. No change was observed in the jejunal uptake of ^{3}H-PG-1 and glucose, and net jejunal secretion of water and sodium was observed after the period of ethanol ingestion. Thus, in this prospective study of four patients, either ethanol ingestion or folate deficiency induced by diet was followed by decreased jejunal uptake of water and sodium, whereas the combination of these two factors resulted additionally in the malabsorption of glucose and ^{3}H-PG-1.

The role of protein deficiency in intestinal folate metabolism and absorption was tested in another study in which rats were fed liquid diets containing no protein and 36 percent of their calories as ethanol (15). Compared to controls fed varied combinations of ethanol and protein, no changes were observed in the uptake of ^{3}H-PG-1 from perfused jejunal loops or in the activity of jejunal dehydrofolate reductase.

Other researchers have shown decreased jejunal uptake of water and sodium chloride during jejunal perfusion of recently drinking alcoholics (6). A recent study has demonstrated that 2-weeks' administration of either ethanol and a folate-deficient diet or of a folate-deficient diet alone to normal volunteers is followed by decreased jejunal uptake of water and sodium (16). The independent role of folate deficiency in altered gut function has been demonstrated in the rat, in which induction of folate deficiency is followed by megalocytic changes in the epithelial surface epithelium (17), decreased uptake of labeled thiamine (18), and net jejunal secretion of water and sodium (19). Thus, the combination of dietary folate deficiency and continued exposure of the small intestine to ethanol may explain the diarrhea and malabsorption of ^{3}H-PG-1 of chronic alcoholism.

Although these data indicate that poorly nourished, folate-deficient alcoholics have impaired intestinal absorption of ^{3}H-PG-1, they are incomplete with respect to the effect of alcoholism on the availability of folates from natural sources. Dietary folates are predominantly a mixture of pteroylpolyglutamates that, during the process of intestinal absorption, are hydrolyzed to pteroylmonoglutamate by an enzyme (folate conjugase) in the small intestine mucosa (20, 21, 22). Thus, folate malabsorption could result from impaired activity of folate conjugase, decreased intestinal transport of PG-1, or a combination of these factors.

We have chosen the nonhuman primate as an animal model in which to study more fully the effect of alcoholism on folate absorption and metabolism. In a previous study of baboons, Lieber

and de Carli showed that the chronic ingestion of ethanol as 50 percent of total calories was followed by a spectrum of hepatic injury, including fatty and inflammatory changes and, ultimately, cirrhosis (23). Although these studies were interpreted to show an injurious effect of ethanol on the liver independent of malnutrition, the data did not exclude altered nutrient absorption as a result of chronic exposure to ethanol. Other earlier studies showed that the gastrointestinal tract of the rhesus monkey is susceptible to dietary folate deficiency (expressed as diarrhea), in association with rapidly developing megaloblastic anemia (24).

Materials and Methods

Monkeys of the species *Macaque radiata*, weighing 3 to 5 kg and of both sexes, were purchased after removal from their natural habitat. Each animal was trained to ingest a totally liquid diet as its sole source of fluid and calories. The diet, identical to that used in the baboon study, was isocaloric and contained 16.6 percent of calories as protein (casein), 21.4 percent as lipid (corn oil and olive oil), and 62 percent as carbohydrate (dextrin-maltose). Vitamins and minerals were present in adequate amounts, including an excess of folic acid (500 mg per liter). Over a period of adaptation, five monkeys received a similar diet in which ethanol was gradually replaced for carbohydrate to a final level of 50 percent of total calories. The animals were paired by sex and initial body weight. The diet was offered as 100 cal/kg to the ethanol group, while each control animal was fed amounts equal to the previous day's intake of its pair. Each monkey was weighed twice weekly, and the average daily caloric intake per kilogram was calculated for each month.

Folate status was monitored by determining serum and red cell folate levels in blood drawn at monthly intervals (26). Additionally, monthly measurements were made of complete blood count and serum levels of total protein, albumin, alkaline phosphatase and glutamic oxaloacetic transaminase (SGOT). At 3-month intervals, the monkeys were transferred to hanging metabolic cages for 5-day collections of urine and feces. This procedure permitted subsequent analysis of excretion of average fecal lipid (27) and nitrogen excretion (micro-Kjeldahl method) and, at the 6-month interval, of complete nitrogen balance.

After 3 months, surgical laparotomy was performed on each animal. About 2 g of liver was removed for microscopy and measurement of folate (26) and protein concentration (28). After

subsequent digestion with exogenous hog kidney folate conjugase, total hepatic folate was measured using two organisms, *Lactobaccillus casei* and *Streptococcus faecalis*. Use of the two organisms permitted an estimate of the methylation of hepatic folates as the difference in values, divided by activity for *L. casei*, multiplied by 100. At laparotomy, the jejunal mucosa of each animal were sampled through an elliptical incision. The mucosa were subsequently analyzed by light and electron microscopy and measurement made of activities of sucrase and lactase (29) and folate conjugase (30).

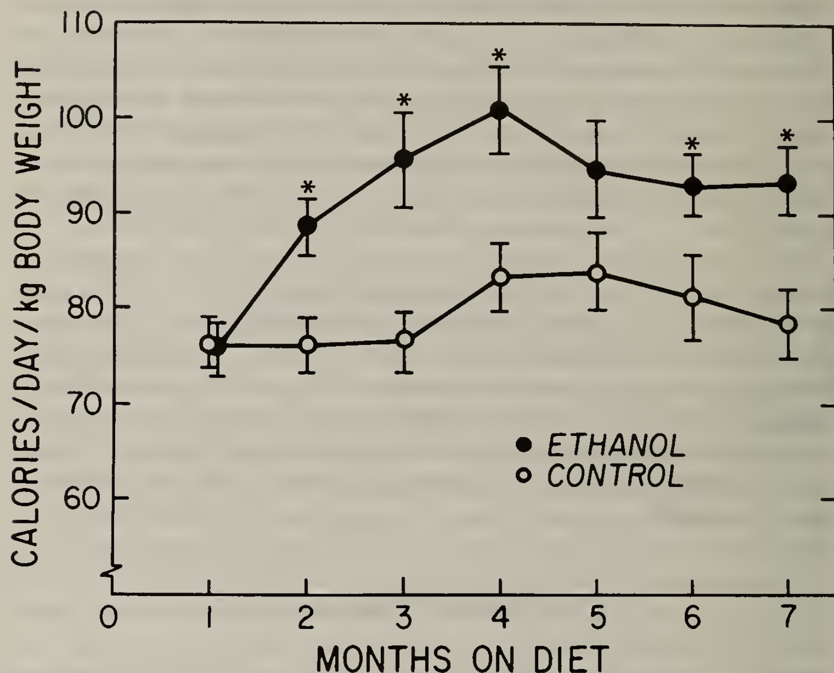
One month after surgery, folic acid absorption was measured as follows. Each fasting animal was administered ^{3}H-PG-1, 25 μg/kg, by stomach tube. The compound was proved pure by previous DEAE column chromatography. Two hours following administration of the labeled folate, each animal received 5 mg of folic acid intramuscularly as a tissue-saturating dose. Isotope recovery was measured in urine collected during the following 72 hours. Counts were corrected for quenching using a quench curve and also by internal standards.

Results and Discussion

After 7 months of feeding, all animals maintained weight or showed a slight gain. However, as shown in figure 1, the daily caloric intake required for weight maintenance, calculated as average daily calories consumed per kg body wt, was significantly greater in the ethanol-fed monkeys from the second month onward. This finding could not be ascribed to intestinal malabsorption, because fecal fat and nitrogen excretion, expressed as a percentage of daily intake, was no different between the groups at 3 and 6 months (table 1). Complete nitrogen balance at 6 months showed no changes between the groups (figure 2). These data contrast with those of Rodrigo et al., who showed increased urinary nitrogen excretion and negative nitrogen balance in rats fed 36 percent of their calories as ethanol for 24 days (31). The findings are consistent with the "empty calorie" concept of ethanol as a food, which Pirola et al. suggest is due to the loss of hydrogen equivalents through the hepatic microsomal ethanol-oxidizing system (32).

Liver specimens obtained by surgical laparotomy at 3 months demonstrated fatty deposition within the parenchymal cells of each ethanol-fed animal. By electron microscopy, in addition to fat droplets, the parenchymal cells showed mitochondrial swelling

Figure 1. Caloric Intake per kg Body wt/d, Based on Average Values Calculated for Each of Seven Months



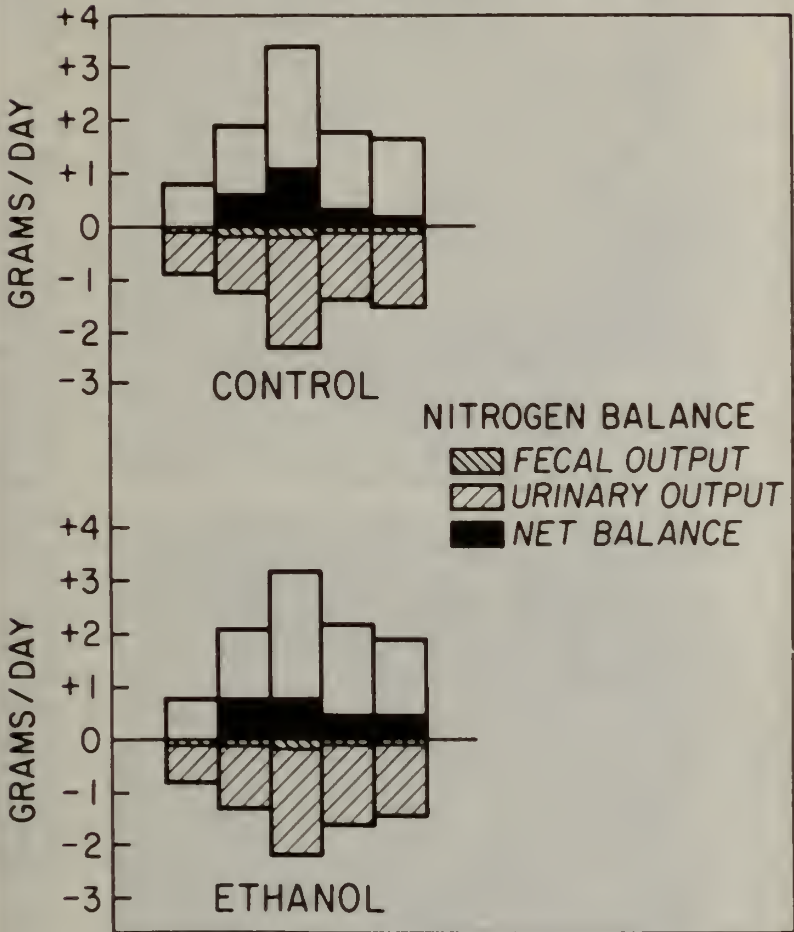
Means ± SEM shown; * indicates significant difference between groups.

Table 1. Fecal Fat and Nitrogen Excretion in Ethanol-Fed and Control Monkeys, as Percentage of Amount Fed per Day

	Fat	Nitrogen	Fat	Nitrogen
Ethanol fed	15.0±1.34	2.4±0.3	10.4±0.9	6.3±0.3
Control fed	17.5±1.58	4.1±0.6	12.3±1.3	6.2±0.5

with prominent cristae and also apparent early collagen deposition within the space of Dissé (figure 3). Further evidence that ethanol feeding was associated with hepatic injury included the observation in the ethanol-fed animals of a 2-fold rise in SGOT at 3 months, followed by a 6-fold rise at 6 months. Alkaline phosphatase was not a sensitive indicator of early hepatic injury (table 2). No changes were observed in serum proteins. Light or electron

Figure 2. Nitrogen Balance at Three Months



Each bar represents a single animal, with pairs shown vertically. Daily data calculated from average during a 5-day collection period.

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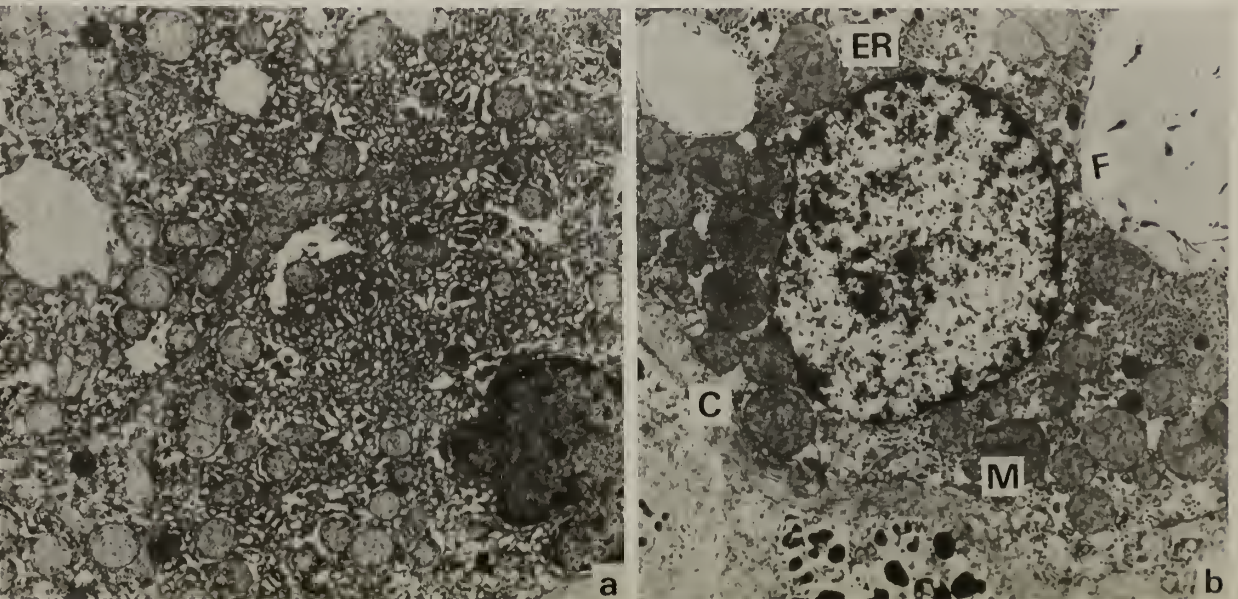
Figure 3. Electron Microscopic Appearance of Liver from Control (left) and Ethanol-Fed (right) Monkeys



Abnormalities shown on the right include mitochondrial enlargement with disordered cristae (M), disorganized endoplasmic reticulum (ER), increased numbers of fat vacuoles (F), and a prominent collagen band (C) in the Space of Dissé. (× 6450)

Table 2. Liver Enzymes at Three and Six Months

	SGOT μ/L		Alkaline phosphatase μ/L	
	3 months	6 months	3 months	6 months
Ethanol fed	77.5±14	188±40.3	321.2±30.6	440±48.6
Control fed	30.4±6.53		308±36.5	

microscopy revealed no differences in the small intestinal mucosa.

The changes in the hepatic parenchyma are similar to those described by Lieber and de Carli, who found fatty changes with mitochondrial enlargement and measured increases in hepatic triglyceride and collagen and activity of the microsomal ethanol-oxidizing system in baboons fed a similar diet (23). Rubin et al. described distorted mitochondria with dilated smooth endoplasmic reticulum in surface epithelial cells of gut mucosa of rats fed ethanol for 9 months and in jejunal biopsy specimens of alcoholic patient volunteers who had received 150 to 200 g of ethanol/d for 2 months (33). The present data suggest that the hepatic parenchymal cell is more sensitive to ethanol, because these cells, but not the epithelial cells of the gut, were affected at this early stage of ethanol feeding.

As shown in table 3, the hepatic folate concentration in the ethanol-fed monkeys was significantly less than that in the control animals ($p < 0.001$). Hepatic protein concentrations were similar in the groups, so it is likely that these data reflect an actual decrease in parenchymal cell folate and not greater contributions of fat to the weight of the livers of ethanol-fed animals. As shown, the percentage of methylated folate was significantly less in the liver tissue from the alcohol-fed animals ($p < 0.01$). Hepatic folate deficiency did not correlate with hematopoietic

Table 3. Hepatic Folate and Protein Concentration at Three Months

	Folate μg/G	% Methylated	Protein mg/G
Ethanol fed	4.28±0.38	55.7±1.8	180.4±13.8
Control fed	7.01±0.59	65.8±3.4	191.8±24.3
p	<.001	<.01	N.S.

Table 4. Serum and Red Cell Folate Levels

	Serum (ng/ml)		Red cell (ng/ml)	
	3 months	6 months	3 months	6 months
Ethanol fed	5.7±.93	5.7±1.09	62.4±7.13	46.0±14.3
Control fed	4.7±.46	3.3±.44	53.0±3.02	34.1±9.56

deficiency: None of the animals became anemic, and serum and red cell folate levels remained unchanged throughout (table 4). The red cell folate levels were considerably lower than normal human levels (34).

Other researchers have described levels in this range in the baboon, suggesting distinct interspecies differences (35). Maintenance of peripheral blood folate status may be ascribed in part to the excessive provision of dietary folate (50μg/kg body wt), even though, as described below, folate absorption was impaired in the ethanol-fed animals. The lack of correlation of the effect of ethanol feeding on hepatic and circulating folate suggests that the latter site is preferentially spared at the same time as hepatic storage capability, and methylation is diminished.

Following administration by gastric gavage of purified ^{3}H-PG-1, followed by an intramuscular tissue-saturating dose of folic acid, significantly less radioactive label appeared in the 72-hour urine collection of the ethanol-fed animals than did in the controls (table 5). These results could reflect altered renal, hepatic, or intestinal function. Creatinine clearances were similar in the two groups. Previous work suggests that the hepatic injury of ethanol results in less hepatic retention of labeled ^{3}H-PG-1, a factor that should enhance, rather than diminish, urinary folate excretion (36). We have previously demonstrated the adequacy of tissue

Table 5. Absorption of ^{3}H-PG-1

	% ^{3}H recovered[a]
Ethanol fed	18.8±1.9
Control fed	43.8±5.8

Note: $p < 0.005$

[a]In 72-hour urine after gastric gavage.

saturation by this method in an alcoholic patient (4) and consider it unlikely that lowered urinary excretion in the ethanol-fed animals represents loss of the isotope into depleted tissue. These data are most consistent with impaired intestinal absorption of ^{3}H-PG-1 in the ethanol-fed monkeys. The activity of jejunal folate conjugase, as well as that of jejunal disaccharidase, was not impaired by ethanol feeding (table 6), so the present data suggest that intestinal hydrolysis of pteroylpolyglutamate is not impaired by chronic ethanol feeding.

Table 6. Jejunal Enzymes After Three Months

	Folate conjugase nM/mg protein	Lactase μ/g protein	Sucrase μ/g protein
Ethanol fed	1.45±.54	25.3±6.1	312±139
Control fed	0.86±.17	23.8±3.7	479±152

Conclusions and Research Needs

A prospective study of the effect of prolonged ethanol administration on hepatic and intestinal folate metabolism has been initiated using a nonhuman primate model. After 3 months of feeding 50 percent of calories as ethanol, the data have shown a greater caloric requirement for weight maintenance in the ethanol-fed animals; however, there was no evidence for dietary fat or nitrogen malabsorption. The decreased folate concentration in the liver may be ascribed to a combination of diminished intestinal absorption of folic acid and altered hepatic metabolism, as suggested by impaired methylation. We anticipate that continued ethanol feeding will result in progressive hepatic injury, which may be associated with more severe impairment of folate metabolism. Further studies on this model will focus on the following areas.

1. Correlation of predictable progression of hepatic injury with altered hepatic synthesis of pteroylpolyglutamate, the storage form of folate. Following absorption as pteroylmonoglutamate, folates are reconjugated to pteroylpolyglutamate in the liver (37). Impaired hepatic pteroylpolyglutamate synthesis can be evaluated by chromatographic analysis of liver folates after parenteral injection of labeled ^{3}H-PG-1 (38). Results between the groups can be correlated with microscopic assessment of hepatic injury.

2. Evaluation of the effect of chronic ethanol feeding on hydrolysis and absorption of pteroylpolyglutamates. The finding of similar activities of mucosal folate conjugase in the two groups does not exclude the possibility that chronic ethanol feeding impairs the intestinal hydrolysis of pteroylpolyglutamates. Microscopic changes in the epithelium may develop with more prolonged feeding of ethanol. A recent study has shown impaired activity of brush border surface disaccharidases in chronic alcoholics (39). We have recently described the presence of two mucosal folate conjugase activities—one intracellular and the other on the brush border surface (40). Our human jejunal perfusion studies indicated that the in vivo hydrolysis of pteroylpolyglutamates is primarily affected by a surface-active folate conjugase (41), so it will be appropriate to study the specific effect of ethanol on the brush border surface enzyme. The availability of synthetic ^{14}C-labeled pteroylheptaglutamate will permit future comparisons of absorption of this compound, administered simultaneously with ^{3}H-PG-1.

3. Evaluation of the effect of dietary induction of folate deficiency on intestinal and hepatic folate metabolism. In view of the morbidity (severe diarrhea) (25) and possible mortality to be expected from induction of folate deficiency in the monkey, we have chosen to save this aspect of the experiment until last. By removing folate from all diets, it will be possible to study the effect of folate deficiency, with and without continued ethanol administration, on peripheral blood folate levels, on intestinal folate absorption, and on hepatic synthesis of pteroylpolyglutamate storage folate, again correlating these data with microscopic evaluation of the injury.

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Discussion of Paper by Halsted et al.

Dr. Schenker: I think I heard you say that it is not completely straightened out whether folate is transported by an active process or not. What seemed to be the difficulties with regard to determining the mechanism of transport of folate? That is my first question. And the second question is, in your monkeys, you expressed your folate concentration in the liver as per unit weight. What about the total size of the liver in the monkeys? Was it larger? If you were to multiply it by that factor, would the difference be smaller?

Dr. Halsted: Well, let me answer the second question first. I do not know what the total weights of the livers are because they are still in the monkeys. I guess this gets down to the problem that everybody deals with, how do you express enzyme activity in the liver? If fat is increasing, weight of the liver also increases. We have also expressed the results on the basis of per mg protein, and there is still a significant difference.

Dr. Schenker: There are some ultrasound techniques whereby you could perhaps determine the total mass of the liver, and that was really what I was getting at. We are using it in patients in terms of determining drug-metabolizing activity, and I think that might be something you might want to consider using.

Dr. Halsted: Thank you. As for mechanisms of folate absorption, I think it is surprising that people have worked in this area for 15 or 20 years, and the answer is still elusive. I think the best evidence is that folic acid absorption is a saturable process, and it can be stimulated with glucose. Congenital folic acid deficiency in humans has been reported, which suggests that there is a specific carrier mechanism. Whether this means that there is an active uphill transport or whether something else is going on is unclear. There is another theory. It holds that the polarity of folic acid is changed as it goes through the unstirred layer because of pH changes, and this somehow affects solubility and transport.

The Effect of Ethanol on Folate Metabolism*

Robert S. Hillman

Studies of the incidence and character of hematological abnormalities in alcoholic patients have directed attention to an apparent toxic effect of alcohol on folate metabolism. This interference with normal folate supply to hematopoietic tissue results in marked disruptions of cellular proliferation, maturation, survival, and function. However, the defect in cellular maturation, that is, abnormal DNA replication and cell division, is the recognized hallmark of folate deficiency. Clinically, it is first recognized by the appearance of megaloblastic erythropoiesis on examination of the bone marrow, followed by the development of a macrocytic anemia.

The direct relationship between alcohol and folate-deficient megaloblastic erythropoiesis was first established by Sullivan and Herbert (16,17). Since then, survey studies of chronic alcoholic patients have shown low serum and/or red cell folate levels in 50 to 80 percent of such individuals, with up to 60 percent having associated hematological abnormalities (6,18). The effect of alcohol ingestion on the serum folate level is dramatic. When normal individuals are placed on a diet containing little or no folate and asked to drink sufficient alcohol to raise their blood alcohol levels to approximately 100 mg/100 ml, they demonstrate a dramatic reduction in serum folate levels. These reach a deficient range within 24 to 48 hours (7). This effect can also be seen with acute intravenous infusions of alcohol. It is quite different from the behavior of serum folate levels in subjects maintained on deficient diets alone (8). It is also significant in terms of tissue folate supply; megaloblastic erythropoiesis will follow within as little as 10 days as a result of this level of alcohol ingestion.

The ability of alcohol to induce a tissue abnormality is closely related to the state of folate nutrition. As demonstrated by the Sullivan and Herbert study (16) and the work of Lindenbaum and Lieber (13), folate supplementation will counteract the toxic

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effect of alcohol on folate metabolism. Furthermore, in a Seattle survey study of chronic alcoholics, the relationship of food and alcohol to the incidence of hematological abnormalities was clearly demonstrated (5). Patients with abnormal hematopoiesis tended to drink the equivalent of more than 4 to 8 ounces of 100-percent ethanol per day and eat less than one meal per day. Thus, any study of alcohol toxicity on folate metabolism must be aware of and control for the level of folate nutrition.

Although simple dietary deprivation of folate plays an important role, it is not the only reason for abnormal folate metabolism in chronic alcoholics. At least two other mechanisms have now been identified. As discussed by Dr. Halsted, alcohol interferes with the absorption of folate in patients with poor folate nutrition. However, similar to the dietary deprivation situation, this toxic effect cannot explain the dramatic fall in serum folate levels and the rapid induction of megaloblastic erythropoiesis. Therefore, attention must be directed toward a toxic effect of alcohol on internal folate metabolism.

When the rapid depression of serum folate levels by alcohol was first appreciated, Eichner and Hillman carried out a series of studies looking for an alcohol-induced error in the measurement of serum folate (7). Alcoholic plasma, alcohol, acetaldehyde, lactate, and triglycerides were tested for their effect on the *L. casei* bacteriological assay system. No inhibitory effect could be detected. In addition, subsequent studies using the milk binder radioisotope assay of serum folate have confirmed the effect of alcohol on folate levels, thereby ruling against a simple measurement error (15). Studies of folate binders in the plasma of alcoholics have also failed to reveal any nonspecific effect of alcohol which might interfere with folate delivery to cells (4).

A more productive area of investigation has been the study of internal folate kinetics. Beginning with the first measurements of tissue uptake and handling of folate, it has been possible, using nonisotopic folate, to show a change in tissue avidity for folate in association with folate deprivation (2,3,9). As the methodology for kinetic measurements has improved, studies carried out on alcoholic humans have demonstrated normal or increased uptake and tissue binding of tritiated pteroylglutamic acid, regardless of the state of folate nutrition or alcohol intake (1,12,19). As estimated by the Johns et al. (11) washout technique, the apparent rate of pteroylglutamate reduction, methylation, and storage as a tissue-bound methyltetrahydrofolate pentaglutamate was similar for normal, folate-deficient, and alcoholic subjects. Perhaps the only suggestion of an alcohol-related defect was a slight increase

in storage and/or a decrease in utilization of ^{14}C methyltetrahydrofolate in alcoholic subjects. However, this finding must be interpreted in the light of the use of a ^{14}C-methyltetrahydrofolate isotope that not only is of low specific activity, but also contains equal amounts of the d-isomer, a form of the vitamin that may not be used by human tissue.

Studies using a rat-animal model have provided much more satisfactory data. McGuffin et al. were able to show that acute alcohol ingestion in the rat results in a rapid depression of serum folate levels, at a time when liver folate stores are relatively intact (14). These results are similar to those experienced with human studies. It has also been possible to establish the isotopic and chromatographic techniques required for measurements of folate clearance from plasma, hepatic uptake and storage, rates of folate congener and polyglutamate formation, and finally, folate release into bile. As a result of this work, a specific toxic effect of alcohol on the hepatic handling of folate can now be demonstrated (10). In the normal rat, the intravenous injection of either ^{3}H pteroylglutamic acid or ^{14}C methyltetrahydrofolic acid is followed by a rapid uptake of 40 to 50 percent of the isotope into liver over a 1 to 3 hour period. Each isotope then follows a distinctly different pathway. In the case of ^{14}C methyltetrahydrofolate monoglutamate, there is an almost immediate excretion of the isotope into bile, so that by 3 hours there is little or no residual activity present in liver. None of the isotope is incorporated into the liver pentaglutamate pool, and the excretion into bile is not associated with a change in the character of the folate. In contrast, following the uptake of ^{3}H pteroylglutamic acid into liver tissue, it is rapidly reduced and methylated to form ^{3}H methyltetrahydrofolate monoglutamate. As a part of this process, 15 to 20 percent of the material is also converted to the pentaglutamate form of methyltetrahydrofolate. The remainder is then excreted into bile, either as methyl- or formyl-tetrahydrofolate monoglutamate, for recirculation through the gut. This then forms a characteristic enterohepatic cycle.

The size and behavior of the enterohepatic cycle in the rat is quite significant. As much as 50 percent of a single tracer dose of isotope will move through the cycle in a 6-hour period. In addition, the role of the cycle in determining the serum folate level is easily demonstrated. Interruption of bile folate flow by common bile duct drainage results in a rapid fall of the serum folate to levels of 40 percent of normal within 4 to 8 hours. Thus, the enterohepatic cycle provides an excellent potential target for

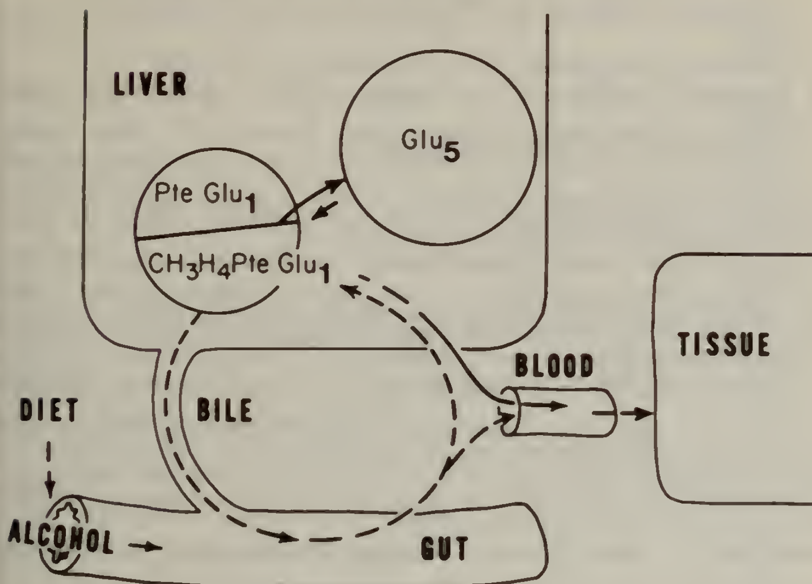
any toxic effect of alcohol on either gut absorption or hepatocyte folate metabolism.

The presence of such a defect has now been demonstrated. When the kinetics of the enterohepatic cycle were studied in animals maintained on a folate-free diet for 3 days and compared to animals on a deficient diet plus alcohol by intragastric instillation, isotope uptake by the liver was normal or increased in both groups of animals. At the same time, the folate-deprived animals demonstrated a reduced incorporation of ^{3}H pteroylglutamic acid into the methyltetrahydrofolate pentaglutamate pool. Instead, the folate appeared to be shunted into a larger than normal methyltetrahydrofolate monoglutamate pool and into bile. These results suggest a normal mechanism in the hepatocyte for mobilizing polyglutamate stores to maintain bile and serum folate levels in periods of folate deficiency.

When folate-deprived animals were treated with alcohol, the behavior of the folate isotopes was quite different. Although ^{14}C methyltetrahydrofolate uptake and excretion into bile were not affected, alcohol ingestion resulted in a major shunting on ^{3}H pteroylglutamic acid into the pentaglutamate storage pool. A major reduction of folate excretion into bile then resulted, and, as confirmed by bacteriological assay, there was a concomitant decrease in the total amount of folate flowing through the enterohepatic cycle. These findings are summarized in figure 1. In the alcoholic animal, any folate coming to the liver for the purpose of reduction and/or methylation will preferentially be shunted toward the pentaglutamate pool or maintained within the hepatocyte as a methyltetrahydrofolate monoglutamate. The result is a reduction in bile folate flow and a drop in the serum folate level.

The mechanism behind this alcohol effect is still unclear. One possibility is the formation of an abnormal type of folate such as ethyltetrahydrofolate, which would be unavailable for normal export into bile. A second might be a specific alcohol effect on the conjugase system of liver, which would interfere with the mobilization of the pentaglutamate storage pool. However, a direct demonstration of reduced levels of carboxypeptidase in alcoholic rat liver has not been possible. An increased use or storage requirement for folate by the hepatocyte as a part of the alcohol induction of the microsomal enzyme system is another possibility. This relationship has been suggested as a reason behind the apparent folate deficiency experienced during diphenylhydantoin (Dilantin) ingestion. Finally, recent work by Dr. Wagner would suggest a direct toxicity of alcohol on the hepatocyte export pathway for folate.

Figure 1. Effect of Alcohol on Folate Circulation



In the alcoholic animal, dietary deprivation of folate and alcohol ingestion reduces folate transport across the gut wall. In addition, alcohol interferes with the kinetics of liver folate storage and excretion into bile and further reduces the normal flow of folate through the enterohepatic cycle. As demonstrated by tracer isotope studies, alcohol ingestion results in a shunting of folates returning to the liver for reduction and methylation into either a pentaglutamate or a methyltetrahydrofolate monoglutamate pool at the expense of bile folate excretion. This action results in a rapid and significant fall in enterohepatic recirculation of folate and the level of serum folate available to tissue.

Our understanding of the relative importance of hepatic folate storage and enterohepatic flow is only beginning. The demonstration of a toxic effect of alcohol on folate kinetics has been restricted to the liver and enterohepatic folate cycle, but the potential importance of such a toxic effect may reach out to all peripheral tissues. Many tissues may, in fact, act similarly to the hepatocyte in accepting various folate congeners, performing the reduction of methylation steps and actively incorporating new folate into pentaglutamate stores. Thus, as methods become available, it may be possible to show that alcohol has a universal toxic effect on folate utilization, resulting in major disruptions in internal folate metabolism at all tissue sites. This possibility is an important and exciting area for future investigation.

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Discussion of Paper by Hillman

Dr. Henderson: What happens to the erythrocyte level of folate when the plasma goes down so precipitously? Does it drop, also?

Dr. Hillman: They are very insensitive to all of this, because most of what you are looking at is a trapped type of polyglutamate in red cells, which is not going anywhere. It is just sitting there, and you have to wait for a whole new population of red cells, produced under these adverse conditions, to appear. If you do that, you wait for 3, 4, 5 weeks, then the red cell folate level goes down. But in terms of the 48-hour to 1-week time, you do not see any change in red cell folate.

Dr. Henderson: Have other tissues been examined in this regard?

Dr. Hillman: That is the problem. For the other tissues, there are a lot of methodology requirements, and a lot of hard work is needed.

The Uptake of Folate by Isolated Hepatocytes

Conrad Wagner, Donald W. Horne, and William Briggs

The following report presents some recent studies on the uptake of folate by isolated hepatocytes and some preliminary data on the effect of ethanol on this process. These in vitro experiments complement the in vivo studies presented by Dr. Hillman and are particularly appropriate to consider at this time.

For the past several years, we have been working with the isolated hepatocyte system in order to establish it as a model for the study of the transport of water-soluble nutrients. This system has been used to measure the transport kinetics of amino acids (1), bile acids (2), and other small molecules (3,4) into the liver cell.

The major form of folate found in human plasma is (l)-5-methyl-H_4 folate. Uptake of this compound by isolated rat hepatocytes (3) follows the time course shown in the first figure. It shows that the rate of uptake is linear for at least 10 minutes, and that uptake continues to increase up to 90 minutes. At this time, tissue-to-medium concentration ratios are greater than 1.0. Other studies have shown that at least 80 percent of the 5-methyl-H_4 folate taken up is unchanged and unbound after this time. The uptake is saturable with increasing substrate concentration, is competitively inhibited by analogs, and is inhibited by various compounds that interfere with energy production. Examples are also shown in figure 1. The presence of 10 mM sodium arsenate (an inhibitor of high-energy phosphate formation) and 0.1 mM 5-formyl-H_4 folate (a substrate analog) inhibits the uptake of 0.25 μM 5-methyl-H_4 folate. On the other hand, 10 mM sodium azide (an inhibitor of electron transport) stimulates the rate of uptake.

Of necessity, uptake measurements of this type represent net flux. A stimulation of the net flux by an agent such as sodium azide may be explained as an actual inhibition of efflux. A similar phenomenon was observed by Goldman, who reported that the uptake of methotrexate by L-1,210 cells was stimulated by sodium azide; this effect was explained by an inhibition of efflux (5). Figure 2 shows that sodium azide inhibits efflux of labeled 5-methyl-H_4 folate from the hepatocytes.

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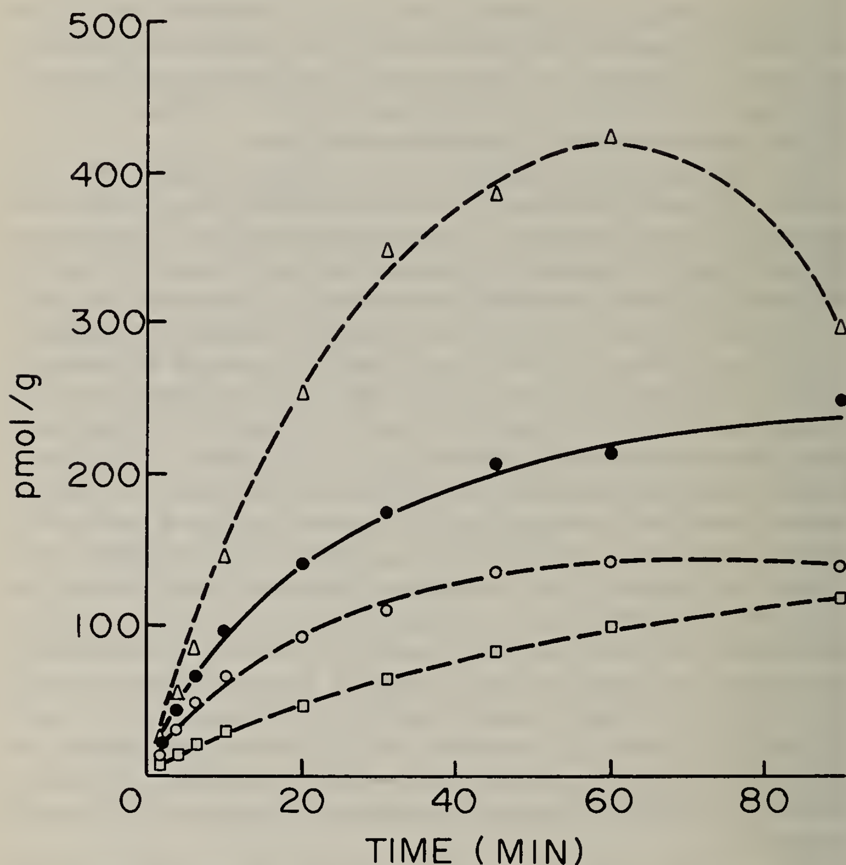
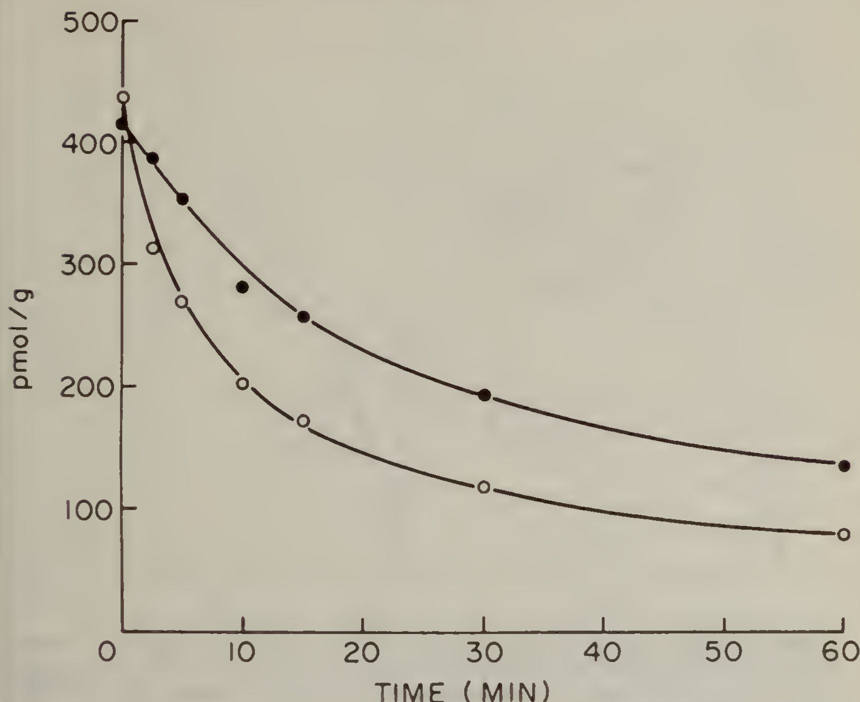
Figure 1. Time Course of 5-Methyl-H_4 Folate Uptake in Isolated Hepatocytes



Labeled 5-methyl-H_4 folate and the test compound were added to the cells simultaneously at zero time. The uptake of 0.25 μM 5-methyl-H_4 [G-^{3}H] folate was measured over the next 90 minutes in control cells (●) and cells with 10 mM sodium azide (△), 10 mM sodium arsenate (○), and 0.1 mM 5-formyl-H_4 folate (□).

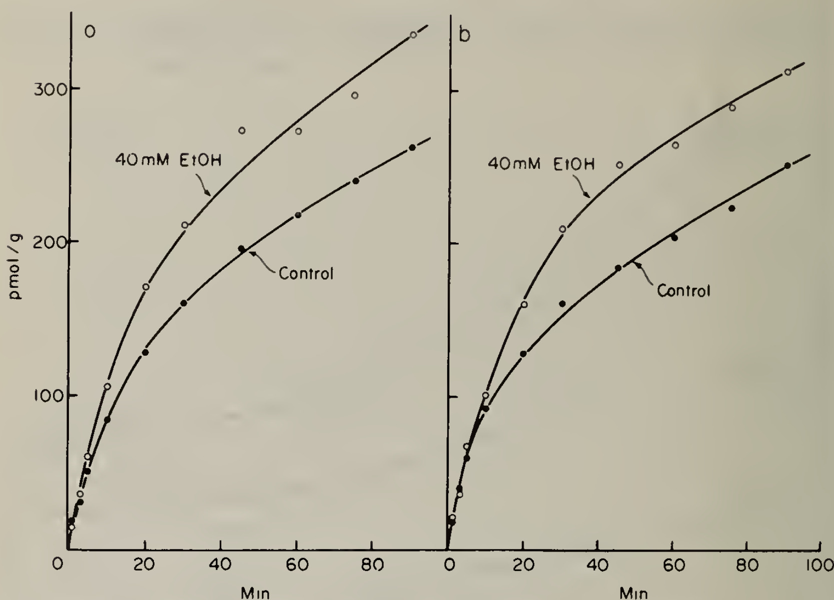
Figure 2. Inhibition of Efflux of 5-Methyl-H_4 Folate from Isolated Hepatocytes



The cells were incubated for 15 minutes in the presence of 1.66 μM 5-methyl-H_4 [G-^{3}H] folate and 10 mM sodium azide. The cells were washed once in 5-methyl-H_4 folate- and azide-free medium at 37° and resuspended in fresh medium with (●) and without (○) 10 mM azide.

The effect of ethanol on this uptake process was investigated next. These results are shown in figure 3. Part A of figure 3 demonstrates that the in vitro addition of 40 mM ethanol to the uptake medium markedly stimulates the rate of 5-methyl-H_4 folate uptake. This stimulation is also seen in part B of figure 3. In these latter experiments, shown in part B, the hepatocytes were isolated from animals that had received 4 gm/kg of ethanol 1 hour prior to sacrifice. The isolated hepatocytes were then incubated with and without 40 mM ethanol. There is no difference in the rate of uptake of the substrate by either the controls of parts A and B or by the ethanol-stimulated cells of parts A and B. This finding indicates that, although the in vitro presence of ethanol enhances the rate of 5-methyl-H_4 folate uptake, prior acute administration of ethanol does not.

Figure 3. Effect of Alcohol on Uptake of $5\text{-}CH_3\text{-}H_4PteGlu$



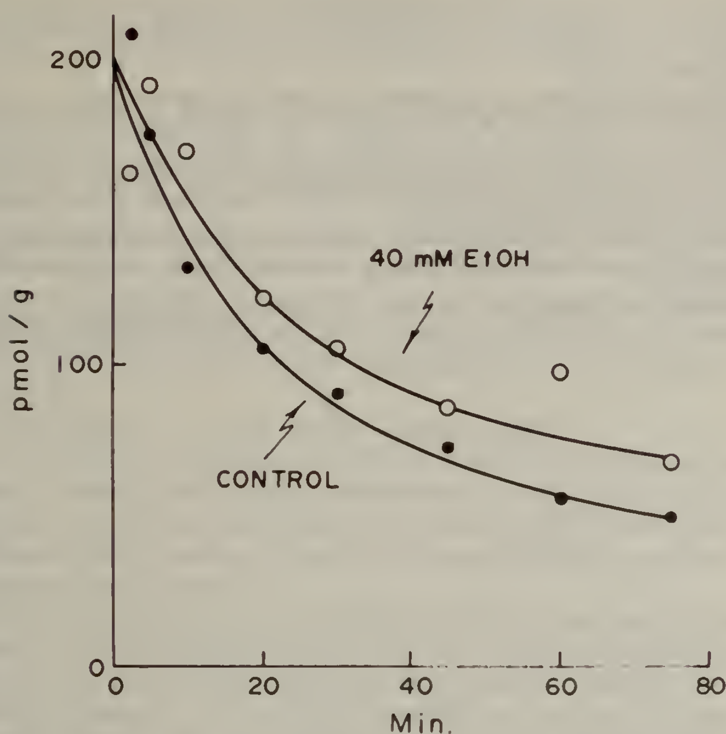
A. Male rats weighing approximately 200 g were used for isolation of hepatocytes. The resulting cells were incubated in the absence (●) or presence (○) of 40 mM ethanol, and the uptake of 0.25 μM 5-methyl-H_4 [G-^{3}H] folate was measured.

B. The uptake was the same as for A, except that the hepatocytes were isolated from animals which had received 4 g/kg of ethanol by stomach tube 1 hour before sacrifice.

It should be noted that stimulation of uptake by ethanol is not immediately apparent. The effect of ethanol is apparent after 20 minutes and is greatest after 40 minutes. Thus, a metabolite of ethanol may be responsible for the stimulated uptake. In other experiments, the substitution of 40 mM acetaldehyde for ethanol in the in vitro experiments did not result in any stimulation.

The increased rate of uptake in the presence of ethanol is reminiscent of the effect of sodium azide (figure 1). We therefore sought to determine if ethanol inhibited efflux. The results of one such experiment are shown in figure 4. There is a small but measurable inhibition of efflux by ethanol. This effect is not always seen and the results are variable, which may be due to the fact that the maximum effect of ethanol on uptake is observed after 40 minutes incubation. When efflux is measured, most of

Figure 4. Effect of Alcohol on Efflux of 5-CH_3-H_4PteGlu



Hepatocytes were incubated for 45 minutes in the presence of 0.25 μM 5-methyl-H_4 [G-^{3}H]-folate. The cells were washed once with 5-methyl-H_4 folate-free buffer at 37° and then incubated in the presence (○) or absence (●) of 40 mM ethanol.

the preloaded substrate has left the cell by 40 minutes, and small differences would not be observed.

These studies have shown that there is a saturable, energy-dependent uptake system for (l)-5-methyl-H_4 folate in the isolated rat hepatocyte. Uptake is stimulated by sodium azide and ethanol. The stimulation by azide can be explained by a primary inhibition of the efflux system. The stimulation by ethanol may also be due to an inhibition of efflux, but this effect is variable.

These results show that efflux of 5-methyl-H_4 folate is not simply a reversal of influx and may be linked to different processes in the cell. These in vitro studies also tend to support those of Hillman on the in vivo effects of ethanol administration on the disappearance of folate from the plasma.

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Discussion of Paper by Wagner et al.

Dr. Lieber: I wonder whether I may have some additional information concerning that remark you made that acetaldehyde did not reproduce the effect. Could you tell us what level of acetaldehyde was used? What was the measurement of the acetaldehyde in vitro and how did you actually perform that experiment?

Dr. Wagner: The uptake experiments were carried out with 40 mmol ethanol. Instead of using 40 mmol ethanol, we used 40 mmol acetaldehyde.

Dr. Lieber: Now 40 mmol acetaldehyde is, of course, a huge amount.

Dr. Wagner: Yet, it is, but we saw no effect.

Dr. Lieber: I do not know what it actually would mean, except that if you add 40-mmol acetaldehyde and do not block its conversion to ethanol with pyrazole, you actually should have the ethanol effect, because most of the acetaldehyde is going to be converted to ethanol.

Let me just point out two major pitfalls of acetaldehyde experiments. This comment pertains both to what we heard today and also what we heard in terms of the effects of acetaldehyde on protein synthesis. If one adds acetaldehyde, in vitro, there are two major pitfalls. One, it is rapidly converted to ethanol unless one blocks that conversion, and, therefore, if one is interested in the effects of acetaldehyde, one should always be careful to block its conversion back to ethanol. And second, even if one does that, the capacity for the cell to metabolize acetaldehyde is such that the level very rapidly falls to almost zero. So it is very important to keep giving acetaldehyde, infusing it continuously or adding it continuously, and monitoring the levels. Otherwise one may well see no effect because there is no acetaldehyde left within a matter of minutes. So these are two important pitfalls that one should probably be aware of if one performs experiments with acetaldehyde.

Intestinal Thiamine Absorption: Normal Characteristics and Effects of Ethanol

Anastacio M. Hoyumpa, Jr.

Abstract

Thiamine deficiency is a common feature of chronic alcoholism in humans and may lead to impaired neurological and cardiovascular functions. To understand the pathogenesis of thiamine deficiency in chronic alcoholism, the normal characteristics of intestinal thiamine transport were studied in rats, and the effect of ethanol was determined. Results indicate the presence of a dual system of intestinal thiamine transport in rats. At low thiamine concentrations ($< 1.0\ \mu M$), transport is a saturable, carrier-mediated, sodium-dependent active process; at higher concentrations ($> 1.0\ \mu M$), transport is a passive process. Ethanol impairs the active, but not the passive, component of thiamine transport by blocking the exit of the vitamin from the cells. Thiamine entry into the cells is not affected by ethanol. Further, there is evidence suggesting that ethanol impedes thiamine transport across the serosal membrane by inhibiting Na-K ATPase activity in the basolateral membrane. Additional studies are needed, however, to confirm whether active thiamine transport is truly dependent on Na-K ATPase activity.

Chronic alcoholism is frequently associated with thiamine deficiency. Poor intake of the vitamin is perhaps the main cause (1,2), but decreased activation of thiamine to thiamine pyrophosphate (3) and reduced hepatic storage of thiamine in patients with alcoholic fatty liver (4) may be important also. Moreover, there is evidence in animals to suggest that other nutritional disorders, such as folate (5,6) or pyridoxine deficiency (7), may contribute to decreased thiamine absorption. Finally, alcohol may impair the intestinal absorption of thiamine (8,9). Because of the last consideration, studies were conducted to determine the characteristics of normal intestinal transport of thiamine in rats and, subsequently, to determine the effect of ethanol.

Methods

Thiamine transport was measured both in vivo and in vitro in rats. First, using isolated, well-vascularized intestinal loops, in vivo, the rate of disappearance of ^{35}S-thiamine hydrochloride from the intestinal lumen was measured by determining the difference between the quantity of thiamine introduced into the loop and the amount recovered from the lumen and wall of the intestinal segment after absorption was allowed to proceed for a period of time. Second, measurements of net transmural flux of ^{14}C-thiamine hydrochloride were obtained in vitro, using everted intestinal segments (10). Movement across a concentration gradient was deemed present when the serosal-to-mucosal concentration ratio became significantly greater than the initial ratio of 1. Third, to further characterize normal thiamine transport, unidirectional tissue uptake of ^{14}C-thiamine hydrochloride across the brush border membrane was measured, using ^{3}H-Dextran as a nonabsorbable marker of adherent mucosal volume (11). The technique was also adapted to determine the transfer of thiamine from the tissue to the serosal compartment (12). Uptake into the tissue, as well as movement from the tissue to the serosal fluid, was shown to be linear with time up to 5 minutes and with dry tissue weight (12). Finally, unidirectional uptake and net transmural flux were measured, in vitro, under short-circuit conditions (13,14).

Characteristics of Normal Intestinal Thiamine Transport

Data obtained from in vivo studies showed that at low concentrations of thiamine, ranging from 0.06 to 1.5 μM, absorption from all segments of the small intestine was a saturable process, with the rate being greatest in the duodenum and least in the ileum (15). In contrast, the rate of absorption in the duodenum, jejunum, and ileum increased in linear fashion as the lumenal thiamine concentration rose from 2 to 560 μM.

These findings were confirmed and extended by in vitro studies (15). Examination of net transmural flux revealed movement of 0.2 μM against a concentration gradient so that the serosal-to-mucosal ratio increased to 1.5; for 20 μM thiamine, the ratio remained 1.0. The difference was significant. Moreover, inhibitors such as pyrithiamine, dinitrophenol, N-ethyl maleimide, and ouabain reduced the serosal/mucosal ratio to 64, 70, 72, and 86

percent of normal. These inhibitors did not affect the net flux of 20 μM thiamine.

The unidirectional jejunal uptake of low concentrations of thiamine was found to be saturable (figure 1A), consistent with the earlier in vivo results. Pyrithiamine, a structural analog of thiamine, exhibited competitive inhibition on the jejunal uptake of low concentrations of thiamine. In contrast, pyrithiamine did not influence the uptake of high thiamine concentrations that were nonsaturable (figure 1B). In addition, uptake of low concentrations of thiamine was significantly reduced in the presence of anoxia, low temperature, and replacement of sodium ion with equimolar mannitol. These conditions had little or no adverse effects on the uptake of high concentrations of thiamine.

In recent years, the water layer adjacent to the lipid membrane of the brush border microvilli has come to be recognized as an important determinant of the kinetics of transport (16). Stirring serves to reduce the thickness of the aqueous barrier and thereby facilitates transport of solutes (16). The effect of mechanical stirring of the incubation media at 900 rpm was therefore examined in relation to thiamine transport. Stirring reduced the km of thiamine from 0.634 μM to 0.374 μM, but Kmax was not affected and the permeability constant, P, remained unchanged.

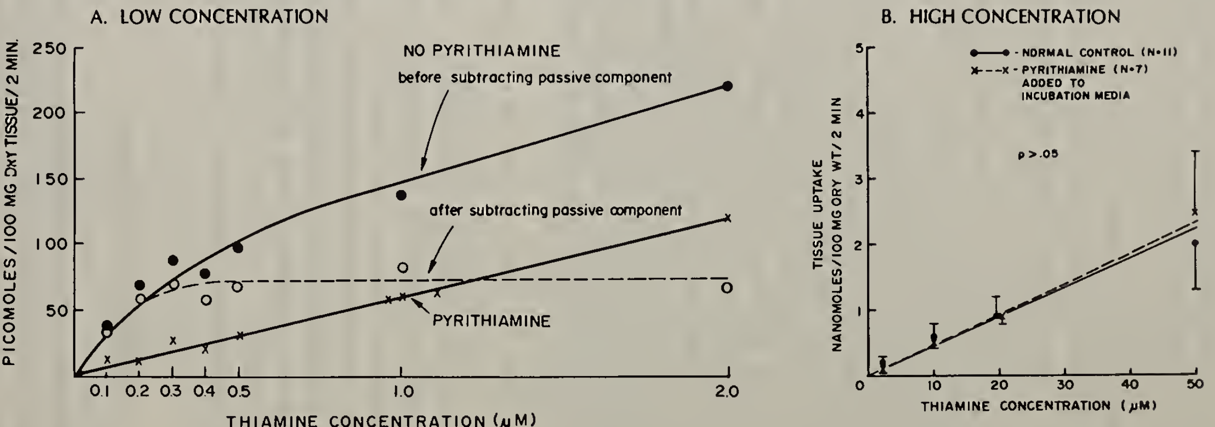
Studies with electrical short-circuiting maneuvers indicate that thiamine at low (but not high) concentrations can be transported against an electrical gradient (15).

Taken together, these findings suggest the existence of a dual system of thiamine transport across the rat small intestine. At low thiamine concentrations (< 1.0 μM), transport is a saturable, carrier-mediated, sodium-dependent active process that proceeds against an electrochemical gradient and is affected by changes in the thickness of the water layer brought about by stirring. In contrast, at high concentrations (> 1.0 μM), transport is predominantly a passive process, unaffected by structural analogs, metabolic inhibitors, or stirring. A similar dual system of intestinal transport has now been shown for folates (17) and vitamin A (18).

Effect of Ethanol

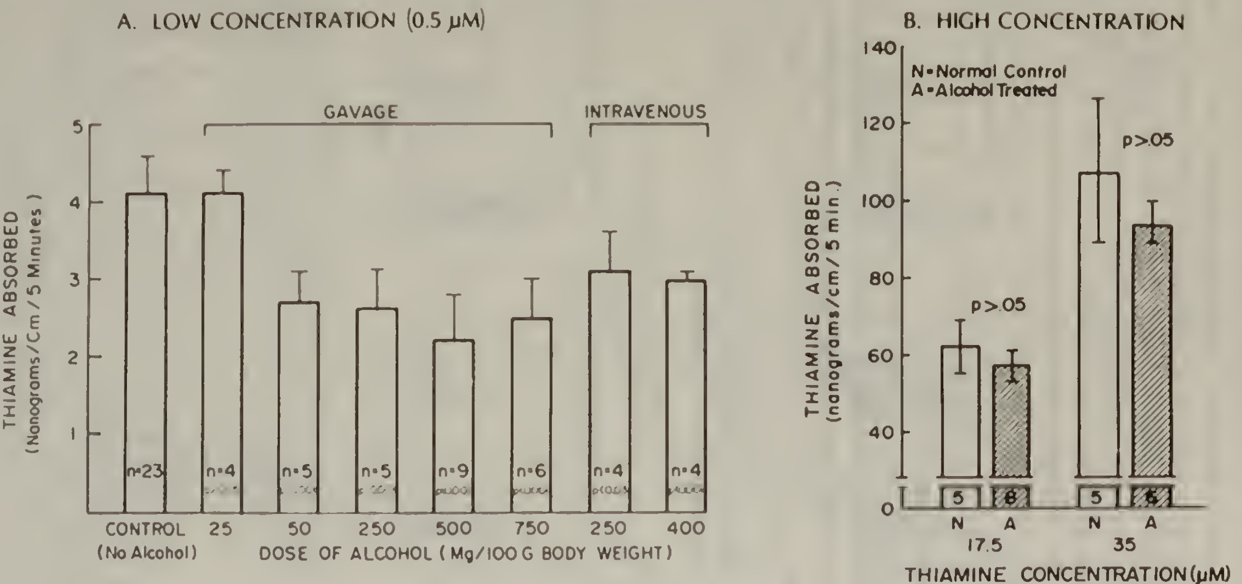
Acute Ethanol Studies. The intragastric or intravenous administration of ethanol in single doses, 50 to 750 mg (but not 25 mg/100 g body weight), produced in 1 hour a significant reduction (average 38 percent) in the absorption of 0.5 μM thiamine from in vivo duodenal loop (figure 2A) (12). Once attained, the degree of

Figure 1. Unidirectional Jejunal Uptake of Low and High Concentrations of Thiamine



Pyrithiamine 2 μM inhibited the uptake of low concentrations of thiamine (panel A); the addition of 200 μM of this analog had no effect on the uptake of high concentrations of thiamine (panel B). Reproduced from reference (15) with publisher's permission. Copyright 1975 by The Williams & Wilkins Co.

Figure 2. **Effect of Alcohol on Absorption from Intact Duodenal Loop of Low and High Concentrations of Thiamine**

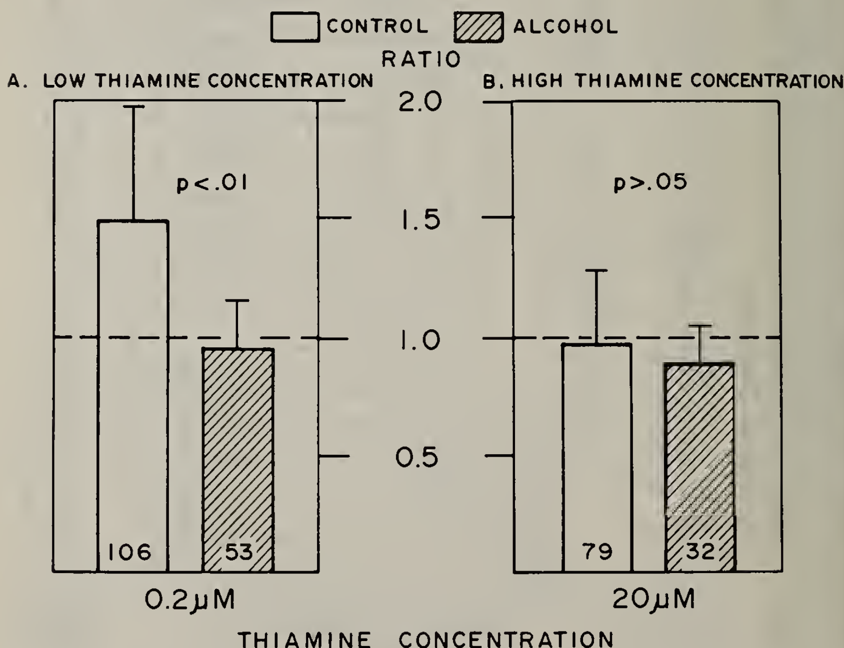


Ethanol reduced the absorption of 0.5 μM (A), but not of 17.5 or 35 μM thiamine (B). In B, ethanol 750 mg/100 G body weight was given intragastrically. Reproduced from reference (12) with publisher's permission. Copyright 1975 by C. V. Mosby Co.

inhibition was not dependent on the ethanol dose, and it was reversible within 24 hours. The smaller the dose given, the quicker was recovery (12). This ability of ethanol to inhibit the absorption of low concentrations of thiamine appeared to be specific, inasmuch as an equal volume of saline or isocaloric amounts of glucose did not influence thiamine absorption. Neither was the effect related to the osmotic action of ethanol. In contrast, the absorption of high concentrations of thiamine, 17.5 and 35 μM, was not affected by the intragastric administration of ethanol 750 mg/100 g body weight (figure 2B). Similarly, the net flux of low thiamine concentration (0.2 μM), but not of high concentrations (20 μM), was inhibited by 2.5-percent ethanol placed in the mucosal and serosal compartments (figure 3).

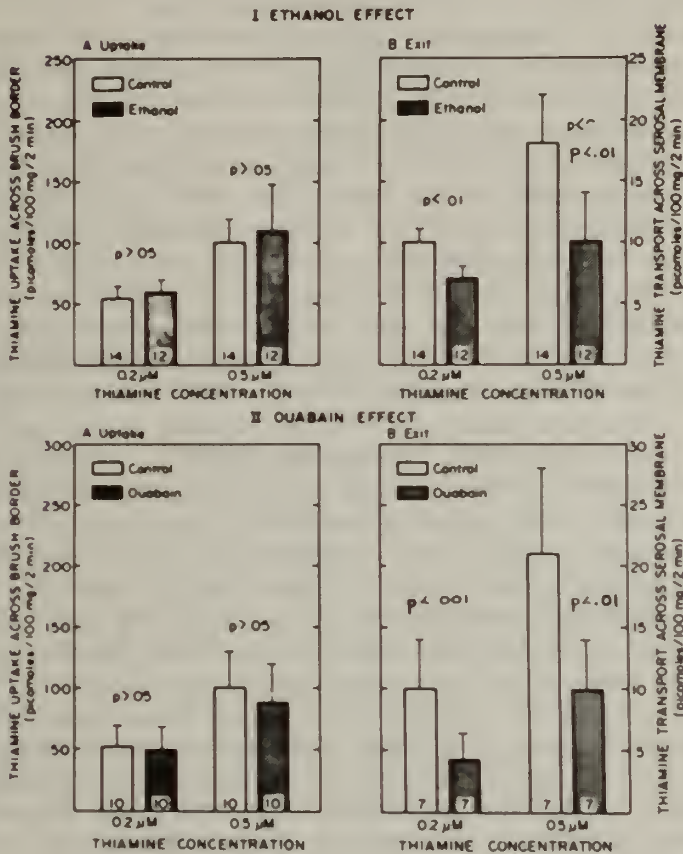
To determine the possible site of ethanol action, the effect of ethanol on thiamine tissue uptake and subsequent transfer from the tissue to the serosal compartment (thiamine exit) was measured. Tissue uptake of low concentrations of thiamine (0.2 and

Figure 3. Effect of Alcohol on Net Flux of Low and High Concentrations of Thiamine



Alcohol reduced the net flux of low (0.2 μM) (panel A) but not of high (20 μM) (panel B) concentration of thiamine. Reproduced from reference (12) with publisher's permission. Copyright 1975 by C. V. Mosby Co.

Figure 4. Similarity Between Ethanol (I) and Ouabain (II) Effect on Thiamine Transport



Both agents allowed uptake of thiamine across the brush border membrane to proceed normally, but they inhibited thiamine cellular exit across the serosal membrane. Reproduced from reference (12) with publisher's permission. Copyright 1975 by C. V. Mosby Co.

0.5 μM) was not affected at all by intragastric administration of ethanol 750 mg/100 g body weight (figure 4, IA). In contrast, ethanol significantly reduced the serosal exit of the same concentrations of thiamine (figure 4, IB). As did ethanol, ouabain allowed tissue uptake of thiamine across the brush border membrane to proceed normally (figure 4, IIA), but it blocked the exit of the vitamin across the serosal membrane (figure 4, IIB). Neither ethanol nor ouabain affected the uptake or exit of high concentrations of thiamine. Ouabain is a known inhibitor of Na-K

ATPase, which is believed to play an important role in active transport, so these results suggest that ethanol may impair thiamine serosal transport by inhibiting Na-K ATPase activity in the basolateral membrane of the intestinal epithelial cells.

The effect of ethanol on basolateral membrane Na-K ATPase activity was therefore directly assessed and correlated with serosal thiamine transport (19). When 0.5 M ethanol was directly added to the membrane preparation, Na-K ATPase activity was reduced by 59 percent. Ethanol 0.1, 0.2, 0.3, 0.5, 0.7, and 1.0 M added in vitro reduced Na-K ATPase to 94, 78*, 58*, 41*, 28*, and 17* percent of control value (*$p < .001$), respectively. In addition, 1 hour after the intragastric administration of ethanol 50, 100, 250, 500, and 750 mg/100 g body weight in vivo Na-K ATPase activity declined to 73*, 52*, 52*, 62*, and 48* percent of control value (*$p < .01$). The same in vivo doses of ethanol decreased thiamine serosal transport to 82, 48*, 50*, and 51* percent of control (*$p < .05$), respectively. The plasma ethanol concentration capable of inhibiting Na-K ATPase and thiamine transport was at least 60 to 70 mg/100 ml. These data clearly show an association between ethanol inhibition of basolateral membrane Na-K ATPase and decreased thiamine serosal transport, but determining whether this association is one of cause and effect requires further study.

Chronic Ethanol Studies. So far, the data cited have been obtained after single acute doses of ethanol were given to normal rats. The relevance of these observations, however, to the pathogenesis of thiamine deficiency in chronic human alcoholism is uncertain. Therefore, the effect of chronic ethanol administration on thiamine transport was studied (20). Rats were fed an ethanol-containing liquid diet as described by DeCarli and Lieber (21). Control animals were fed a similar mixture, except that ethanol was replaced by isocaloric amounts of dextrin-maltose. One liter of each mixture contained 0.725 mg of thiamine. After 6 to 8 weeks, thiamine tissue uptake and serosal exit were measured and correlated with basolateral membrane Na-K ATPase activity. Results showed that chronic ethanol feeding did not alter the rate of thiamine uptake or exit, and Na-K ATPase activity remained normal (20). Furthermore, the thiamine pyrophosphate content of the liver, heart, brain, and jejunum of rats fed the DeCarli-Lieber diet for as long as 4 months was normal (20). It was noted, however, that at the time of the transport and Na-K ATPase measurements, the ethanol concentrations in the plasma and intestinal lumen were only 40.2 ± 19.4 ($n = 16$) mg/100 ml, and 39.3 ± 15.3 ($n = 14$) mg/100 ml, respectively. From serial measurements of tail vein blood, it was found that there was considerable fluctuation in

ethanol plasma concentrations throughout the data, reflecting the nocturnal feeding habit of the animals. High ethanol levels considered legally inebriating in humans were noted toward midnight, whereas low levels were found toward noon when the transport and Na-K ATPase measurements were obtained.

Some alcoholics are known to consume more alcohol at certain times than at others. To simulate this clinical condition and to increase ethanol concentrations to levels attained at night, rats fed the ethanol diet for 6 to 8 weeks were given an additional single dose of ethanol 250 mg/100 g body weight. One hour later, thiamine transport and Na-K ATPase activity were measured. There was a 56-percent and a 44-percent decrease in serosal transport of 0.2 and 0.5 μM thiamine with a corresponding 68-percent fall in basolateral membrane Na-K ATPase activity (19). The ethanol concentrations in the plasma and gut lumen were 185 and 318 mg/100 ml, respectively. Tissue uptake of thiamine remained normal.

The difference between the normal thiamine transport and Na-K ATPase activity following chronic ethanol administration on one hand, and the inhibition of thiamine transport and Na-K ATPase activity after the additional dose of ethanol on the other hand, may lie, at least partly, in the difference in ethanol plasma concentrations at the time of the transport and enzyme measurements. It should also be noted that these studies only measured thiamine transport rate across a segment of the jejunum during a very brief period of the day. They were not designed to evaluate overall absorption of thiamine from the entire gastrointestinal tract. Such a study was done by Balaghi and Neal (personal communication). Rats were given 10-percent alcohol as the only source of drinking liquid for 8 weeks, and the excretion of thiamine in the feces and urine was measured. These investigators noted increased fecal excretion of thiamine and decreased urinary excretion of thiamine, consistent with malabsorption of the vitamin; yet, as we did, they found the tissue thiamine content to be normal.

Conclusions

Based on studies in rats, intestinal thiamine transport is clearly governed by a dual system: active for low or physiological concentrations and passive for high pharmacological concentrations. Ethanol inhibits the active, but not the passive, component of thiamine transport. Uptake or transport across the brush border

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proceeds normally in the presence of ethanol, but thiamine exit or transport across the serosal membrane is blocked and is associated with inhibition of Na-K ATPase activity. We did not study the mechanism by which ethanol reduces Na-K ATPase activity, but it is under active investigation by others. Available data suggest that ethanol may adversely affect Na-K ATPase activity by altering the microenvironment of this membrane-bound enzyme (22). The parallel reduction in thiamine serosal transport and Na-K ATPase activity supports the hypothesis that ethanol impedes thiamine transport by inhibiting Na-K ATPase. However, to establish that Na-K ATPase truly modulates thiamine transport, it will be necessary to show that increasing Na-K ATPase activity also enhances thiamine serosal transport. The observation that vasopressin treatment increases both Na-K ATPase and intestinal thiamine absorption in chicks (23) is an important bit of supportive evidence, although it is not known from that study which transport step is affected.

In chronic ethanol administration, inhibition is dependent more on the ethanol concentration than on the duration of exposure to ethanol. This animal model may be intermittent, with normal transport taking place when the ethanol is low and noninhibitory. Under such circumstances, thiamine content in various tissues may be maintained at normal levels for at least 4 months, as seen in our study. However, when high ethanol concentrations are reached, malabsorption may predominate. Any intermittent malabsorption may become more significant if associated with marginal thiamine intake, as is often the case with alcoholic patients.

Future Areas of Study

From these studies, it is apparent that multiple steps are involved in thiamine intestinal transport. However, we have investigated only some aspects of the entry and exit steps. Other aspects of thiamine transport, which have not been discussed here, require further investigation. These should include the characterization of thiamine-binding protein as a possible important component of thiamine uptake, the study of thiamine phosphorylation and dephosphorylation as intracellular events that may be affected by alcohol, the investigation of the possible role of cyclic AMP, and the manipulation of Na-K ATPase activity to determine if it truly modulates thiamine serosal transport.

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Discussion of Paper by Hoyumpa

Dr. Halsted: Your group has previously shown that rats made folate-deficient have poor thiamine transport. Have you looked at basolateral ATPase activity with folate deficiency?

Dr. Hoyumpa: Yes, we are currently setting up the study. We have done some preliminary studies, and unfortunately I am not prepared to give you the results yet.

Effect of Ethanol on Vitamin B_6 Metabolism

Lawrence Lumeng

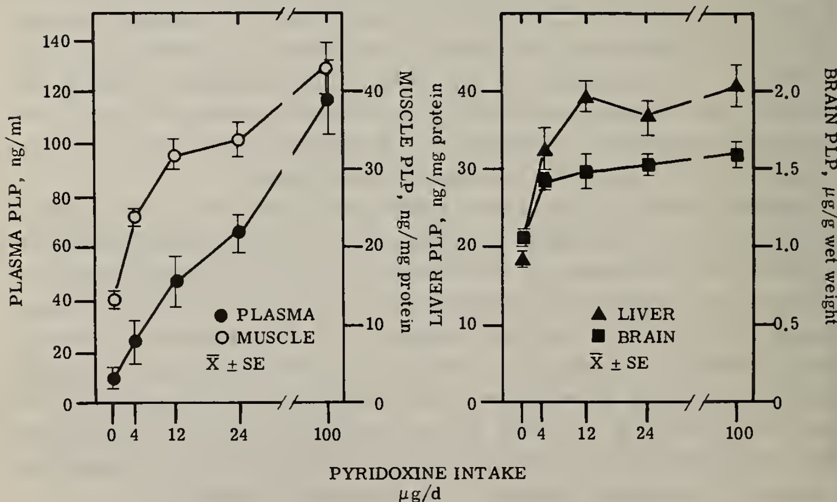
My assignment is to discuss the problem of vitamin B_6 deficiency in chronic alcohol abuse and to review the present knowledge of the nature of this abnormality. In the alcoholic patient, two factors are important in facilitating the early development of a primary vitamin B_6 deficiency. One is that alcoholic beverages contain negligible amounts of this vitamin (1,2), even though ethanol contributes substantially to total caloric intake (3). The other is that, based on recent dietary record analyses, a high percentage of healthy Americans actually ingest less vitamin B_6 than the recommended dietary allowance (4). Moreover, as will be discussed, chronic alcohol abuse also produces impairment in the metabolism of vitamin B_6, thus leading to an increased requirement. In the face of an already marginal intake, these conditioning factors can become crucial determinants.

It is unfortunate that, in the clinical evaluation of vitamin B_6 deficiency, the findings tend to be nonspecific and to occur only when the deficiency is already severe. The most specific is probably sideroblastic anemia, reversible by vitamin B_6 administration (5). The incidence of this finding in chronic alcoholics has been estimated to be 20 to 30 percent. However, even the development of this abnormality is probably the end result of multiple factors (6,7,8,9). Therefore, the clinical evaluation of vitamin B_6 deficiency in the alcoholic is imprecise and insensitive, and more accurate assessment must rely on the measurement of either the functional integrity of vitamin B_6-dependent pathways or of the content of the vitamin in tissues and body fluids.

Recent studies indicate that measurement of the concentration of vitamin B_6 compounds in plasma is the most reliable indicator of the state of vitamin B_6 nutrition (10,11,12). The predominant form of the vitamin B_6 compounds in plasma is pyridoxal phosphate, PLP (13). As we have reported, it is derived almost entirely from hepatic synthesis and behaves as a relatively stable, albeit small, circulating storage pool of the vitamin B_6 compounds in the body (14). It is firmly bound to albumin and its concentration correlates with a high degree of certainty with the intake of vitamin B_6 in normal animals (12), including humans (15).

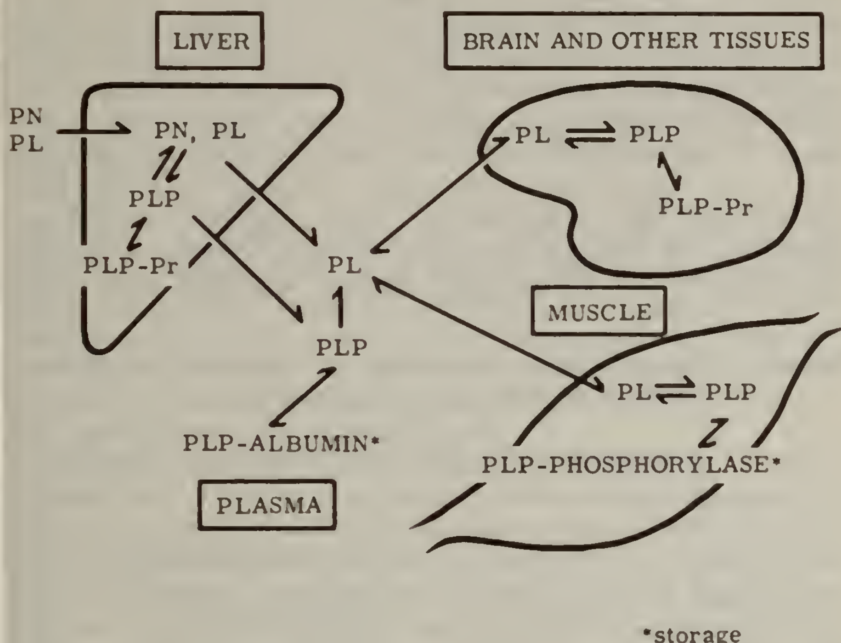
Figure 1 shows the results of a study in which weanling rats were fed diets containing 0 to 100 μg pyridoxine daily for 9 weeks. Growth curve analysis indicated that 24 μg/day is the optimal intake for these animals. Plasma PLP content increased as a function of dietary intake of vitamin B_6, over the entire range examined. In tissues, PLP and pyridoxamine-P (PMP) are the stable storage forms of the vitamin (13). Skeletal muscle, by virtue of its total mass, is by far the largest tissue storage depot (16). As is apparent, plasma PLP concentration exhibits a very high degree of correlation with muscle PLP content. Accordingly, the measurement of plasma PLP should represent a reliable and sensitive indicator of the state of vitamin B_6 nutrition. Its concentration reflects not only the degree of undernutrition, but also of storage. On the other hand, PLP in brain and in liver has already reached saturating levels with relatively low amounts of pyridoxine intake, 4 and 12 μg/day, respectively. These results demonstrate that the measurement of plasma PLP is a reliable and sensitive indicator of the state of vitamin B_6 nutrition, because its concentration reflects not only the degree of undernutrition but also the degree of storage. The postulated interrelationship of PLP in plasma to that in other tissues and organs and the role of protein binding in the body economy of this coenzyme are depicted in figure 2.

Figure 1. Pyridoxine Intake μg/d



Varying pyridoxine intake affects the PLP content of plasma, muscle, liver, and brain from rats.

Figure 2. Postulated Interrelationships of the Vitamin B_6 Compounds in Various Body Compartments



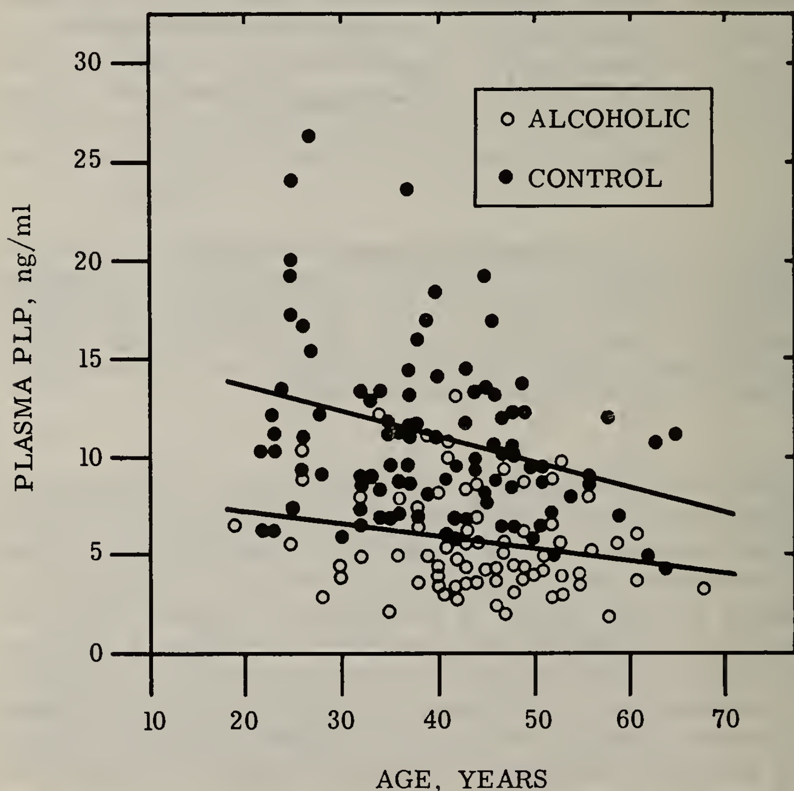
Pyridoxine (PN) and pyridoxal (PL) are presented to the liver via the portal circulation. Binding of PLP and PMP by extracellular and intracellular proteins (Pr) protects these coenzymes against hydrolysis. The principal PLP-binding protein in plasma is albumin and, in skeletal muscle, glycogen phosphorylase. Phosphorylase, alanine aminotransferase, and aspartate aminotransferase are major PLP-binding proteins in liver. In plasma, PLP is the major vitamer; PMP is only a minor component.

Baker and coworkers (17) were among the first to examine the problem of vitamin B_6 deficiency in alcoholics by blood concentration measurements. They studied 172 hospitalized alcoholics and correlated the circulating concentration of a number of vitamins with the degree of liver disease. Abnormally lowered serum folic acid was the most prevalent, followed by B_6 and then B_1. For these studies, total B_6 content of serum was measured by protozoan assay. The incidence of low serum B_6 was almost 40 percent in patients with alcoholic cirrhosis, 30 percent in those with fatty liver, and about 20 percent in those with normal liver histology.

Since that time, a number of studies with more precise methods have firmly established that a high percentage of chronic alcoholics

with or without liver disease exhibit abnormally lowered plasma PLP levels (9,18,19,20,21,22). Figure 3 shows the results of a study from our laboratory, designed specifically to examine the incidence of vitamin B_6 abnormality in acknowledged alcoholics who were free of liver disease (20). The plasma PLP content of 66 such subjects was compared with that of 94 control subjects. As shown by the lower regression line, the mean plasma PLP concentration of the alcoholic population was significantly lower than that of the control group, the upper regression line. This relationship held true for all the age groups examined, even though plasma PLP in healthy individuals normally declines with age. Thirty-five of the 66 alcoholic subjects exhibited PLP values

Figure 3. Plasma PLP Concentration of Alcoholic and Nonalcoholic Men, Plotted as a Function of Age



The upper line is the regression line of the control group (solid circles) and the lower, of the alcoholic group (open circles).

below 5 ng/ml, which, in our laboratory, is the low limit of normal. Thus, depending on the population surveyed, the incidence of abnormally lowered plasma PLP in alcoholic subjects may be as high as 50 percent, even when they are devoid of liver disease. This finding has been confirmed by Davis and Smith (21). However, in agreement with the studies of Baker and coworkers (17), these investigators have shown that the incidence of low plasma PLP in alcoholics with liver disease is higher, and may be as high as 80 to 100 percent.

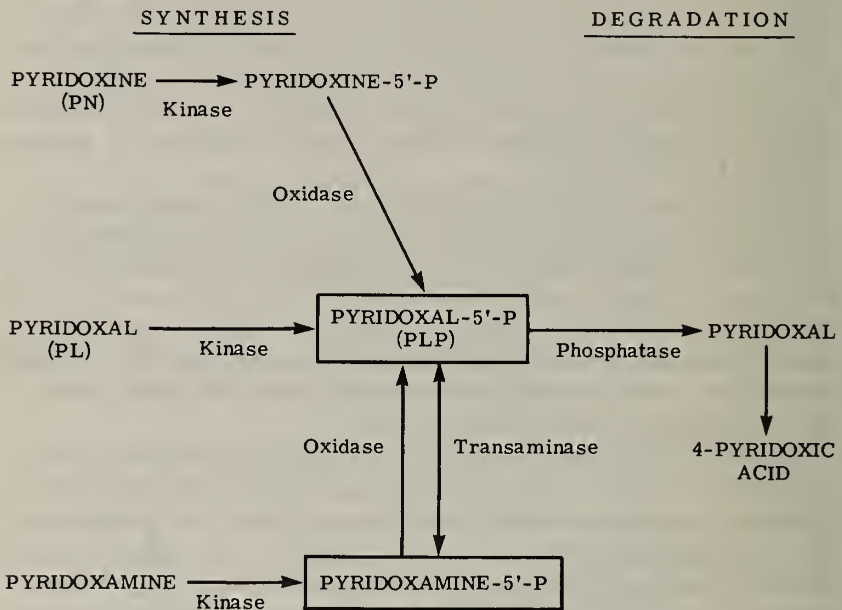
Because decreased serum PLP levels tend to occur more frequently in the setting of prolonged alcohol abuse and to associate with other manifestations of malnutrition, it is probable that inadequate dietary intake of vitamin B_6 is a major cause of this abnormality. However, there is now good evidence that alcohol oxidation itself, and also liver disease, can profoundly alter the metabolism of vitamin B_6 and PLP and, thus, can contribute to the development of a deficiency state.

In 1970, Hines and Cowan observed that the net conversion of pyridoxine to PLP is impaired in drinking alcoholic subjects (7). Although the effect of liver disease was not ruled out in this study, the findings suggested that alcohol itself (or its oxidation) may directly interfere with the metabolism of vitamin B_6. Figure 4 shows the principal pathways of PLP synthesis and degradation in mammalian tissues. Synthesis of PLP from pyridoxal is catalyzed by pyridoxal kinase. PLP synthesis from pyridoxine is dependent on both pyridoxal kinase and pyridoxine-P oxidase activities. Pyridoxamine-P is synthesized from pyridoxamine, catalyzed by pyridoxal kinase. The conversion of PMP to PLP is dependent on pyridoxine-P oxidase and various aminotransferases. The degradation of PLP is mediated by alkaline phosphatase. In the liver, pyridoxal can be further oxidized to form pyridoxic acid, catalyzed by aldehyde oxidase.

In order to study the effect of ethanol itself or its oxidation on the metabolism of vitamin B_6, we began investigation first with human red blood cells. Erythrocytes possess the full complement of enzymes for the synthesis of PLP, as well as for its degradation to pyridoxal (20). In the red cell, pyridoxic acid is not the end product of PLP degradation. Hence, with this exception, the entire metabolic pathway for vitamin B_6 metabolism can be studied in erythrocytes. Moreover, because erythrocytes do not metabolize ethanol, the effects of ethanol and its oxidation product, acetaldehyde, can be examined separately.

As shown in figure 5, the intact erythrocyte, when incubated with pyridoxine for 2 hours, accumulates substantial quantities of

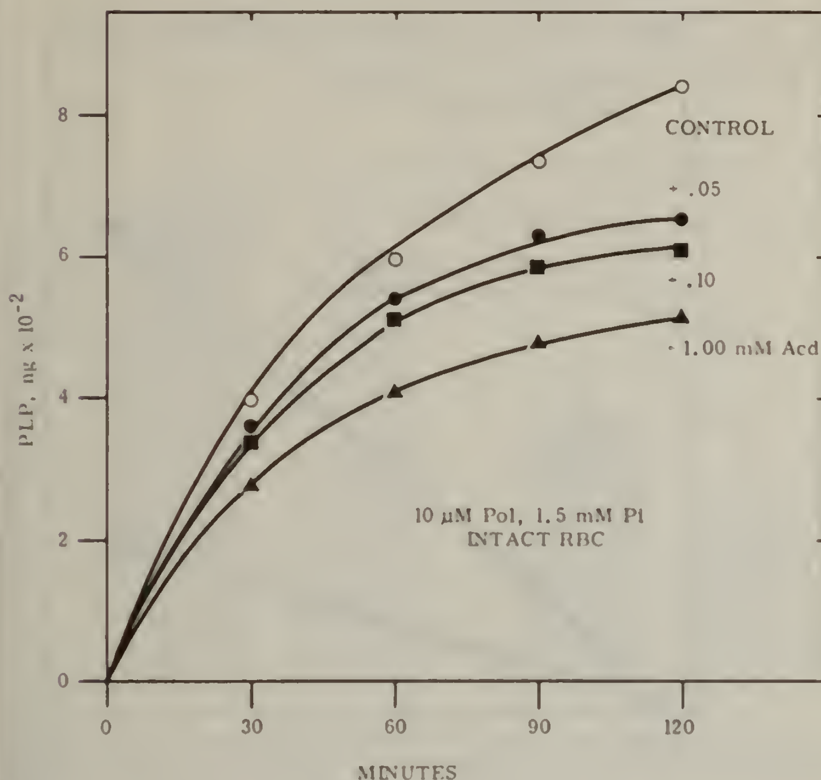
Figure 4. Known Pathways for the Synthesis and Degradation of PLP in Mammalian Tissues



PLP. The time course is nonlinear, because, concurrent with synthesis, there is also degradation of PLP, catalyzed by a membrane-bound phosphatase. Ethanol, in concentrations as high as 70 mM, did not affect the net formation of PLP at all. However, as shown here, acetaldehyde, in concentrations as low as 0.05 mM, significantly lowered the accumulation of PLP in the erythrocyte. Similar results were obtained when pyridoxal or pyridoxine-P was the substrate.

The effect of acetaldehyde can be due either to inhibition of PLP synthesis or to accelerated degradation. In order to separate these events, the experiment was repeated with hemolyzed erythrocytes. Because the phosphatase which breaks down PLP is bound to the erythrocyte cell membrane, it can be removed by centrifugation. PLP synthesis can then be examined, in the hemolysate supernatant, unhampered by degradation. As shown in figure 6, the synthesis of PLP from pyridoxal was not affected by acetaldehyde. Similar results were obtained with other substrates—for example, pyridoxine-P—and also with intact cells, when phosphatase activity was inhibited by 80 mM Pi. These results indicate that acetaldehyde does not inhibit PLP synthesis.

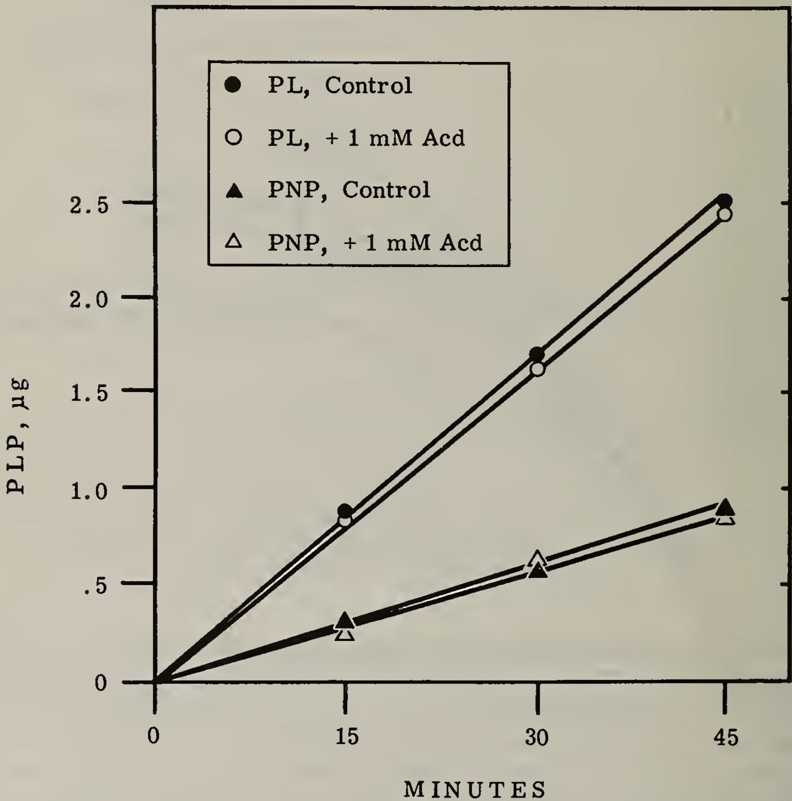
Figure 5. The Effect of Acetaldehyde (Acd) on the Net Synthesis of PLP from Pyridoxine (Pol) by Human Erythrocytes



We therefore arrived at the hypothesis that acetaldehyde mediates the derangement in vitamin B_6 metabolism associated with ethanol abuse by accelerating the degradation of PLP. However, because the liver is the principal organ responsible for the oxidation of ethanol, as well as for the synthesis of PLP from dietary precursors, validation of the hypothesis requires that the effect of acetaldehyde be demonstrable in that tissue. It must also be demonstrated that the effect is independent of the nutritional status of vitamin B_6 and that it can be blocked when ethanol oxidation by the liver is inhibited. Moreover, the effect should be observed both chronically and acutely.

To test the above hypothesis, experiments were then performed on rats. One group was fed a liquid diet containing an excess of vitamin B_6, and another, a diet deficient in vitamin B_6. In each group, the experimental animals received 36 percent of their

Figure 6. The Effect of Acetaldehyde (Acd) on the Net Formation of PLP by the Supernatant Fraction of Hemolyzed Human Erythrocytes

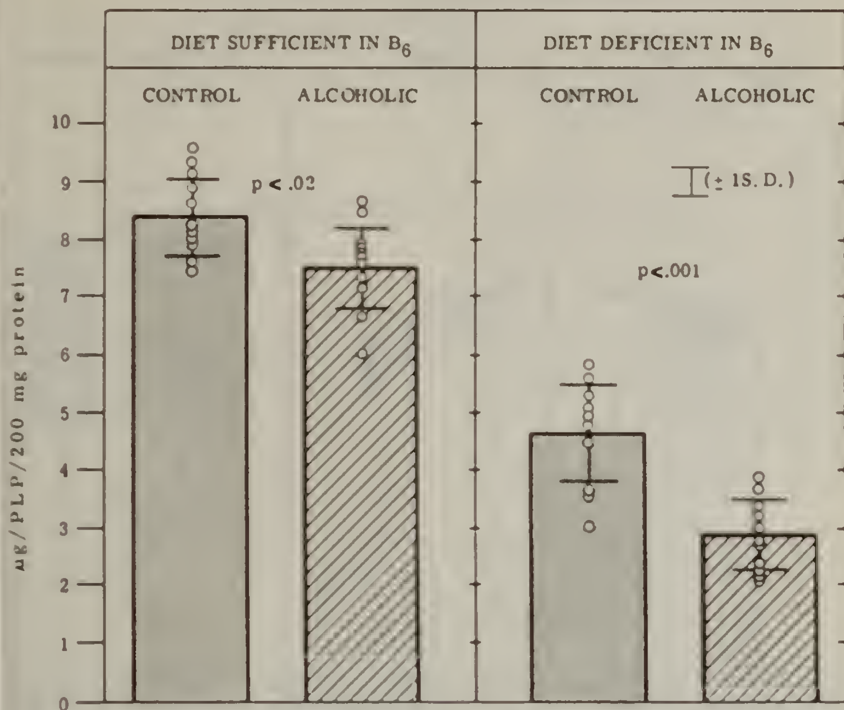


The substrates are pyridoxal (PL) and pyridoxine-P (PNP).

calories as alcohol, while the control animals were pair-fed diets with ethanol isocalorically replaced by dextrin-maltose (figure 7). After 45 days, all animals were sacrificed and liver PLP content was measured. The experimental, alcohol-fed animals in both groups exhibited significantly lower liver PLP levels than their corresponding controls. Therefore, chronic alcohol ingestion lowered liver PLP content, irrespective of whether the diet was sufficient or deficient in vitamin B_6.

As indicated, the effect of ethanol oxidation should also be demonstrable acutely. This factor was examined with the use of isolated perfused liver. Figure 8 shows the effect of perfusing

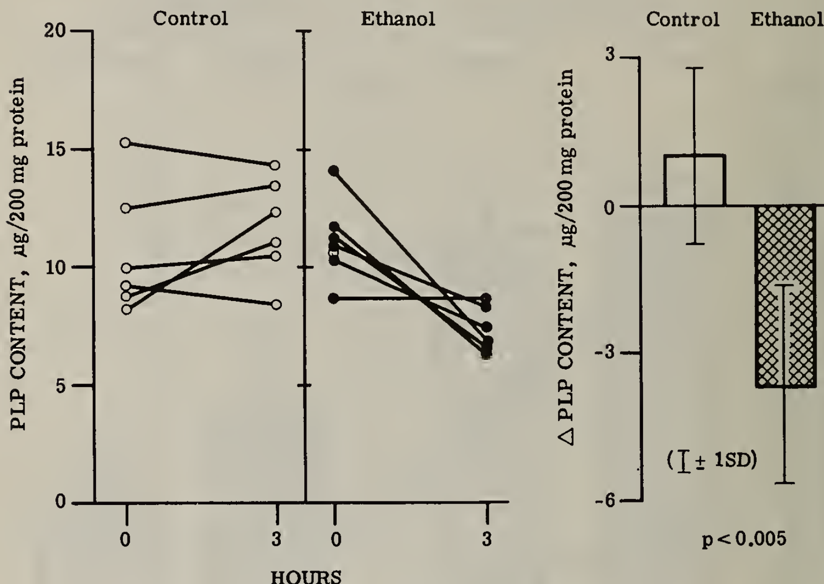
Figure 7. The Effect of Chronic Ethanol Ingestion on the Hepatic PLP Content of Pair-Fed Rats



Animals fed the vitamin B_6-sufficient diet consumed 50 μg of pyridoxine daily. The vitamin B_6-deficient diet contained no pyridoxine. The liver PLP content of the vitamin B_6-sufficient rats was measured after 52 days and that for the vitamin B_6-deficient group, after 42 days.

livers isolated from vitamin B_6-sufficient animals with ethanol. In the absence of ethanol, little or no change in liver PLP content occurs during 3 hours of perfusion. By contrast, perfusion with 18 mM ethanol lowered liver PLP in almost every instance. As shown by the bar graph on the right in figure 8, the mean decrease in liver PLP content produced by ethanol was more than 3 μg/200 mg protein. Interestingly, other laboratories have reported that the administration of ethanol to rats increases the urinary excretion of nonphosphorylated vitamin B_6 compounds (23) and that the perfusion of rat livers with ethanol increases the release of nonphosphorylated vitamin B_6 compounds into the perfusate (24). The findings here presented are consistent with and provide an explanation for these observations.

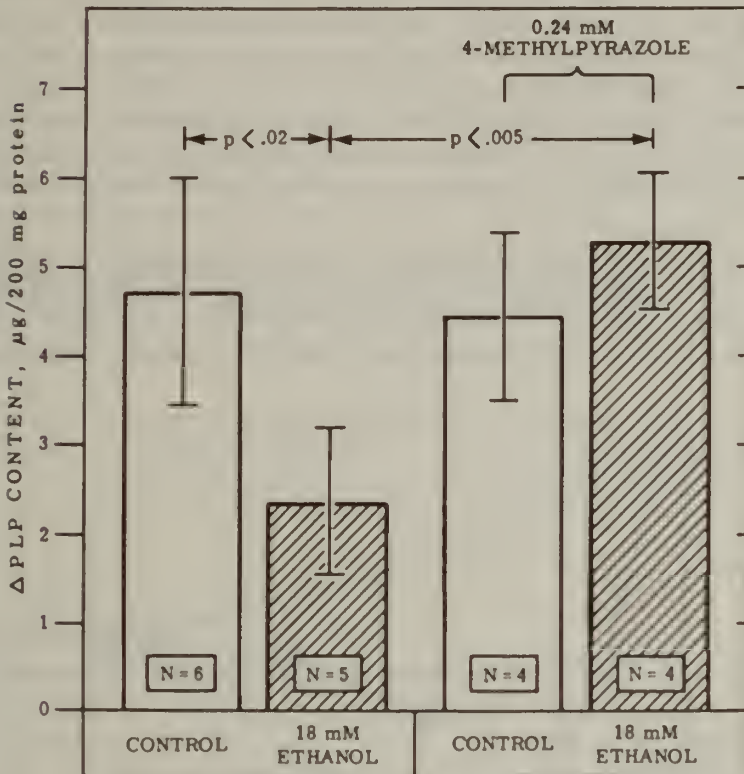
Figure 8. The Effect of Ethanol (18 mM) on the Hepatic PLP Content of Isolated Perfused Livers from Vitamin B_6-Sufficient Rats



Perfusions were performed without added pyridoxine. The PLP contents of livers perfused without (○-○) and with ethanol (●-●) were measured at the beginning and at the end of the perfusion. The bar graph compares the mean (±SD) changes in hepatic PLP content for the two groups.

A deleterious effect of alcohol on PLP metabolism could also be demonstrated in perfused livers isolated from vitamin B_6-deficient rats. Because the animals are B_6-deficient, it is experimentally more convenient to measure PLP formation by the liver in the presence of a vitamin precursor. As shown in figure 9, perfusion with 1.2 mg percent pyridoxine increased liver PLP content by more than 4 µg/200 mg protein in 2 hours. The inclusion of 18 mM ethanol in the perfusate significantly reduced liver PLP accumulation. The effect of 4-methylpyrazole was then examined. This compound is a potent inhibitor of alcohol dehydrogenase and prevents the oxidation of ethanol to acetaldehyde. It had no effect on PLP formation in the control situation. However, it completely abolished the effect of ethanol. Therefore, when alcohol oxidation is inhibited, PLP metabolism is restored to normal. These data substantiate the results shown earlier with the human erythrocytes and indicate that it is acetaldehyde, not

Figure 9. The Effect of Ethanol (18 mM) in the Absence and Presence of 4-Methylpyrazole on the Hepatic PLP Content of Isolated Perfused Livers from Vitamin B_6-Deficient Rats



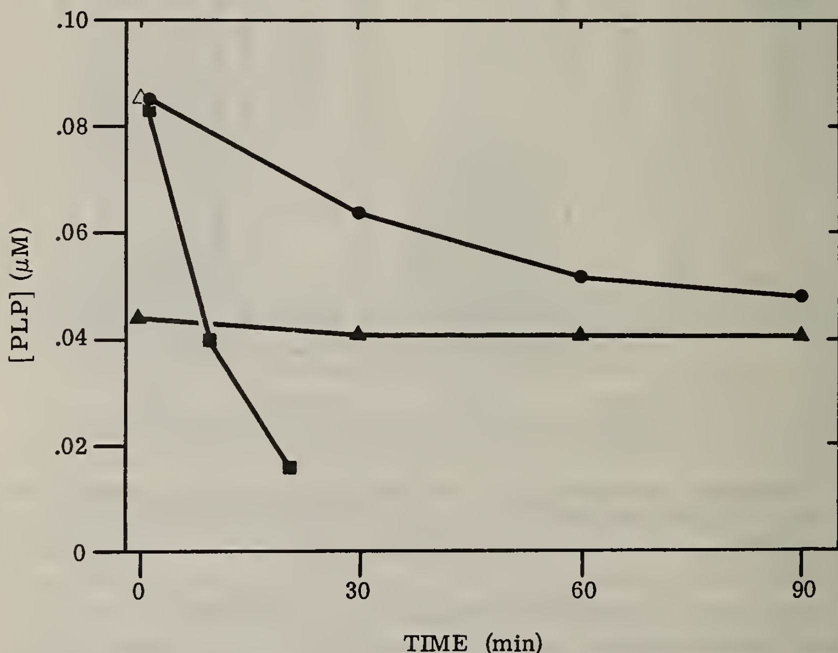
The livers were perfused with 1.2 mg of pyridoxine added to 100 ml of medium. The mean (±SD) changes in hepatic PLP contents are shown.

ethanol, that mediates the deleterious action of ethanol oxidation on PLP metabolism.

As indicated previously, the action of acetaldehyde appeared to be one of stimulation of PLP degradation and not of inhibition of synthesis. How this might occur has recently been elucidated. The enzyme principally responsible for PLP degradation is the plasma membrane-bound alkaline phosphatase (25). Studies with this enzyme showed that acetaldehyde does not increase its specific activity. Thus it appeared that the stimulatory effect of acetaldehyde must be to increase the availability of PLP for hydrolysis by

alkaline phosphatase. Most of the PLP in liver cytosol is tightly bound to proteins (27). However, a small but significant amount of it is either free or loosely bound, as this portion can be removed by dialysis or gel filtration. Whether or not PLP is protein bound, and how it is bound, greatly affects its susceptibility to hydrolytic cleavage. As shown in figure 10, PLP existing freely in solution is rapidly hydrolyzed by alkaline phosphatase, present in the liver plasma membranes. In contrast, PLP that is tightly bound to cytosolic proteins and that is not removed by dialysis is almost not hydrolyzed at all. Therefore, protein-binding of PLP protects it against degradation. Moreover, a considerable degree of protection

Figure 10. The Effect of Dialysis and Plasma Membrane-Associated Phosphatase on the PLP Content of Hepatic Cytosol

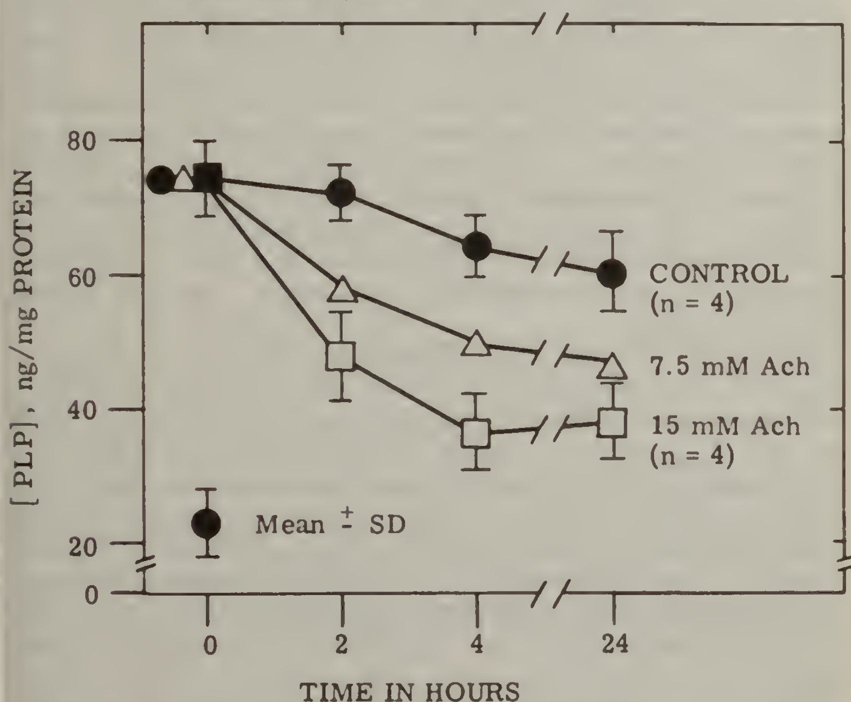


The incubation mixture contained 50 mM TEA-HCl (pH 7.4), 5 mM $MgCl_2$, and, where indicated, 0.72 mg cytosolic protein, and/or 0.23 mg plasma membrane protein. The concentration of PLP in the presence of undialyzed cytosol is indicated by the open triangle (△). The level of PLP does not change significantly in the presence of dialyzed cytosol plus plasma membranes (▲-▲). PLP (200 ng), in the free form, is rapidly hydrolyzed by the plasma membrane-associated phosphatase (■-■). The addition of dialyzed cytosol to 100 ng PLP protects significantly the hydrolysis of PLP mediated by the phosphatase activity in plasma membranes (●-●).

against degradation appears to be extended also to PLP added to cytosol that is only loosely bound to proteins. These and other studies have led us to believe that protein-binding and the hydrolysis of unbound PLP are important mechanisms for regulating the body content of PLP. In the normal state, the tissue content of PLP is determined principally by protein binding. PLP synthesized in excess of this binding is rapidly hydrolyzed by alkaline phosphatase. Thus there is a dynamic equilibrium between synthesis and degradation, with protein binding serving as an intracellular buffer and the modifier of the rate of PLP degradation (26).

The manner by which acetaldehyde acts to increase the availability of PLP for hydrolysis is shown in figure 11. When rat liver cytosol is dialyzed against 30 volumes of buffer for 24 hours, only about 20 percent of the PLP is removed. In the presence of 7.5 and 15 mM acetaldehyde, considerably more PLP is removed in dialysis. Similar results have been obtained with PLP and

Figure 11. The Effect of Acetaldehyde (Acd) on the PLP Content of Rat Liver Cytosol



Cytosol was dialyzed against 30 volumes of a buffer containing different concentrations of Acd; the PLP remaining in the dialysis bags was measured as a function of time of dialysis.

erythrocyte hemolysates. Thus acetaldehyde, at high concentrations, displaces or facilitates the dissociation of PLP from protein-binding and, in this manner, promotes PLP degradation. In agreement with this interpretation, we have also demonstrated that acetaldehyde will inhibit the activation of purified apotyrosine aminotransferase by PLP. The kinetics of the inhibition show a mixed competitive-noncompetitive pattern. The activation of ornithine decarboxylase apoenzyme by PLP is similarly inhibited by acetaldehyde. We infer from these studies that not only does alcohol oxidation itself accelerate the degradation of PLP, but that, with chronic excessive alcohol ingestion, the body storage capacity for vitamin B_6 is also lowered. Both processes would contribute to the development of vitamin B_6 deficiency.

Chronic alcohol abuse and alcoholic liver disease can also result in other abnormalities of vitamin B_6 metabolism. Hines and Cowan have reported that chronic and heavy alcohol consumption may cause an impairment of erythrocyte pyridoxal kinase activity (28). They have also reported the appearance, in the serum of drinking alcoholics, of a large molecular weight substance that inhibits erythrocyte and hepatic pyridoxal kinase activity. The significance of these findings is difficult to evaluate because (1) their findings have not been reproduced by other laboratories; (2) the origin of the serum inhibitor and its relationship to pyridoxal kinase, which is an intracellular enzyme, are unknown; and (3) it is unknown to what extent the level of pyridoxal kinase activity in erythrocytes and other tissues contributes to the regulation of the cellular and body economy of PLP. It is known, however, that the total and specific activity of pyridoxal kinase of erythrocytes in humans can vary widely among races (29).

As noted previously, the incidence of vitamin B_6 deficiency is higher in alcoholic patients with liver disease than in those without liver disease. There is now evidence that liver disease itself will impose additional stresses on vitamin B_6 metabolism, by mechanisms other than those already discussed. Thus Baker and coworkers (30) recently reported that chronic alcoholics with liver disease exhibit an abnormality in their ability to use food as a source of folate, thiamine, and B_6. More important, Mitchell et al. (22), Rossouw et al. (31), and Labadarios et al. (32) have recently reported that PLP degradation is accelerated in the presence of liver disease.

In summary, I have reviewed the different pathways by which the excessive use of alcohol can produce stress on nutritional balance and on vitamin B_6 metabolism. Because alcoholic beverages

have high caloric value but are empty in nutritional value, they promote the development of primary nutritional deficiency by preempting food intake. Furthermore, alcohol ingestion and alcoholic liver disease can interfere with the normal processes of vitamin B_6 metabolism, e.g., absorption, storage, conversion to biologically active forms, and degradation. These conditioning factors can occur concurrently, thereby accelerating the development of vitamin B_6 deficiency. Evidence has been presented that acetaldehyde, the oxidation product of ethanol, interferes with the metabolism of vitamin B_6 by promoting the degradation of PLP. It may also lower the tissue storage capacity for vitamin B_6. In patients with alcoholic liver disease, intestinal absorption of vitamin B_6 from food sources is impaired, and PLP degradation is accelerated. Because of these conditioning factors, discontinuation of excessive alcohol ingestion is a necessary step in treatment.

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Discussion of Paper by Lumeng

Dr. Hillman: The recent work done by Drs. Pierce and Solomon appears to confirm what Dr. Lumeng has just talked about. Dr. Pierce, in a series of alcoholics from Seattle, was able to show a fairly high incidence of low PLP values in serum. But he could show no correlation between the low PLP values and the incidence of so-called ring sideroblastic anemia, said to occur in the tissue because of the low values. And I think, therefore, that we have very little faith that this measurement actually, from a hematological standpoint, was telling us very much.

Also, Dr. Solomon had tried to repeat Hines, and Cowan's work in terms of pyridoxal kinase levels in red cells in alcoholic anemias, both megaloblastic, with rings, without rings, every variety you could think of. After a long struggle, really, he demonstrated that there are a lot of other factors you have to worry about. Not only is there the nutritional status, which people did not control for at all in previous work, there is the problem of racial characteristics, as Ernie Beutler has shown. Blacks have much lower kinase levels than whites, and you have to know the racial makeup of the group you are studying.

There is also something that I think everybody should have expected, and that is, if you have a severe anemia, you have a young population of red cells out in circulation. And just like any enzyme, whether it is hexokinase or any red cell enzyme, pyridoxal kinase is very high in young cells. Therefore, if you look at the very severely ill alcoholic, you will find high pyridoxal kinase levels in the circulating erythrocytes. The final answer always comes down to, what about the PLP level inside the cell? And you have to realize, at least for the red cell work, nobody's been measuring the key cells back in the marrow. You are measuring a cell that has lost all of its mitochondria and nucleus and everything that was important to pyridoxine, so you are measuring a residue.

At least, in terms of looking at something as simple as saturated transaminase, there is clearly no deficiency of PLP in that denucleated cell that arrives in circulation. I do not know what to say about the cell inside the marrow, and perhaps Larry would like to comment. It seems to me that the key factor is whether

PLP is getting inside the mitochondria of the cell that is trying to make porphyrin. We have not really learned much of anything until we actually study that transport or the content of PLP inside the mitochondria.

Dr. Lumeng: Thank you for your comments. As I indicated, sideroblastic anemia, no doubt, is a result of multiple factors, and B_6 deficiency is probably only one of multiple factors. The problem, of course, as you indicated, is that nobody has looked at B_6 metabolism of the erythroblasts or immature red cells. I think much work needs to be done in that area. You are interested in Δ-ALA synthetase, and that enzyme, of course, is intramitochondrial, so it is therefore important to study how B_6 ever gets into the mitochondria. Being hepatologists, we have been interested mainly in the liver, but I think we probably should look at the red cell precursors as well.

Dr. Henderson: Larry, in the case of liver, how do you envision acetaldehyde influencing PLP degradation or dephosphorylation?

Dr. Lumeng: We envision acetaldehyde as an agent that will release free PLP from its bound form. Free PLP can then be readily hydrolyzed by membrane-associated PLP phosphatases.

Dr. Schenker: I will make two very brief comments about Larry's remarks concerning some of the work going on in our laboratory. There is no question that, in the presence of liver disease of almost any type—whether it be chronic, like cirrhosis; acute, like viral hepatitis; or obstructive, like carcinoma of the pancreas—independent of alcohol, and independent of nutrition, apparently, there is an abnormal handling of plasma PLP. This abnormality, I think, has been adequately shown. Interestingly, it appears that a major component of this abnormal handling of plasma PLP is an increased degradation of the PLP. So if one infuses a bolus of PLP into patients and kinetically follows the disappearance curve, one finds that there is a major increase in PLP degradation.

The question is why this happens. There are several possibilities. And, actually, the reason for my sabbatical here is to try to find out with Drs. Lumeng and Li precisely why it happens. One possibility is that there is an abnormal binding, a decreased binding if you will, of PLP to plasma proteins. Using nonradioactively labeled PLP, we were unable, by equilibrium dialysis experiments, to show a decrease in the binding of PLP. However, PLP in plasma is bound very, very avidly, to the extent of about 99.8 percent, so it is obvious that we might easily have missed a very small decrease in binding without radioactively labeled PLP. We are now hoping to synthesize some radioactive PLP. We hope it will be stable

enough, and we can run these experiments in cirrhotic plasma and find out if there is or is not an abnormality in PLP binding in cirrhotic plasma.

The second possibility, which is not exclusive of the first, is that there may be an increased metabolism of PLP by the cirrhotic liver—if the liver is very important in degradation of PLP. Unfortunately, no one (to my knowledge) knows just how important the liver is to the handling of PLP. Again, we are hoping to develop these kinds of studies over here using the hepatectomized dog model that Drs. Lumeng and Li have worked on previously.

My last comment is that, by using dogs, unanesthetized dogs, without any nutritional deficiency, and by infusing a single dose of alcohol, 3g/kg, we have been able to show that there is a decreased net formation of plasma PLP from pyridoxine. There is no question about it. The question is why this happens. Studies are in progress in our unit, and we hope to have more data later.

Deficiency of Carnitine in Cachectic Cirrhotic Patients*

Daniel Rudman

Carnitine is synthesized from lysine and methionine. Lysine is methylated at its ϵ amino group by S-adenosyl-methionine, to form trimethyllysine, and this compound is then decarboxylated to form γ-butyrobetaine. In the rat, steps one and two occur in most tissues in the body. However, the final step, the hydroxylation of γ-butyrobetaine to carnitine, is essentially restricted to the liver. To produce a state of carnitine deficiency in the rat, one has to satisfy two conditions: First, one has to exclude exogenous carnitine from the diet, and second, one has to reduce the intake of dietary lysine and methionine to well below the minimum daily requirements. When that occurs, the rat can readily be made carnitine deficient. According to studies in the literature, carnitine deficiency in the rat leads to growth retardation and fatty liver.

So far, there have really been only a handful of reported cases of carnitine deficiency in humans. Most reports have appeared in the neurological literature, and these usually have presented as myopathy, sometimes with muscle atrophy. The histology shows lipid-filled vacuoles in the muscle. These are explained in terms of the physiological role of carnitine in providing an essential step in the transport of fatty acids from the cytosol into the mitochondria, the sites of fatty acid oxidation.

So the current view is that carnitine deficiency in humans is a rare syndrome, usually related to some problem either in the biosynthesis of carnitine; or in the transport of carnitine to its site of action within the muscle cells; or, in some cases perhaps, to a problem in the acyl carnitine transferase that is essential for carnitine to perform its function of fatty acid oxidation.

The origin of our study is the recognition that protein calorie malnutrition is quite prevalent within hospitals. Our surveys in

*Presented in its entirety in *The Journal of Clinical Investigation*, 60:716–723, 1977. Copyright 1977 by American Society for Clinical Investigation.

several hospitals have shown that, on general medical and surgical services, we are now approaching a 20- or 30-percent prevalence of significant protein calorie malnutrition. In view of this high prevalence of nosocomial protein calorie malnutrition, we undertook a study to see if there were also a prevalence of carnitine deficiency within the hospital population. The study proceeded in three phases.

The first phase consisted of a survey of about 240 patients with various disease categories, as well as of 16 normal individuals, for free and total serum carnitine. The levels were measured using the method of Marquis and Fritz (1) in a fasting, morning blood sample. In the normal individuals (table 1), the mean ±SE total carnitine was 79 ± 3 μM and free carnitine 49 ± 3 μM. None of the disease categories we looked at differed significantly from the normal serum carnitine levels, with the single exception of 36 alcoholic cirrhotic individuals who, as a group, showed hypocarnitinemia. In 14 of these 36 individuals, the carnitine levels were correlated with certain nutritional characteristics, such as the creatinine/height ratio, midarm muscle circumference (MAMC), triceps skinfold thickness (TSF), and plasma albumin, as well as certain liver tests—bilirubin and prothrombin time (table 2). The 14 hypocarnitinemic cirrhotics had substantial protein calorie malnutrition. Their creatinine to height ratio was only 68 percent of normal; they showed profoundly reduced midarm muscle circumference; the triceps skinfold was just about gone; and, in addition, they had advanced hepatocellular disease. The latter was reflected by low serum albumin, high serum bilirubin, and prolonged prothrombin time. One might argue about the degree of hypoalbuminemia, to what extent it reflected their protein calorie malnutrition, and to what extent it reflected their severe liver disease.

We went on to the second phase, aimed at pinpointing the mechanism of the hypocarnitinemia. Six severely hypocarnitinemic cirrhotics were drafted for the second phase of the study. Their clinical characteristics (table 3) consisted of cirrhosis of long duration (6 years or more), severe anemia, hypoalbuminemia, jaundice, prolonged prothrombin time, extensive ascites in most instances, as well as severe malnutrition (indicated by the creatinine/height ratio and triceps midarm muscle circumference). These patients were severely anorectic; they were spontaneously consuming only about 600 or 800 calories a day and only about 18 or 20 grams of protein a day. These patients were studied for 18 days in the clinical research center and compared to normal controls (table 4). On days 1 to 6, the spontaneous dietary intake

Table 1. Phase A of the Study: Fasting Serum Carnitine Concentration in 17 Clinical Categories

Group	Number	Male/Female	Age		Fasting Serum Carnitine (mean ±SE) (total/free)
			Average	Range	
			yr		*μM*
Normal	16	8/8	42	21-50	79±3/49±3
Back pain	12	7/5	46	30-55	85±12/53±8
Uterine myoma	10	0/10	53	32-50	67±10/41±7
Rheumatoid arthritis	12	4/8	40	25-54	75±6/42±6
Ischemic heart disease	14	10/4	63	41-63	71±5/41±4
Chronic obstructive lung disease	16	12/4	58	43-65	84±7/51±7
Carcinoma of the breast (stage I)*	21	0/21	54	34-63	80±4/54±5
Carcinoma of the breast (stage IV)†	14	0/21	50	37-66	63±15/39±10
Carcinoma of the colon (stage I)*	15	9/6	53	47-64	85±6/56±8
Carcinoma of the colon (stage IV)†	12	6/6	56	42-62	62±10/38±12
Malignant melanoma (stage I)*	9	4/5	38	24-42	65±9/49±8
Malignant melanoma (stage IV)†	11	6/5	40	33-56	63±12/38±7
Acute myelocytic leukemia	14	10/4	36	27-48	78±9/40±8
Alcoholic cirrhosis	36	24/12	52	39-67	51±9§/32±5§
Regional ileitis	9	6/3	31	26-4	67±11/40±6
Cerebral vascular disease	12	6/6	67	48-71	83±10/53±9
Cholelithiasis	14	6/8	62	41-76	80±11/52±6

Diet was uncontrolled.

*Localized lesion without detectable involvement of regional nodes or distant metastasis.

†One or more metastatic lesions.

§ $P < 0.05$ for comparison with normal group.

Table 2. Comparison of Nutritional Indexes and Plasma Albumin, Bilirubin, and Prothrombin, in 16 Normals, 14 Hypocarnitinemic Cirrhotics, and 22 Normocarnitinemic Cirrhotics

Group	Serum Carnitine (total/free)	Creatinine/ Height Ratio	MAMC	TSF	Plasma Albumin	Plasma Bilirubin	Prothrombin Time
	μM		% of Standard		g/100 ml	mg/100 ml	S
Normals	79±3/49±3	108±5	108±5	114±5	4.5±0.1	0.8±0.2	11.9±0.7
Hypocarnitinemic cirrhotics	36±4/17±3*	68±6*	71±5*	23±7*	1.4±0.3*	6.1±0.7*	19.3±1.1*
Normocarnitinemic cirrhotics	66±9/34±6	79±6*	84±5*	90±5	2.9±0.6*	3.8±0.6*	13.3±0.6

*Differs from normal with $P < 0.05$.

Table 3. Phase B of the Study: Clinical and Nutritional Evaluation of Six Normal Subjects and Six Hypocarnitinemic Cirrhotic Patients

	Normal (average ±SE)	Cirrhosis					
		1	2	3	4	5	6
Age/sex	31±3 (4 M/2F)	51/F	63/F	43/F	52/M	48/F	65/M
Known duration of disease, yr	—	9	6	8	12	7	10
Hematocrit, %	38±2	21	23	30	25	27	26
Albumin, g/100 ml	4.5±0.3	1.6	2.4	2.5	1.3	2.0	2.2
Bilirubin, mg/100 ml	0.6±0.1	3.5	5.3	7.7	4.9	5.0	8.2
Prothrombin time, s	12±0.05	15	17	20	16	13	15
Creatinine clearance, ml/min	118±8	52	74	48	71	83	44
Ascites*	0	+++	++	+++	++++	+++	++
MAMC, % of standard	103±4	70	65	67	70	64	64
TSF, % of standard	115±6	15	22	11	30	15	20
Creatinine/ht index, % of standard	110±4	43	52	56	41	53	62
Spontaneous daily intake:							
calories	2,780±115	920	1,120	830	610	1,160	850
protein	86±6	20	31	21	18	20	16
Serum carnitine (total/free), μM†	76±5/52±6	20/6	30/15	26/11	18/5	33/17	19/12

*Ascites was graded as follows:
+, positive "puddle" sign;
++, flank dullness up to anterior axillary line;
+++, dullness extends medial to anterior axillary line; abdomen distended but not tense; fluid wave; umbilicus flat;
++++, abdomen tense with fluid wave and everted umbilicus; diaphragm elevated.
†During phase A of the study.

Table 4. Average Composition of Diets in Phase B for Six Normal (N) and Six Cirrhotic (C) Subjects*

Nutrient Day	RDA	Days 1–6		Days 7–12		Days 13–18	
		N	C	N	C	N	C
Calories	2,400	2,790	1,068	2,430	2,210	2,410	2,180
Carbohydrate, g	—	334	155	289	325	284	338
Fat, g	—	110	40	105	63	105	57
Amino acids, g†	56	91	22	82	86	82	79
Lysine, mg	800	4,800	400	3,230	3,400	3,230	3,160
Methionine, mg	1,400	2,400	205	2,050	2,170	2,050	2,040
L-carnitine, μmol	0	410	29	< 10	< 10	250§	250§

*For a 70-kg adult male.
†Either as dietary protein, infused amino acids, or both.
§ This value assumes zero carnitine in the "carnitine-free" diet.

of carnitine, lysine, and methionine was measured, together with the simultaneous serum and urine levels of carnitine. During days 1 to 6, our normal controls were eating healthy amounts of calories, carbohydrate, and fat, spontaneously consuming 91 grams of protein and more than the minimum daily requirements of lysine and methionine. They were eating about 410 μmol carnitine a day. In contrast, the six hypocarnitinemic cirrhotics, were spontaneously eating only about 1,000 calories a day and taking in only 22 grams of protein. Their intake of lysine and methionine was only one-half to one-fifth the recommended daily allowances. Their spontaneous intake of carnitine was only 29 μmol a day. Thus, during the first 6 days, we established that our hypocarnitinemic cirrhotics had a subnormal intake of lysine and methionine, the two precursors for endogenous carnitine synthesis. They also had a nearly zero intake of exogenous preformed carnitine. Both of these factors, of course, would predispose to carnitine depletion.

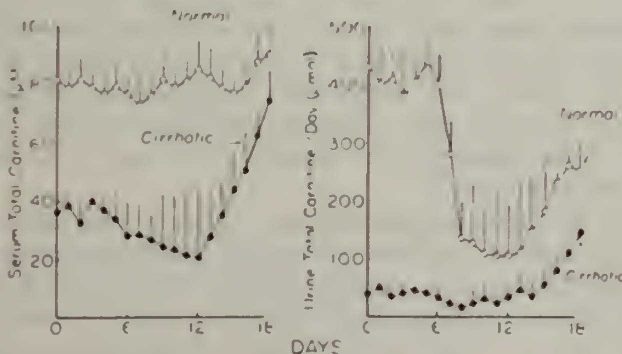
Because there is no information in the literature on the carnitine content of food, we took 25 foods and measured the quantity of carnitine in one serving of each. Meat (animal muscle) is rich in carnitine, but most other foods have vanishingly low levels. One of the implications of these data is that when a hepatologist puts cirrhotic patients on low-protein diets, in order to

protect them from encephalopathy (a maneuver that is very often done), the patients' source of exogenous carnitine is removed.

During days 7 to 12, we put both groups of patients on a carnitine-free diet. By infusions of standard amino acid solutions in the cirrhotics, we brought their daily intake of lysine and methionine up to the same levels as those of the normals. The latter procedure was carried out to see whether, when these patients were brought up to a normal intake of lysine and methionine, they would then correct their state of carnitine deficiency.

Finally, in the last part of the metabolic study, with the dietary intake held the same as during the second period, both groups received a supplement of 250 μmol of carnitine per day by mouth. The results of these studies are summarized in figure 1. During the spontaneous intake (days 1 to 6), normal controls excreted about 400 μmol of carnitine a day. In contrast, the hypocarnitinemic cirrhotics had virtually no carnitine or very low levels of carnitine in the urine. Now during days 6 to 12, both the controls and the cirrhotic patients were placed on a carnitine-free diet, rich in lysine and methionine. The normal controls maintained their normal serum carnitine levels. Their urine carnitine levels dropped to about 100 μmol per day and stabilized there. The cirrhotics showed no improvement in their carnitine levels on the lysine- and methionine-rich diet. Their serum carnitine levels drifted down as low as 20 μmol/l, and there was some further reduction in the

Figure 1. Fasting Serum and Urine Total Carnitine Values (average ±SE) in Six Normal and Six Cirrhotic Subjects During Phase B of the Study



Nutritional intake during days 1-6, 7-12, and 13-18 is described in table 3.

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urine carnitine. During days 12 to 18, when we gave the cirrhotic patients supplements of 250 μmol of L-carnitine a day, we were able to correct the carnitine deficiency state, at least the chemical state of carnitine deficiency. For the first time, their serum carnitine levels rose to the normal range and there was a sharp increase in their urine carnitine levels as well.

In developing conclusions from this study, I want to first point out that, from the rat work, we know that the turnover rate of the body pool of carnitine is about once every 15 or 20 days, and that urine carnitine is a metabolic end product. So urinary excretion of carnitine per day is essentially equal to the daily intake of dietary carnitine plus the endogenously synthesized carnitine. The picture of carnitine metabolism we see in normal human subjects is that they are eating about 400 μmol a day on a typical American diet. They are excreting about 400 μmol a day, and, apparently, endogenous synthesis from lysine and methionine is turned off. When these normal individuals are put on a carnitine-free, lysine- and methionine-adequate diet, their urinary excretion drops to about 100 μmol a day, which seems to represent the endogenous production of carnitine under these circumstances. This amount represents an adequate amount of carnitine, because on the carnitine-free diet humans show no signs of carnitine deficiency.

Now what about the cirrhotics? We have shown that within our hospital there is about a 30-percent prevalence of carnitine deficiency in the cirrhotic population. This carnitine deficiency results from three factors. First, because of the profound anorexia of these patients, often compounded by the prescription of low-protein, meat-free diets, the patients are taking in virtually no exogenous carnitine. Second, because of their anorexia, and sometimes because of their low-protein diets, their intake of lysine and methionine, the precursors for endogenous carnitine synthesis, is subnormal. And third, in the advanced cirrhotics we studied, the capacity for the third step of carnitine synthesis within the liver—from lysine and methionine—apparently has been lost. Even when supplied with adequate amounts of lysine and methionine, these patients were unable to restore their serum or urinary carnitine levels.

Now, what might the clinical significance of this finding be? In the inborn cases of carnitine deficiency, the clinical picture is one of periodic neurological symptoms, with an abnormal EEG, some hepatocellular disturbance, and a fatty myopathy. I think hepatologists will agree that all three of these features are seen from time to time in cirrhotic patients. So we have to ask how many of these clinical manifestations of the natural history of cirrhosis may be

contributed to by carnitine deficiency as a mechanism. To whatever extent, it should be readily correctable because, as we have shown, with carnitine supplements one can restore normal serum and urine carnitine levels within a few days in these end-stage cirrhotics.

The last thing I would like to point out is that there is a very recent paper by Kahn in *Clinica Chemica Acta* reporting from India on a group of Kwashiorkor children with abnormally low serum carnitine levels. The data are comparable in degree to the serum carnitine levels we found in our hypocarnitinemic cirrhotics.

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Discussion of Paper by Rudman

Dr. Knochel: I think your observations are very important. However, in the last 20 or so patients with overt alcoholic myopathy, we found that the fat content of their muscle is not increased. My question is did any of your patients have elevated serum creatine phosphokinase (CPK) activity?

Dr. Rudman: We did not look at the CPK value in our patients. To whatever extent alcoholic myopathy has a hypocarnitinemic etiology, you would expect to see lipid-filled vacuoles, because they are a classic histology in carnitine-deficiency-induced myopathy. Therefore, the alcoholic myopathy you are seeing is not the lipid-vacuole variety. Your comment would tend to suggest that most cases of alcoholic myopathy are not primarily due to carnitine depletion.

Dr. Schenker: The first question that I have is have you tried to see whether patients with acute viral hepatitis would make carnitine from the precursors? Then you could use them as their own controls. Second, do you have to have a cirrhotic lesion in order to see carnitine-deficiency myopathy?

Dr. Rudman: No. We have not made any observations on acute viral hepatitis. Where we showed patients of the other 15 categories, the impression we got is that it is hard to produce carnitine deficiency in humans. The subjects included some very sick people, some very malnourished groups, and some with metastatic carcinoma, people who had been on carnitine-free diets because of IV feedings for weeks. Yet their serum carnitine levels were normal.

We concluded that the mechanism for endogenous carnitine synthesis, including the last hepatic step, would protect an individual against carnitine deficiency, even if the individual were on a totally carnitine-free diet for a long time. We also concluded that a cirrhotic lesion or a very severely damaged liver is required to produce carnitine deficiency.

Dr. Lumeng: The major function of carnitine in skeletal muscle is fatty acid oxidation. Have you examined this aspect in the cirrhotic patients?

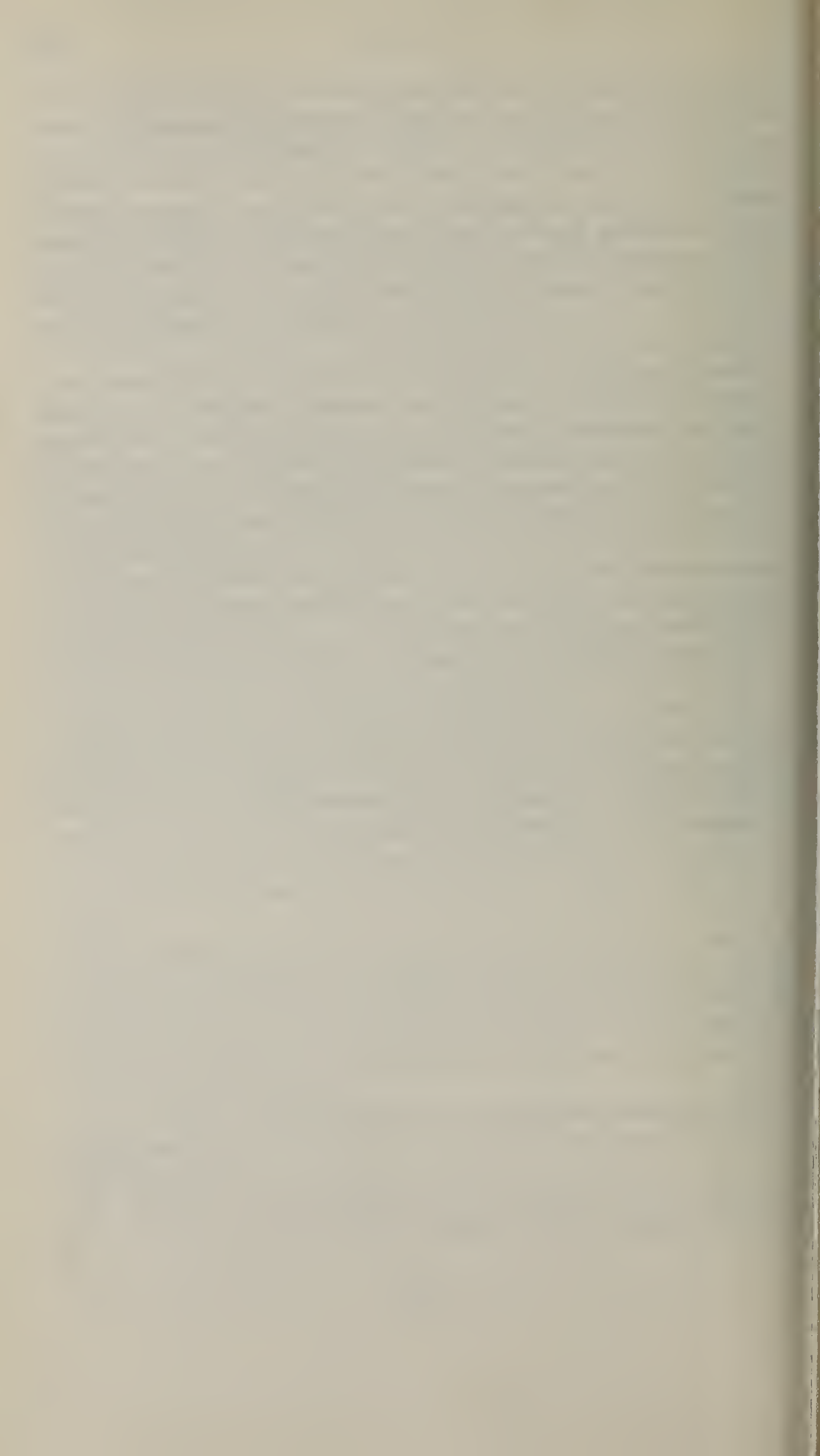
Dr. Rudman: No, we have not done that yet. You would predict that. We have followed several of these cirrhotics with

hypocarnitinemia to the autopsy table and compared their tissue carnitine levels with those of eight normally nourished individuals who had died (usually from acute cardiovascular causes), after an acute illness of less than 3 days. The hypocarnitinemic end-stage cirrhotics invariably exhibited subnormal tissue levels of carnitine in skeletal muscle, heart, liver, kidney, and brain. We saw only one-quarter to one-third as much carnitine in these tissues as in the normal tissues. So this finding verified our conclusion, from serum and urine data, that these patients truly were carnitine depleted.

Now that comes to your point that the main function of carnitine is to make it possible for the various tissues to use long-chain fatty acids as a metabolic fuel. And you would predict that these tissues would be impaired in their ability to use fatty acids as a fuel. The patients with carnitine deficiency as a whole should show a high respiratory quotient (RQ), being unable to lower the RQ with fasting. Because of the depletion of carnitine in the liver, the patient should have lost the ability for ketogenesis, so that in the starved state ketosis would not develop. As yet, we have not tested these important parameters.

Dr. Wagner: Have you looked for excretion of γ-butyrobetaine or accumulation of the precursor?

Dr. Rudman: Thanks for the suggestion. If our hypothesis is correct, that there is a block at the third step, then there should be an accumulation of one or two of the preceding intermediates, γ-butyrobetaine or perhaps the trimethyllysine. We have not looked for either of those yet, but we will.



Section IV: Effects of Alcohol on Protein and Amino Acid Metabolism

Plasma Amino Acid Measurements for the Detection of Alcoholism: Current Status*

Spencer Shaw and Charles S. Lieber

Abstract

Studies of the effects of alcohol on amino acid metabolism in the baboon have led to the observation that, following chronic heavy alcohol consumption, the level of plasma α-amino-n-butyric acid (AANB) is increased markedly. Similarly, increases in plasma AANB were observed in human volunteers given alcohol along with an adequate diet under metabolic ward conditions. The measurement of plasma AANB provides a sensitive and highly specific biochemical test to detect and assess chronic heavy drinking. It is especially useful for screening ambulatory populations and for evaluating the outcome of alcoholism rehabilitation.

Studies of the effects of alcohol on amino acid metabolism in the baboon have lead to the observation that, following chronic heavy alcohol consumption, the level of plasma α-amino-n-butyric acid (AANB) is increased markedly (1). Similarly, increases in plasma AANB were observed in human volunteers given alcohol along with an adequate diet under metabolic ward conditions (2). The application of the measurement of plasma AANB as a biochemical test for chronic heavy drinking in the alcoholic, however, was complicated by the fact that the plasma level of AANB is affected by dietary factors. Indeed, dietary protein restriction or deficiency results in a decrease in the plasma level of AANB (3,5) and such abnormalities frequently may be present in the alcoholic. Therefore, in order to use plasma AANB levels to assess chronic heavy drinking, it was necessary to control for nutritional status. Control was achieved by simultaneously measuring plasma branched-chain amino acids that are known to reflect dietary protein intake (3,5). For convenience, a representative branched-

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chain amino acid, leucine, was selected, but similar results were obtained with each of the other branched-chain amino acids.

The level of AANB expressed relative to leucine was found to be increased approximately twofold among active alcoholics when compared to that of controls (6). This increase was reversible within 1 week after cessation of drinking. The rise occurred among ambulatory as well as hospitalized subjects. Furthermore, there was a statistically significant positive correlation between the level of AANB/leucine and the degree of alcoholism assessed by National Council on Alcoholism criteria of alcoholism, alcohol intake histories, and a psychological questionnaire (6).

Further investigation of the relationship of AANB to leucine in alcoholics and nonalcoholics revealed that the increase in AANB/leucine due to alcohol consumption was not linear over the entire range of leucine values. Therefore, we recommended the replacement of the use of a simple ratio with experimentally derived curves (7). In addition, it was found that the sensitivity of this test for chronic heavy drinking could be improved by the simultaneous measurement of γ glutamyl transpeptidase (GGTP). Using this modification, along with the experimentally derived curves, one is able to detect approximately 80 percent of active alcoholics sampled within 7 days of drinking, with only a 2-percent rate of false-positive determinations among controls (7). This test was found to be much more sensitive than a measurement of blood alcohol level and also more useful because blood alcohol does not distinguish acute from chronic alcohol consumption. Furthermore, it was as sensitive as, but much more specific than, GGTP.

The usefulness of the measurement of AANB to detect chronic heavy drinking among patients with nonalcoholic liver disease is more limited because of the frequency of false-positive tests (8,2,9,10). However, among these patients, this test is less frequently abnormal than is GGTP (8).

Recently, the increase in AANB due to chronic alcohol consumption has been questioned and has been attributed to nonspecific liver damage (9,10). Other research groups, however, have confirmed a reversible increase in AANB relative to leucine following chronic alcohol consumption (Ellen Gordon, personal communication). Furthermore, in the rat model of alcohol consumption, which does not develop liver damage other than steatosis, an increase in AANB/leucine has been reported by several groups (10,11). In liver slices, this rise has been shown to be due, at least in part, to increased hepatic production of AANB by alcohol (12).

In patients with severe liver damage unrelated to alcohol, the level of AANB relative to leucine may be increased due to low

leucine values. This situation has frequently been observed in patients with advanced liver damage such as cirrhosis (13,14,15) and may be attributed, at least in part, to portal systemic shunting associated with hyperinsulinemia and hyperglucagonemia (15). Such patients with clinically overt liver disease do not usually represent a diagnostic problem with respect to latent alcoholism. Furthermore, in the majority of patients with moderately severe liver damage unrelated to alcohol, increases in AANB/leucine were not observed by our group (2,6) or others (16).

Measurement of AANB as a biochemical test for alcoholism has been found extremely useful in detecting latent alcoholism among ambulatory subjects (including white-collar workers), as well as in monitoring the success of alcoholism rehabilitation. Among such subjects, we are able to detect approximately 80 percent of the alcoholics. In many instances, the presence of heavy drinking was not suspected, but was later corroborated after a positive blood test, family investigations, or determinations of blood alcohol levels. To date, we have studied 350 subjects and have observed 121 positive tests among 150 alcoholics, and only 4 false-positive tests among 200 controls.

In conclusion, measurement of plasma AANB provides a sensitive and highly specific biochemical test to detect and assess chronic heavy drinking. It is especially useful for screening ambulatory populations and for evaluating outcome of alcoholism rehabilitation.

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Ethanol Consumption and Free Amino Acids of Rat Plasma and Liver

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Recent studies have indicated that the plasma ratio of α-amino-n-butyric acid to leucine (A/L ratio) was elevated in ambulatory and hospitalized alcoholics (1). This finding suggests that the increased A/L ratio may become an objective empirical marker for the detection and assessment of alcoholism (1). In order to test this hypothesis, we have looked at the A/L ratio and other amino acid concentrations in the plasma and liver of rats following chronic ethanol ingestion.

Experiment

Two-month-old male rats of the Sprague-Dawley strain were housed individually in stainless steel cages at room temperatures of 25°-28°C. They were divided into three groups of six rats each and were fed Purina Chow. The first group (control rats) received tapwater and Chow ad libitum. The second group received 20-percent (V/V) ethanol as their sole drink and Chow, ad libitum. The rats in the third group received a solution of sucrose (which has an equivalent caloric value to the 20-percent ethanol) and were pair-fed to the ethanol-treated rats.

After 6 months on the dietary regimens, the rats were fasted for 18 hours and sacrificed by cardiac puncture. The fasting blood was collected in a heparinized centrifuge tube and the plasma was separated and stored at −25°C. The whole liver was perfused with saline-isotonic solution, removed, and weighed. An aliquot of each tissue was homogenized in distilled water to yield a 5-percent homogenate. Then followed centrifugation, after which the tissue supernatants and the plasma were deproteined with sulfosalicylic acid. Free amino acids were analyzed by ion-exchange chromatography with a Technicon automated amino acid analyzer. The data were analyzed statistically by means of Student's t test.

Plasma-Free Amino Acids

Table 1 shows the results of free amino acid concentrations in rat plasma following chronic ethanol consumption. Ethanol had no effect on the values of essential amino acids. However, the rats drinking sucrose solution for the isocaloric substitution of ethanol had a decreased level of isoleucine as compared to that of the rats consuming ethanol. Among the nonessential amino acids, the most striking difference was the marked increase in the concentration of α-amino-n-butyric acid in the ethanol-treated rats. The growth was approximately threefold over the control rats and fivefold over the sucrose-drinking animals. Chronic ethanol consumption also resulted in a pronounced increase of free ornithine and glutamic acid over control values. On the other hand, the

Table 1. Free Amino Acids in Rat Plasma Following Chronic Ethanol Consumption

Amino Acids	Control	20% Ethanol	Sucrose
		(μM/100 ml	
Essential			
Arginine	18.13± 2.42[a]	14.26± 3.54	13.17± 3.62
Histidine	6.21± 2.89	6.69± 1.35	7.95± 1.19
Isoleucine	11.93± 2.92	15.48± 2.12	9.78± 1.12[e]
Leucine	20.78± 4.86	24.05± 3.01	19.19± 1.92
Lysine	37.90± 3.41	31.63±11.01	36.85± 6.85
Methionine	5.18± 0.20	6.61± 1.22	6.49± 1.02
Phenylalanine	8.86± 1.38	9.95± 1.08	10.44± 1.50
Threonine	20.97± 3.25	32.30±10.55	28.86±10.40
Tyrosine	10.41± 1.61	10.34± 1.32	11.24± 3.00
Valine	22.80± 5.04	27.80± 3.00	19.24± 2.56
Nonessential			
Alanine	40.53± 6.17	48.66±12.48	115.51±23.55[d,e]
AABA[b]	2.88± 1.28	10.52± 3.39[c]	1.93± 0.75
½ Cystine	2.87± 0.87	2.78± 0.64	1.08± 0.43
Glutamic acid	36.36± 4.64	53.72± 9.46[c]	69.82± 2.56[d,e]
Glycine	36.99± 8.51	34.05± 9.83	34.81± 3.51
Ornithine	5.49± 1.81	10.92± 2.98[c]	12.73± 1.35[d]
Proline	6.57± 1.91	7.22± 3.94	8.10± 1.63
Serine	18.83± 4.49	22.98± 1.62	36.69± 3.63[d,e]
Taurine	50.29±17.33	52.87±18.92	37.60±10.02

[a] Mean of six rats ±S.D.
[b] α-amino-n-butyric acid.
[c] $P < 0.01$ or $P < 0.05$ versus control.
[d] $P < 0.01$ versus control.
[e] $P < 0.01$ or $P < 0.05$ versus ethanol.

sucrose solution as the sole drinking fluid increased the amount of free serine, glutamic acid, and alanine when compared to the rates of the other two groups. There was also an increased ornithine level of the sucrose-drinking group over the controls.

Liver-Free Amino Acids

The liver-free amino acid levels of the rats receiving 20-percent ethanol, or isocaloric substitute of sucrose solution for ethanol, and the control animals receiving tapwater are summarized in table 2. As with plasma, the values of all individual essential

Table 2. Free Amino Acids in Rat Liver Following Chronic Ethanol Consumption

Amino Acids	Control	20% Ethanol	Sucrose
		(μM/100 mg dry wt.)	
Essential			
Arginine	0.071±0.011[a]	0.072±0.054	0.076±0.020
Histidine	0.397±0.060	0.461±0.109	0.360±0.063
Isoleucine	0.643±0.179	0.646±0.141	0.455±0.084
Leucine	1.120±0.340	1.247±0.263	0.783±0.126
Lysine	1.366±0.358	1.557±0.228	0.927±0.122[d,e]
Methionine	0.281±0.109	0.265±0.095	0.188±0.077
Phenylalanine	0.594±0.172	0.641±0.138	0.444±0.084
Tyrosine	0.263±0.103	0.330±0.134	0.261±0.089
Valine	1.050±0.283	1.173±0.261	0.831±0.187
Nonessential			
Alanine	3.919±0.400	4.074±0.974	4.078±0.385
AABA	0.149±0.130	0.429±0.158[c]	0.162±0.109[e]
Aspartic acid	0.995±0.213	1.178±0.318	1.001±0.256
½ Cystine	0.063±0.032	0.056±0.036	0.076±0.032
GABA[b]	0.765±0.187	1.607±0.171[c]	0.912±0.262[e]
Glutamic acid	1.849±0.422	1.258±0.113[c]	1.157±0.255[d]
Glycine	2.698±0.450	3.345±0.563	2.594±0.617
Ornithine	0.969±0.428	1.591±0.442[c]	0.934±0.109[e]
Proline	0.582±0.251	0.427±0.165	0.429±0.164
Serine	1.284±0.291	1.438±0.168	1.331±0.312
Taurine	2.872±1.717	4.062±1.885	0.508±0.132[d,e]

[a] Mean of six rats ±S.D.
[b] γ-amino-n-butyric acid.
[c] $P < 0.01$ or $P < 0.05$ versus control.
[d] $P < 0.01$ or $P < 0.05$ versus control.
[e] $P < 0.01$ or $P < 0.05$ versus ethanol.

amino acids in the ethanol-treated rats were about the same as those in control rats. On the other hand, the rats drinking sucrose solution tended to have low values of the three branched-chain amino acids. In addition, free lysine levels were significantly reduced as compared to either those of the controls or those of the ethanol-treated rats.

Of the nonessential amino acids, chronic ethanol consumption resulted in a marked elevation of both α-amino-n-butyric acid and γ-amino-butyric acid levels. Also, there was an increase in ornithine and a decrease in glutamic acid. The rats receiving sucrose solution showed a marked drop of taurine and a significant reduction of glutamic acid.

Ratio of α-Amino-n-Butyric Acid to Leucine (A/L)

The data in table 3 indicate that the A/L ratios in both plasma and liver were markedly increased after ethanol feeding. These changes appear to be specific, inasmuch as the A/L ratios were normal in sucrose-drinking and pair-fed rats.

Table 3. Ratio of α-Amino-n-Butyric Acid to Leucine (A/L)

Tissue	Control	Ethanol	Sucrose
Plasma	0.139[a]	0.437[b]	0.101
Liver	0.129	0.344[b]	0.207

[a]Mean of six rats.
[b]$P < 0.01$ versus control or sucrose.

Conclusion

Our findings support the earlier observation of Lieber and others (1) indicating an increase in plasma α-amino-n-butyric acid to leucine ratio in the hospitalized alcoholics and in baboons fed alcohol as 50 percent of their total caloric intake. In addition, the parallel increases of the A/L ratios in the plasma and liver further suggest that the increase of liver α-amino-n-butyric acid is the cause of the increased plasma α-amino-n-butyric acid, which in turn results in an elevation of the A/L ratio. However, the mechanisms whereby long-term alcohol consumption results in amino acid abnormalities are still unknown. Further experiments are needed to disclose the nature of the metabolic derangement.

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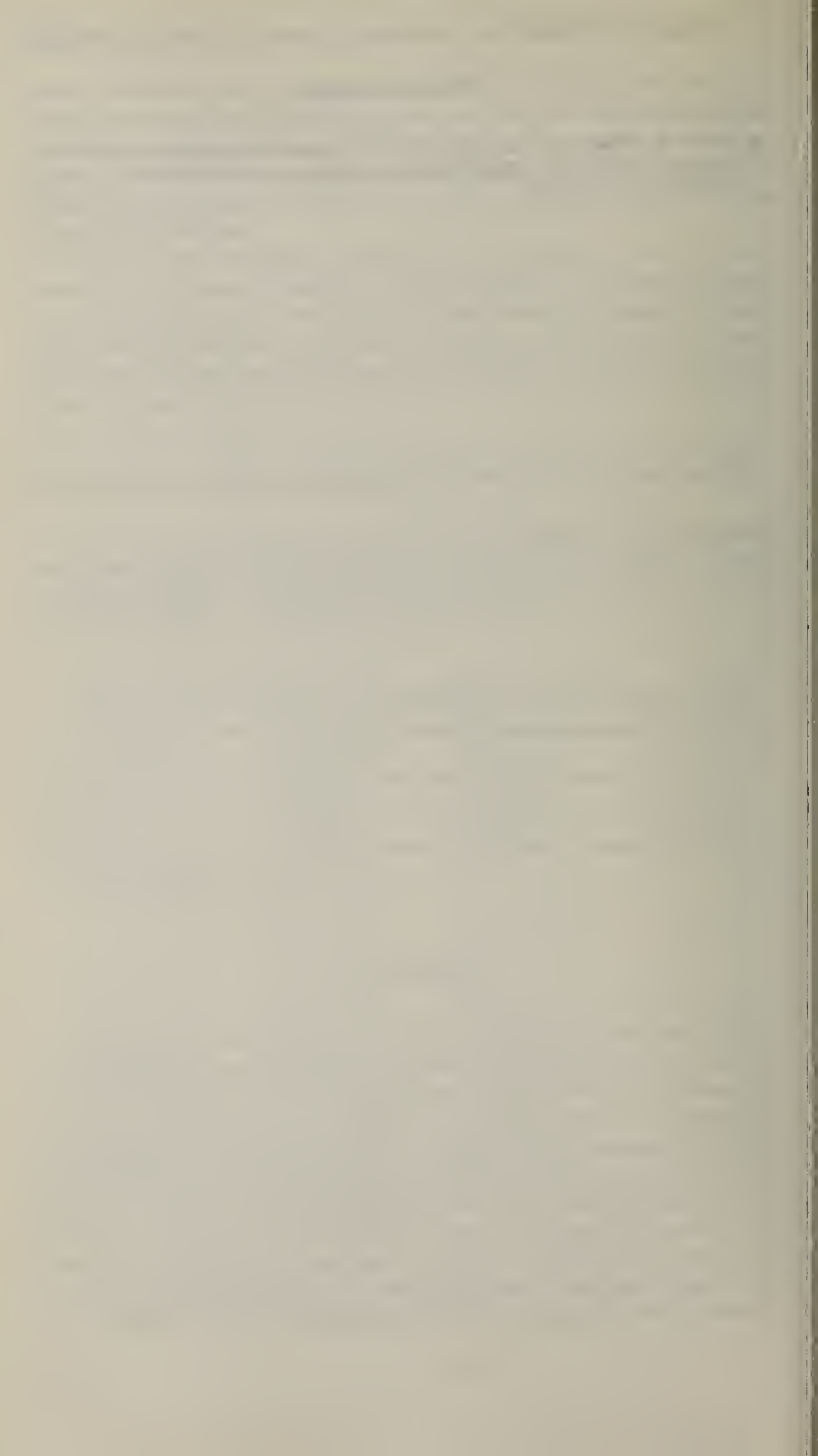

Alcohol, Acetaldehyde, and Albumin Synthesis*

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Introduction

Ethanol is metabolized sequentially to acetaldehyde and to acetate. During this process, protein synthesis has been shown to be inhibited (1,2,3,4). However, the mechanism whereby the metabolism of ethanol results in these alterations in protein synthesis is not clear, and the offending agent has not been identified. Increased levels of acetaldehyde have been noted in patients with chronic liver disease secondary to ethanol intake, and acetaldehyde (and not ethanol) has been shown to interfere with cardiac muscle protein synthesis. In fact, acetate itself has been suggested as the mediator of ethanol toxicity in isolated cell systems. The present review summarizes the acute effects of ethanol and acetaldehyde with and without the metabolic inhibitors 4-methylpyrazole and disulfiram, in livers derived from fed or fasted donors. The extremes of nutrition are thus contrasted in terms of acute effects of the metabolic responses to ethanol.

Donors

Fed or 24-hour-fasted rabbits, weighing 1.2 to 1.4 kg, were used in all studies. The standard rabbit chow (Wayne Rabbit Ration, Allied Mills, Inc., Chicago, Ill.) consisted of 15 percent protein, 2.5 percent fat, and 18 percent fiber; the average intake was 80 to 120 g per day.

Perfusion Solution

The perfusion solution consisted of washed rabbit red cells made up to a final hematocrit value of 25 to 27 percent with Krebs-Henseleit bicarbonate buffer containing 3 g/100 ml of rabbit or bovine albumin, 0.08-percent glucose, and amino acids as listed in table 1.

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Table 1. Standard Perfusion Solution

Parameter	Concentration
Amino Acids (L-form)	
alanine	0.40 mM
aspartate	0.05 mM
cysteine	0.30 mM
glutamine	0.69 mM
glutamate	0.15 mM
glycine	0.30 mM
proline	0.12 mM
phenylalanine	0.10 mM
serine	0.15 mM
arginine	0.15 mM
histidine	0.06 mM
leucine	0.35 mM
lysine	0.15 mM
threonine	0.20 mM
tyrosine	0.02 mM
valine	0.31 mM
isoleucine	0.05 mM
methionine	0.03 mM
tryptophan	0.05 mM
Glucose	1 g/liter
Albumin	30-35 g/liter
Heparin	10,000 U/liter
pH	7.4
Gas	95% O_2-5% CO_2
Hematocrit	26%
Buffer	Krebs-Henseleit

In the alcohol studies, 95-percent ethyl alcohol was added to the perfusion solution at an initial concentration of 0.22 percent by volume; this level was maintained by the constant infusion of ethanol during the experimental period.

Perfusion

The techniques for removal of the liver and its perfusion have previously been described in detail (5). Briefly, under light ether anesthesia, the livers were exposed; the portal vein was cannulated proximally while the liver was in situ; and perfusion was started immediately. The inferior vena cava was cannulated below and severed above the diaphragm; the liver was removed and reoriented on a platform in a heated, humidified box; and the portal vein inflow cannula was transferred to a pump system.

Perfusion was directed into the portal vein at a rate of 1.0 to 1.4 ml/g of liver per minute. The perfusion volume of 140 to 170 ml was recirculated and oxygenated by a disc oxygenator that received the output from the inferior vena cava. Bile was collected from the cannulated biliary duct.

Albumin Synthesis

The [^{14}C] carbonate technique was used to label the hepatic-arginine intracellular pool. Arginine is the immediate precursor, not only of the arginine residue in albumin, but also of urea, so a direct product precursor relationship exists, as has been described by Swick, Reeve et al., and McFarlane (6,7,8). This technique has been examined in detail in numerous publications.

After 30 minutes of perfusion (control and experimental), 100 μCi of [^{14}C] carbonate (specific activity, 5 mCi/mmol) were injected directly into the inflow tube to the portal vein, and the perfusion was continued for 2-1/2 hours.

Albumin synthesis was determined by the following formula:

$$\text{Albumin synthesis} = \frac{\text{albumin guanidino C specific activity}}{\text{synthesized urea C specific activity}} \times \text{perfusate albumin}$$

Synthesized urea carbon-specific activity is presumed to equal the precursor arginine-guanidino carbon-specific activity.

Analytical Methods

The total protein in the perfusion solution was measured by a biuret method, and albumin partition by a Kern microelectrophoresis unit.

Albumin was isolated from the perfusion solution by preparative acrylamide gel electrophoresis. The perfusion solution was treated with a large excess of nonradioactive urea to ensure that the isolated albumin was not contaminated with high specific activity [^{14}C] urea. To ensure purity, samples of the isolated albumin were examined by qualitative polyacrylamide electrophoresis and immunoelectrophoresis at a 6-percent protein level.

In those perfusions where bovine albumin was used, albumin was isolated by two alcohol-trichloroacetic acid separations with intervening dialyses. These techniques have been shown to result in clean albumin preparations with the same [^{14}C] guanidino

carbon-specific activity as those isolated by immunochemical or acrylamide gel methods.

Lactate, pyruvate, and alcohol levels were obtained with Sigma Chemical Company kits 846B, 726, and 331 (Sigma Chemical Co., St. Louis, Mo.). Samples of perfusion solution blood were obtained at 30-minute intervals, and the concentrations of lactate and pyruvate were determined on each sample.

In order for the [^{14}C] carbonate method to be valid, the rates of synthesis of albumin and urea should remain constant during the experimental period. Otherwise, situations may arise wherein the major portion of urea is synthesized when the specific activity of the arginine is different from that when the albumin molecule is synthesized. This situation would lead to falsely low or high values for albumin synthesis.

Urea synthesis was monitored at 5- to 15-minute intervals during the perfusion, and livers that did not have a stable urea synthetic rate were not used. (Eleven perfusions were discarded for this reason.)

Furthermore, in at least one study in all groups, albumin synthesis was measured by an immunochemical method along with the $^{14}C\text{-}CO_2$ method, using high-titer monospecific antibody against rabbit albumin.

Immunochemical quantitation of the newly synthesized albumin was determined by the method of Mancini et al. (8a). In six replicates, the 0.95 error ($P = 0.05$) of the measured diameters averaged ±7 percent. A plot of the square of the diameter versus concentration of antigen standards was made on linear graph paper, and the unknowns were determined from the graph. All batches of antiserum were tested against various dilutions of rabbit serum to ensure the presence of only a monospecific antibody. Also, the antiserum was tested against bovine albumin to test for the absence of cross-reaction.

The data derived by the two independent methods agreed quantitatively in 24 of 32 combined studies, and qualitatively in the other 8 studies (9).

Because alcohol might inhibit the release of newly synthesized albumin, the rate of release of labeled albumin was studied in both alcohol and control perfusion solutions. Labeled albumin was detected in the alcohol and control perfusion solutions by at least 35 minutes after the [^{14}C] carbonate injection, indicating no delay in release of the labeled albumin. The total albumin carbon [^{14}C] guanidine activity was achieved by 90 to 100 minutes in both alcohol and control groups; by 150 minutes, no additional increase in activity was noted. If release had been delayed by

alcohol, the peak total ^{14}C activity of the released albumin would have occurred later. In addition, the increment, in rabbit albumin levels and in the perfusion solutions containing bovine albumin, was steady during the control and alcohol studies, further supporting the conclusion that alcohol did not impede the release of preformed albumin.

DNA-RNA Determinations

After perfusions, the liver was chilled by the gentle injection of 25 ml of ice-cold 0.25 M sucrose (RNase-free, Schwartz/Mann, Orangeburg, N.Y.) in TKM buffer (50 mM Tris-HCl, pH 7.5-25 mM KCl-5 mM $MgCl_2$). The liver was weighed, minced, and homogenized in 2 vol of the same buffer in a glass homogenizer with a loose-fitting Teflon pestle. DNA was determined by the indole method of Ceriotti, as modified by Keck (10). The determination of RNA was essentially that of Fleck and Begg (11). Total protein was determined by the method of Lowry et al. (12).

Polysomal Isolation

Polysomes were isolated from the whole liver, after the 2-1/2- to 3-hour perfusion, employing the techniques described by Blobel and Potter, as modified below. After homogenization in 2 vol of 0.25 M sucrose-TKM buffer, nuclei, debris, and mitochondria were separated by a 10-minute spin at 15,000 g. The supernate was layered over a 1.38 to 2 M sucrose discontinuous gradient in TKM buffer containing cell sap as an RNase inhibitor, as suggested by Blobel and Potter (13). After a 20-hour spin at 105,000 g (Spinco 40 rotor), the bound polysomes sedimenting into the 1.38 M sucrose layer were removed, treated with 1/4 vol of 20-percent Triton-5 percent sodium deoxycholate solution, and recentrifuged through 2 M sucrose in TKM-cell sap for 20 hours, as above. The pellet obtained from this interface has been shown to represent the polysomes that had been bound to the endoplasmic reticulum, and the purity of this fraction was confirmed.

The unbound or free polysomes sedimented through the 2 M sucrose and were harvested as a pellet after the initial 20-hour spin. The pellet was frozen at −20°C and treated identically with the bound pellet obtained 24 hours later.

In livers from fed donors, the large quantity of glycogen present prevents effective isolation of the free polysomes. Thus, in these livers, amylase was added to the postmitochondrial supernatant at

a final concentration of 70 U per ml. After 30 minutes at 4°C with gentle stirring, the same quantity of amylase was added, and gentle stirring was continued for another 30 minutes at 4°C. In studying fasted free polysomes isolated with and without amylase treatment, no effect of this amylase treatment on polysomal aggregation was noted.

Polysome Analysis

The polysomes, bound and free, were suspended in 1 ml of cold distilled water, and 16 to 20 absorbance units (260 nm) were layered over a 34 ml linear sucrose gradient (0.3 to 1.1 M in TKM over a 2 ml cushion of 60-percent sucrose). These gradients were spun at 25,000 g in a SW 27.1 rotor at 4°C for 2 hours, and the resultant gradient was analyzed in an Isco ultraviolet analyzer (Instrumentation Specialties Co., Lincoln, Neb.) at 254 nm (model UA-4).

Polysomal Labeling

The pattern and extent of polysomal RNA labeling was determined by the addition of [^{3}H] uridine; 1.5 mCi (26 μCi per mmol) in the perfusion solution during the 2½-hour perfusion. The isolated factions (bound and free) were analyzed as above in an Isco ultraviolet analyzer, and 1 ml fractions were collected; 0.1 ml was plated on Whatmann No. 3MM discs. The discs were treated sequentially with ice-cold 5-percent trichloroacetic acid containing nonradioactive uridine for 10 minutes, washed with ice-cold 7-percent trichloroacetic acid, cold ethanol, ethanol-ether (1:1 v/v), and ether, and then air dried. The dried discs were suspended in 5 ml of 0.7-percent butyl-PBD in toluene and counted in a liquid scintillation counter, appropriate corrections being made for ^{14}C contamination in the ^{3}H channel.

Results

Influence of Specific Amino Acids on Albumin Synthesis

The acute effects of exposure of livers from fed donors to ethanol results in a 50-percent decrease in the rate of albumin synthesis and a significant and similar decrease in the synthesis of urea. Polysome disaggregation is marked with particular destruction of the endoplasmic membrane-bound polysome. These results can

be counteracted effectively with 10 mM levels of arginine, tryptophan, ornithine, and lysine, the same amino acids that stimulated albumin synthesis in livers from fasted donors in the absence of exposure to another stress such as ethanol. Not all amino acids were effective in the latter situation; histidine, leucine, methionine, and valine were ineffective in stimulating either urea or albumin production (table 2). These observations suggested that the urea cycle might play a more important role in regulating protein synthesis than had been heretofore imagined. Furthermore, ornithine (figure 1), an amino acid in the Krebs-urea cycle, is not incorporated into albumin, but ornithine too resulted in a marked stimulation of albumin production, both in livers from fed donors perfused with ethanol and in livers exposed to the single stress of fasting. Ornithine is the immediate product following the cleavage of arginine into urea and is also the precursor of the polyamines putrescine, spermidine, and spermine. These polyamines have been suggested as playing important roles in maintaining the integrity of the polysomal system.

Thus, studies were conducted to determine if the effects seen with high levels of amino acids could be duplicated by adding 1 mM levels of spermine, a physiologic level found in vivo. To this end, albumin synthesis was measured in livers from fed and fasted donors perfused with ethanol 200 mg%, ethanol plus 1 mM spermine, and ethanol plus arginine 10 mM and 1 mM spermine (table 3). The results indicate that, in fed donors perfused with ethanol and in fasted donors, spermine can result in significant

Table 2. Effects of Amino Acids on Albumin Synthesis in Livers From Fed Donors

Perfusate	Albumin Synthesis (% of control values)	Urea Synthesis (% of control values)	Degree of Bound Polysome Aggregation
Control	100	100	100
Ethanol	37	32	37
Ethanol + A.A.	70	61	70

Bound polysome aggregation (> trisome) varies from 66 to 76 percent.

Control albumin synthesis has varied over the years from 16-18 to 20-22 mg/100 g wet liver wt/hr.

Control liver synthesis has varied over the years from 32-34 mg/100 g wet liver wt/hr.

A.A. = 10 mM levels of either Trp, Orn, Lys, Arg.

Ethanol 200 mg%.

Figure 1. Relationship of Ornithine to the Urea Cycle and to the Synthesis of Polyamines

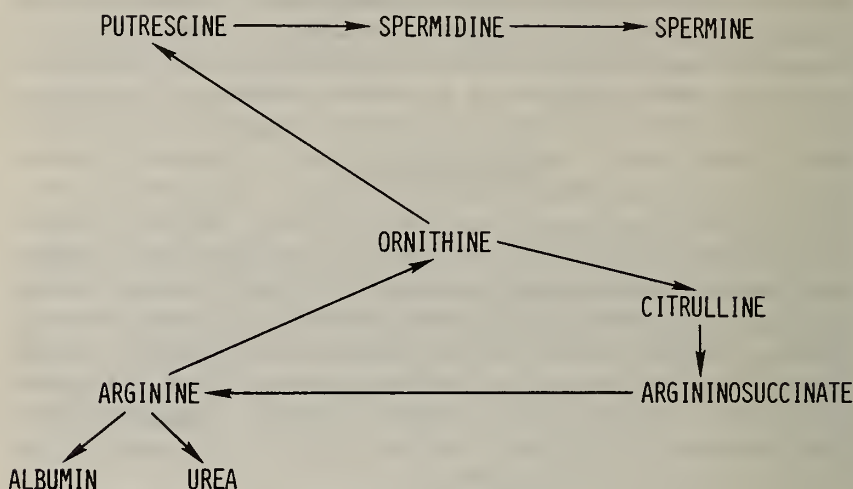

Table 3. The Effects of Arginine and Spermine on Albumin Synthesis in Livers From Fed Donors*

Perfusate	Albumin Synthesis (% of control values)	Urea Synthesis (% of control values)	Degree of Bound Polysome Aggregation
Control	100	100	100
Ethanol	35	34	37–43
Ethanol-arginine	70	78*	77
Ethanol-spermine	53	62	67
Ethanol-spermine-arginine	100	169	77

Ethanol 200 mg%.

Spermine 1 mM.

*It should be noted that urea synthesis from arginine only requires arginase, and ethanol and acetaldehyde do not inhibit this enzyme. The lack of increment in urea synthesis after the addition of arginine, but in the presence of ethanol, is striking, but the cause is not known.

polysome reaggregation. However, in order for albumin and urea synthesis to be effectively stimulated, it is necessary to add arginine as well. These results suggested that arginine might be the rate-limiting amino acid, as it is present in the liver in only trace quantities. Spermine, or the other polyamines, would therefore play a role in effectively binding the programed polysome to the endoplasmic reticulum for the synthesis of proteins for export. The ability of spermine to overcome even the combination of the stresses of fasting and ethanol exposure in terms of polysome aggregation is marked.

Ethanol Versus Fasting

So far, these studies have shown that although both fasting and acute exposure to ethanol reduce albumin synthesis and cause significant polysome disaggregation, the mechanism of the action of ethanol is certainly different from that of fasting, primarily because fasting reduces the total quantity of RNA dramatically within the first 24 to 48 hours. However, acute exposure to ethanol did not cause any detectable alteration in the total amount of hepatic RNA (table 4). Further, studies employing [^{3}H] uridine and cytidine have shown a specific inhibition of entrance of newly labeled RNA into the endoplasmic membrane-bound polysome of ethanol-exposed livers. These results, likewise, appear to indicate a difference in the mechanism of action between ethanol and fasting, although both stresses appear to

Table 4. Albumin Synthesis in the Perfused Rabbit Liver

Parameters	Fasted Donor Control Perfusate	Fed Donor Alcohol Perfusate
Albumin synthesis	Decreased by 40-50%	Decreased by 40-50%
Loss of RNA	30%	9%
Bound polysome	Disaggregated	Disaggregated
Free polysome	Unaffected	Unaffected
Response to amino acids	Albumin synthesis increased to rates exceeding control; partial to complete reaggregation of bound polysome	Albumin synthesis increased to rates approaching control; partial reaggregation of bound polysome

produce a depression in the degree of endoplasmic membrane-bound polysome aggregation and an equivalent alteration or depression in albumin synthesis.

When the stresses of fasting and ethanol exposure are combined (table 5), the depression in albumin synthesis and urea synthesis is even more marked than that seen with either stress alone. The endoplasmic membrane-bound polysome is disaggregated, and so is the free polysome. The amino acids that were effective in reversing either stress alone are not ineffective, although a combination of spermine and arginine did produce significant recovery, not only in terms of polysome aggregation, but also in terms of the capacity for albumin synthesis.

In order to study the effects of acetaldehyde on albumin and urea synthesis, as well as the degree of polysome aggregation, various techniques were employed. Acetaldehyde was infused continuously to maintain the input level at approximately 2 mg% by an infusion rate of acetaldehyde 3-percent weight by volume of 83.3 μl/100 ml portal perfusion rate. At time zero, the concentration of acetaldehyde at the outflow from the liver averaged approximately 0.6 mg/100 ml and, at the end of the perfusion

Table 5. The Effects of the Combination of Fasting Plus Ethanol on Albumin Synthesis*

Perfusate	Albumin Synthesis (% of control values)	Urea Synthesis (% of control values)	Degree of Bound Polysome Aggregation
Control	100	100	100
Ethanol	59	38	10-48
Ethanol-A.A.	59	31-65	No reaggregation
Ethanol-spermine	180	56	90
Ethanol-spermine-arginine	260	167	100

Ethanol 200 mg%.

Arginine and A.A. 10 mM.

Spermine 1 mM.

The degree of bound polysome aggregation averaged 48%.

*Values for albumin synthesis in livers from fasted donors averaged 8-9 mg and 42-62 mg/100 g wet liver wt/hr respectively.

Table 6. Effects of Acetaldehyde on Albumin Synthesis in Livers From Fed Donors

Perfusate		Albumin Synthesis	Urea Synthesis	Degree of Bound Polysome Aggregation
		(% of control values)		
Control	(11)	100	100	100
Ethanol	(6)	50	64	37-63
Acetaldehyde	(8)	50	53	100
4-MP	(3)	109	140	102
Ethanol + 4-MP	(6)	55	55	84
Acetaldehyde + 4-MP	(6)	64	75 (NS)	95

Number of studies indicated by ( ).

Ethanol 200 mg%.

Acetaldehyde 2 mg%.

4-MP = 4-methylpyrazole 1.5 mM.

Control Values:

Albumin and urea synthesis were 22 and 36 mg/100 g wet liver wt/hr respectively.

The degree of bound polysome aggregation averaged 62 percent.

NS not significant compared to the acetaldehyde group. The absolute figures were for acetaldehyde, 19 ± 3 mg/hr/100 g; for acetaldehyde + 4-MP, 27 ± 3 mg/100 g/hr.

2.5 hours later, was approximately 0.8 mg/100 ml. This level of acetaldehyde decreased albumin and urea synthesis but did not alter polysome aggregation in livers from fed donors.

In a second method, 4-methylpyrazole (MP), 1.5 mM, was added to the infusion-containing ethanol, 200 mg%. This level of 4-MP reduced ethanol oxidation by 85 to 95 percent but failed to improve albumin synthesis over the values seen with ethanol alone. Further, when acetaldehyde and 4-methylpyrazole were perfused together, there was no improvement in albumin or urea synthesis. Thus, in livers from fed donors, acetaldehyde did prove toxic to albumin and urea synthesis. The addition of 4-methylpyrazole to acetaldehyde or to the ethanol-containing perfusates failed to improve either parameter significantly, although the degree of polysome aggregation was significantly improved with the combination of 4-MP plus ethanol over the aggregation seen with ethanol alone.

Livers derived from fasted donors, however, yield results significantly different from those seen with fed-donor liver preparations. The effects of inhibiting ethanol oxidation with 4-methylpyrazole reversed the ethanol inhibition of urea synthesis, and albumin synthesis returned to the levels seen in livers from fasted donors per se (in the original, or naive, state). Likewise, the degree of polysome disaggregation was reversed and found to be the same as that observed in livers from fasted donors. In the absence of ethanol, the effect on albumin and urea synthesis and polysome aggregation of the combination of 4-methylpyrazole plus acetaldehyde was essentially the same as that observed with acetaldehyde per se. Thus, in contrast to results seen in livers from fed donors, the addition of 4-methylpyrazole, an agent capable of inhibiting ethanol oxidation, appears to reverse the major effects of ethanol on albumin and urea synthesis as well as the effects on polysome disaggregation.

Table 7. Effects of Acetaldehyde on Albumin Synthesis in Livers From Fasted Donors

Perfusate		Albumin Synthesis (% of control values)	Urea Synthesis (% of control values)	Degree of Bound Polysome Aggregation
Control	(6)	100	100	100
Ethanol	(5)	63	32	53
Acetaldehyde	(7)	100	100	100
4-MP	(4)	100	100	108
Ethanol + 4-MP	(5)	100	84	86
Acetaldehyde + 4-MP	(6)	130	90	90

Number of studies indicated by ( ).

Ethanol 200 mg%.

Acetaldehyde 2 mg%.

4-MP = 4-methylpyrazole 1.5 mM.

Fasted control values for albumin and urea synthesis averaged 11 and 50 mg/100 g wet liver wt/hr respectively; the average degree of bound polysome aggregation was 49%.

Discussion

Acute and chronic toxicity from ethanol consumption is obviously a major health hazard throughout the world, and in order to understand the effects of ethanol intake on various aspects of intermediate metabolism, it will be necessary to focus on specific points. The long-term exposure of the whole animal to ethanol intake, even if dietary management is carefully regulated, is still a highly complex picture. Many factors, including nutrition and alcohol, hormone levels, and blood flow, as well as protein degradation, immunological changes, and genetic predisposition, may affect the end parameter that one is trying to assay (14). It is obvious that experimental models—whether they be isolated perfused livers from a variety of species or hepatocyte cell cultures—differ from each other and also differ from the in vivo situation. But it is only through systems such as these that we will be able to uncover the specific effects of the metabolism of ethanol.

During the metabolism of ethanol, there is disaggregation of the endoplasmic membrane-bound polysome, a decreased rate of synthesis of serum albumin, and a decreased rate of incorporation of other labeled amino acids into proteins for export (15,16,17,18,19). The synthesis in urea is markedly inhibited, although the mechanism of this action is not known. Krebs and his associates have indicated that the metabolism of ethanol diverts ammonia from alanine (or from added ammonia) from the synthesis of urea to the synthesis of aspartate, glutamate, and glutamine and have shown that excess ornithine may abolish the accumulation of aspartate (20). Any inhibition at any step in the urea cycle will obviously decrease the rate of synthesis of urea; hence this would be one explanation.

Further, the data presented in this paper indicate that spermine plays an important role in the protein synthetic mechanism; thus, a decrease in the availability of ornithine, a precursor to the synthesis of the polyamines, would decrease the synthesis of spermine. The polyamines have been shown to play important roles in many aspects of protein synthesis and cellular regeneration (20,21,22,23,24,25,26,27,28). Obviously, there must be many other possible explanations for the effects of ethanol on urea and/or on albumin synthesis; this is simply one hypothesis.

The acute effects of ethanol on the liver's ability to synthesize albumin depend on the nutritional state of the liver. In livers from fed donors, ethanol decreases both albumin and urea as well as the synthesis of other proteins for export. These effects

on urea and albumin synthesis can be reversed by some, but not all, amino acids administered to the liver in 10 mM quantities. Furthermore, the acute effects of ethanol can also be reversed simply by transferring the liver to a perfusate that no longer contains ethanol. These observations certainly indicate that the acute effects of ethanol are transient, and that, although the mechanism of action of excessive amino acids can only be speculated upon at present, permanent damage to the protein-synthesizing system for proteins for export is not caused by exposure of the liver to even these high levels of ethanol.

However, when the liver is derived from a fasted donor, the effects of the combined stresses of fasting and ethanol are much more severe. The addition of excess amino acids, which were capable of reversing the effects of ethanol in livers from fed donors, is no longer effective. The endoplasmic membrane-bound polysomes are disaggregated, and the free polysomes are also disaggregated. It is quite possible that these free polysomes are responsible for the synthesis of the pre- and pro-peptide portions of the albumin molecule, a peptide thought to play a role in the signal hypothesis. This hypothesis suggests that the pre- and pro-peptide that initiate the attachment of the ribosome to the endoplasmic membrane to provide a means of egress for a protein destined for export (29,30,31,32,33,34). Spermine and arginine are likewise ineffective in completely reversing these effects, although spermine does result in some reaggregation of the bound polysome. Spermine plus arginine in livers from fasted donors exposed to ethanol does, however, result in significant improvement of the rates of albumin production, again pointing up the importance of these two substances in the schema of protein synthesis.

Not only are the effects of ethanol dependent on the nutritional status of the liver; acetaldehyde shows an even greater nutritional dependence. Acetaldehyde per se inhibits albumin synthesis in livers from fed donors, but it does not have any effect in livers from fasted donors in terms of reducing albumin synthesis. Further, 4-methylpyrazole does not reverse the alcohol-induced inhibition of albumin synthesis in livers from fed donors, but it does reverse the effect in livers from fasted donors. These observations certainly point up the importance of a clear definition of the nutritional status of the liver, if effects of ethanol and acetaldehyde are to be understood and clearly elucidated.

Another question raised by these particular studies is the role of acetaldehyde in mediating the inhibitory effects that ethanol produces in albumin and urea synthesis. Acetaldehyde reduced

albumin and urea synthesis in livers from fed donors, but it failed to cause the endoplasmic membrane-bound polysome disaggregation seen with ethanol. Further, in livers from fasted donors, acetaldehyde had no effect in lowering albumin and urea synthesis below the levels seen with fasting per se; ethanol was even more toxic in these livers. These results certainly do not support the concept that acetaldehyde is the direct mediator of the toxic effects of ethanol.

The data with 4-methylpyrazole inhibition of ethanol oxidation likewise provide evidence favoring a separate mechanism of action for ethanol and for acetaldehyde. Using livers from fed donors, 4-MP inhibition of ethanol oxidation prevented endoplasmic membrane-bound polysome disaggregation, but albumin and urea synthesis remained depressed. This same result was also found with acetaldehyde infusions. However, with livers from fasted donors, 4-MP decreased the effects of ethanol, a finding that might support the conclusion that acetaldehyde was responsible for ethanol toxicity in this model—except that, in the fasted state, acetaldehyde per se was without toxicity. These studies thus provide no evidence to support the concept that acetaldehyde per se mediates the acute toxic effects of ethanol on albumin synthesis and on urea synthesis. The metabolic interconversion from ethanol through acetaldehyde to acetate involves a variety of mitochondrial and cytosol energy and electron transport changes. It is quite conceivable that these intermediate metabolic consequences of the liver's ability to metabolize ethanol preferentially are responsible, in some fashion, for the alterations in the parameters noted.

These acute studies should in no way be confused with the long-range effects of ethanol consumption on the development of hepatic disease. Cirrhosis of the liver is a highly complex hemodynamic disorder, characterized not only by altered protein production for export, but also by increased collagen synthesis (35,36,37,38,39); by possible changes in total hepatic cellular nitrogen, which may actually increase; by the development of markedly distorted intravascular channels; by portal hypertension; and by the disastrous consequences of all these results. However, first steps must be made, and effective models must be chosen to evaluate, as carefully as possible, the specific effects of any one toxic or metabolic agent.

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Section V:
Alcohol Metabolism: Including the Effects of Chronic Alcohol Ingestion and Nutritional States

Rate-Determining Factors for Ethanol Oxidation in Vivo and in Isolated Hepatocytes

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and Richard L. Veech

Abstract

A literature survey indicates that the rate of ethanol metabolism in rats in vivo is about 3 μmol/min/g liver,* and similar rates are observed when isolated hepatocytes from fed or 48-hr-starved rats are incubated with substrates such as lactate and pyruvate. In the absence of substrates, however, hepatocytes from starved rats oxidize only 0.75 μmol of ethanol/min/g, and with cells from fed rats the rate is 1.9 μmol/min/g. Glucose production can be blocked by tryptophan or quinolinate without affecting the substrate-stimulated ethanol oxidation, so the latter does not depend on an increased ATP utilization for glucose synthesis. Metabolite measurements indicate that substrates return the rate of ethanol metabolism to that seen in vivo by causing a restoration of the malate-aspartate shuttle intermediates depleted during cell preparation. It is emphasized that, although the malate-aspartate shuttle may be rate determining for ethanol metabolism by hepatocytes under some conditions, this is not the case in vivo. Alcohol dehydrogenase (ADH) is present in rat liver at 1.5 times the activity required to account for the rate of ethanol metabolism in vivo, suggesting that the level of ADH could be a major rate-determining factor. To test this suggestion, we conducted a kinetic characterization of rat liver ADH and found that ethanol oxidation via this enzyme is sufficient to account for observed rates of elimination in rats in vivo. Some implications of our results for ethanol metabolism in humans are discussed.

It is generally agreed that alcohol dehydrogenase (ADH) is the major enzyme catalyzing ethanol oxidation in mammalian liver, but some uncertainty remains concerning the cellular factors that determine the rate of ethanol metabolism in vivo. One proposal is

*Wet weight.

that ethanol oxidation is ultimately limited by the rate of NADH reoxidation in the mitochondrial respiratory chain (26,36). This proposal is based, in part, on the observation that substrates for glucose synthesis accelerate ethanol oxidation, and the acceleration is thought to result from the increased ATP utilization for glucose production. Alternatively, it has been suggested that ethanol oxidation, particularly in the fasted state, is limited by the activity of the shuttles that transfer reducing equivalents from the cytosol into mitochondria (36). It is implicit in both of these proposals that the rate of ethanol oxidation is limited by level of cytosolic NADH and, therefore, by the rate of the ADH reaction. However, largely because of poor correlations between ADH levels measured in vitro and rates of ethanol metabolism in vivo, it has often been stated (20,44,52) that the level of ADH activity is not a major rate-determining factor. This report summarizes experiments in which we have examined the roles of ATP turnover, the malate-aspartate shuttle, and the level of ADH activity in determining the rate of ethanol metabolism in the rat.

Ethanol Metabolism in Isolated Hepatocytes

Previous experience with isolated hepatocytes has shown that these cells, when well prepared, retain the metabolic capacities of the intact liver (see, e.g., 25). To have some idea of the rate to be expected with isolated hepatocytes, we compiled 20 values for the rate of ethanol metabolism in vivo (table 1). Two of those values are from separate studies in our laboratory, and the remainder are published values from other laboratories. We take the mean of all 20 values, 3.3 μmol/min/g liver, to indicate the rate of ethanol metabolism in vivo. However, as seen in table 2 and elsewhere (9,26,36), when hepatocytes prepared from fasted rats were incubated without other substrates, the rate of ethanol metabolism was only 0.7 to 0.8 μmol/min/g wet weight of cells. The addition of lactate to these incubations increased the rate 2-1/2 times, and pyruvate restored the rate to that seen in vivo.

To test the suggestion that lactate or pyruvate stimulates ethanol oxidation by creating an ATP demand for gluconeogenesis, we conducted experiments with quinolinate and tryptophan. These compounds block gluconeogenesis in the rat by inhibiting phosphoenolpyruvate carboxykinase, thereby preventing two-thirds of the ATP utilization required in the synthesis of glucose from lactate or pyruvate (43,48). In our experiments (table 2), quinolinate and tryptophan both effectively decreased glucose

Table 1. Rates of Ethanol Metabolism in Rats in Vivo

Reference	Sex	Strain	Dietary State	Rate (μmol/min/g liver)	±SEM
41	Female	Sprague-Dawley	Fed	3.33	
34			Fed	4.21	
39			Fed	3.25	
46			Fed	3.94	
24			No information	2.25	
16		Wistar	Fed	3.87	
					3.48 ± 0.29
32	Male	Sprague-Dawley	Fed	3.1	
31			Fed	3.15	
27			Fed	2.91	
1			Starved 18 hr	2.65	
38			Starved 24 hr	3.25	
47			Starved 48 hr	4.	
16		Wistar	Fed	3.23	
19			Fed	3.6	
21			Fed	3.37	
13			Fed	3.6	
23			Starved 17 hr	3.7	
49			Starved 16-18 hr	3.12	
17			Starved 24 hr	2.4	
35		Wistar x Piebald	Starved 12 hr	2.91	
					3.21 ± 0.11
					3.29 ± 0.12

synthesis, but neither inhibitor had any effect on the lactate- or pyruvate-stimulated rate of ethanol metabolism.

We obtained equivalent results with hepatocytes prepared from fed rats (table 3). In these cells, also, lactate stimulated ethanol oxidation and increased glucose production; quinolinate blocked the lactate-stimulated glucose production but had no effect on the rate of ethanol oxidation. These results indicate that the acceleration by lactate of ethanol oxidation does not depend on an increased ATP demand for glucose synthesis.

The data in tables 2 and 3 are in contrast to those of Meijer et al. (36), who reported that quinolinate decreased the lactate-stimulated rate of ethanol oxidation. We are unable to explain this discrepancy. However, the conclusion drawn from our quinolinate and tryptophan data is supported by results of experiments in which ethanol oxidation and glucose synthesis were measured at various lactate concentrations (figure 1). The major stimulation

Table 2. Effects of Substrates and of Quinolinate or Tryptophan on Glucose Synthesis and Ethanol Oxidation in Hepatocytes From Starved Rats

Additions	Rates (μmol/min per g wet wt of cells)		
	Ethanol Oxidation	Glucose Synthesis	
		−Ethanol	+Ethanol
None	0.75±0.09	0.07±0.004	0.02±0.002
Lactate	1.86±0.07	0.59±0.03	0.39±0.03
Lactate, quinolinate	1.83±0.09	0.16±0.02	0.04±0.003
Lactate, tryptophan	1.88±0.07	0.21±0.03	0.08±0.02
Quinolinate	0.93±0.07	—	0.02±0.002
Tryptophan	1.01±0.10	—	0.02±0.005
Pyruvate	2.78±0.14	0.61±0.03	0.83±0.03
Pyruvate, quinolinate	2.76±0.08	0.35±0.02	0.41±0.01
Pyruvate, tryptophan	2.52±0.18	0.34±0.03	0.47±0.03

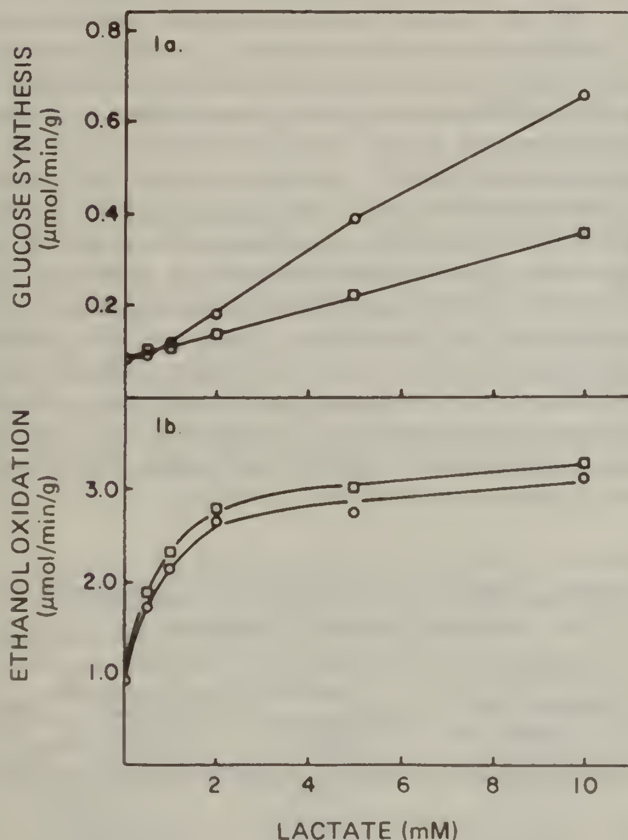
Cells, prepared from 48-hr starved, male Wistar rats, were incubated for 60 min. Initial concentrations of substrates and inhibitors were lactate, 10 mM; pyruvate, 5 mM; tryptophan, 1 mM; quinolinate, 5 mM; ethanol, 8 mM, where rates of ethanol oxidation or glucose synthesis in the presence of ethanol were measured. Rates are means ±SEM for 3–6 cell preparations.

Table 3. Ethanol Oxidation and Glucose Production in Hepatocytes From Fed Rats

Additions	Rates (μmol/min per g wet wt of cells)	
	Glucose Production	Ethanol Oxidation
None	1.47±0.09	—
Ethanol	1.86±0.13	1.89±0.12
Ethanol, quinolinate	1.84±0.21	1.79±0.28
Ethanol, lactate	2.06±0.21	2.53±0.23
Ethanol, lactate, quinolinate	1.72±0.20	2.37±0.20

Cells were prepared from rats fed ad libitum on standard chow. Other conditions are as listed in Table 2.

Figure 1. The Effect of Varying Lactate Concentration on Rates of Glucose Synthesis and Ethanol Oxidation



Cells from 48-hour starved rats were incubated for 20 minutes. Controls (O) contained 8 mM ethanol, lactate at the concentrations indicated, and pyruvate at 0.1 of the lactate concentration. Other incubations contained, in addition, 5 mM quinolinate (□). Each point represents the mean of determinations on at least 3 different cell preparations.

in ethanol oxidation occurred at low lactate concentrations (0.5 mM and 1.0 mM) where, because of the presence of ethanol, glucose synthesis was not stimulated at all. At higher lactate levels (2.0 mM to 10.0 mM), where rates of glucose synthesis increased with increasing lactate concentration, ethanol oxidation rates remained almost constant. The separation between the two processes was even more striking when quinolinate was present (figure 1). As with glucose synthesis, oxygen uptake continued to increase at higher lactate levels (table 4), indicating that an increased mitochondrial rate of reoxidation of NADH did accompany the increasing ATP utilization for gluconeogenesis. There was no concomitant increase in the rate of ethanol oxidation, which again indicates that NADH reoxidation via the mitochondrial electron transport chain is not a limiting factor for ethanol metabolism in isolated hepatocytes. In this regard, it should be noted that, from the stoichiometries of ethanol oxidation, oxidative phosphorylation, and glucose synthesis, the maximum increase in ethanol oxidation that could be caused by ATP utilization for gluconeogenesis would be 1 mole of ethanol oxidized per mole of glucose produced. On comparing increments due to the presence of glucose precursors (table 2 and figure 1), it can be seen that the observed increment in ethanol oxidation is as much as 50 times the increment seen in glucose synthesis.

Table 4. Effects of Substrates and Ethanol on Oxygen Uptake by Hepatocytes

Lactate Added (mM)	Relative Rates of O_2 Uptake	
	−Ethanol	+8 mM Ethanol
0	1.00	1.00
0.5	1.34±0.05	1.18±0.05
1.0	1.43±0.07	1.31±0.02
2.0	1.44±0.07	1.41±0.03
5.0	1.42±0.07	1.58±0.03
10.0	1.42±0.07	1.71±0.03

Oxygen uptake was measured with a YSI model 53 oxygen monitor, and the rate in the absence of added substrates was assigned a value of 1.0. Calibration (3) of the monitor indicated that the absolute rate in the absence of substrates was 2.3–2.5 μmol O_2/min per g wet wt of cells. Addition of 8 mM ethanol alone had no effect on the rate. Pyruvate was added to all incubations to give an initial [lactate]/[pyruvate] = 10. Rates are given as means ±SEM for four experiments.

Role of the Malate-Aspartate Shuttle

It is well known that some components of the malate-aspartate shuttle are depleted during hepatocyte preparation (table 5 and Ref. 8). These metabolite losses are restored following the addition of appropriate substrates, and the metabolism of isolated hepatocytes becomes more like that of the intact liver (8). The results in table 5 and figures 1 and 2 show that the effects of substrates on ethanol oxidation are closely paralleled by increases in α-ketoglutarate and, especially, glutamate. These data suggest that lactate accelerates ethanol oxidation in isolated hepatocytes by increasing the rate at which reducing equivalents are transported into the mitochondria via the malate-aspartate shuttle. Exchange of cytosolic glutamate for mitochondrial aspartate is an essential process in the operation of this shuttle (2,45). The exchange is catalyzed by a specific carrier, for which a glutamate K_m of about 6mM has been determined with isolated mitochondria (45). In our experiments, total cellular levels of glutamate ranged from 1.3 μmol/g wet wt to 4.0 μmol/g wet wt. (table 5, figure 2). Assuming uniform distribution of glutamate throughout the cell, these levels of glutamate would correspond to cytosolic concentrations of 2mM to 5mM. These levels are

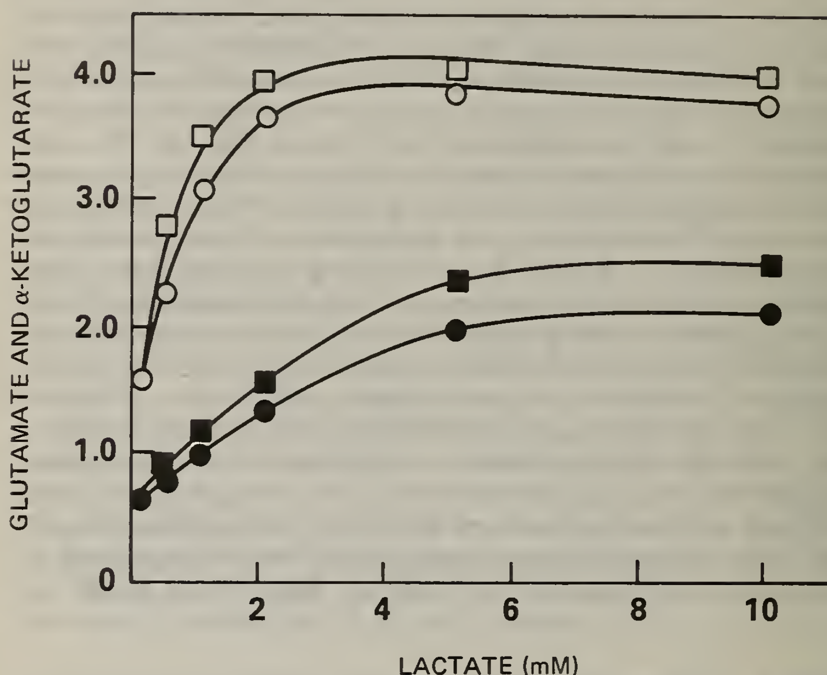
Table 5. Content of Malate-Aspartate Shuttle Components in Freeze-Clamped Rat Liver and in Isolated Hepatocytes

Dietary State	Shuttle Component	Content[a] (μmol/g wet wt)		
		Freeze-Clamped Liver	Hepatocytes	
			Fresh	Incubated[b]
Fed				
	Glutamate	3.21±0.10	0.84±0.14	3.76±0.42
	α-Ketoglutarate	0.28±0.01	0.16±0.02	1.40±0.09
	Aspartate	0.55±0.04	0.63±0.09	0.21±0.03
	Malate	0.29±0.02	0.12±0.02	1.45±0.11
Starved 48 hr				
	Glutamate	2.32±0.16	0.36±0.04	3.80±0.11
	α-Ketoglutarate	0.06±0.006	0.09±0.03	2.05±0.06
	Aspartate	0.60±0.05	0.08±0.01	0.19±0.03
	Malate	0.27±0.04	0.04±0.01	1.72±0.14

[a]Values are means ±SEM for 9 freeze-clamped livers or 6 cell preparations from male Wistar rats.

[b]Hepatocytes were incubated 20 min with 10 mM lactate, 1 mM pyruvate, and 10 mM ethanol; see also figures 1 and 2.

Figure 2. The Effect of Varying Lactate Concentration on Glutamate and α-Ketoglutarate Contents



Incubation conditions and substrate concentrations were as listed for figure 1. (○, ●) were control incubations, and (□, ■) contained quinolinate. Glutamate is indicated by (○, □) and α-ketoglutarate by (●, ■).

below the glutamate K_m for the glutamate-aspartate carrier, so it is possible that the rate of operation of the malate-aspartate shuttle and the resultant level of free cytosolic NADH are dependent on the glutamate concentration. This would explain the particularly close correlation between glutamate concentration and rates of ethanol oxidation with varying lactate levels.

We have also observed that lysine, in the presence of lactate, will increase the rate of ethanol oxidation. It was previously reported that lysine accelerates the recovery of cellular glutamate following hepatocyte preparation, thereby stimulating glucose synthesis from 10 mM lactate and reversing the ethanol-induced inhibition of gluconeogenesis (8). In the present experiments, the addition of 2 mM lysine to hepatocyte incubations containing 10 mM lactate and 8 mM ethanol increased the rate of ethanol oxidation from 1.9 to 2.5 μmol/min/g wet wt. This observation

supports the conclusion that the stimulatory effect of substrates on ethanol oxidation results from increased activity of the malate-aspartate shuttle.

On relating our results or those from other studies with isolated cells to ethanol metabolism in vivo, it is important to note that a variety of conditions or substrate combinations will accelerate ethanol oxidation in hepatocytes; but, even in the presence of accelerators, the highest rates are similar to those in vivo, i.e., 2.6 μmol/min/g to 3.3 μmol/min/g (see, for example, tables 1, 2, and 3, figure 1). Only at that point—where the hepatocyte rate is similar to the rate in vivo—does it become possible to draw inferences about factors that are rate determining in vivo. We know of no experimental condition, either from our studies or from others, that will cause rates of ethanol metabolism in hepatocytes to be greater than those in vivo, suggesting that, when maximally stimulated, the rate of ethanol oxidation by hepatocytes is determined by the same factors operating in vivo. From hepatocyte experiments showing high rates of both gluconeogenesis and urea synthesis (25), it can be estimated that the rate of the malate-aspartate shuttle can be at least as high as 8 μmol/min/g to 10 μmol/min/g cells, or about 3 times the rate of ethanol oxidation in vivo. Moreover, although starvation does not greatly affect hepatic contents of glutamate, aspartate, or malate, hepatocyte preparation causes these to be drastically depleted (table 5). Thus, although the activity of the malate-aspartate shuttle appears to be rate limiting for hepatocytes in some instances, that is probably not the case in vivo where extensive depletion of shuttle metabolites does not normally occur and where, as discussed below, rates of ethanol metabolism are as fast as the level of ADH will allow with the prevailing substrate concentrations.

The Role of Alcohol Dehydrogenase in Vivo

It has been reported previously that the content of ADH in rat liver is either much greater (52) or less (28,29,30,37) than the amount required to account for ethanol metabolism in vivo. In contrast (table 6), we find that the activity is sufficient to catalyze ethanol oxidation at a rate of 5 μmol/min/g liver or only 1.5 times the rate in vivo (table 1). The rat liver enzyme appears to be more labile than other ADH's, and recovery of full activity in homogenates requires the presence of a sulfhydryl reagent such as dithiothreitol (10,33). The view that hepatic ADH activity is excessive may stem partly from a reported activity of

Table 6. Alcohol Dehydrogenase Activity in Rat Liver Homogenates

A. Forward reaction
Ethanol + NAD^+ → Acetaldehyde + NADH + H^+
Activity: 5.05 ± 0.21 (n = 26)

B. Reverse reaction
Acetaldehyde + NADH + H^+ → Ethanol + NAD^+
Activity: 25.5 ± 3.0 (n = 9)

C. Ratio of B/A: 5.02 ± 0.35 (n = 9)

Alcohol dehydrogenase activity is given in units of μmol of substrate converted/min/g wet wt of liver.

150 μmol/min/g, which, however, was determined with lactaldehyde, an artificial accelerator of the reaction (42). Another source of this view could be the frequent measurement of ADH activity in the direction of acetaldehyde → ethanol, for which the rate is 5 times greater than in the direction of ethanol oxidation (table 6).

The similarity between our measured levels of ADH activity and the average rate of ethanol elimination in vivo suggested to us that ADH could be an important rate-determining factor (9,10), and Plapp (40) has recently reviewed evidence that led him to make the same suggestion. In order to test this suggestion, we conducted a kinetic characterization of rat liver ADH that, like other ADH's (54), shows the pattern of product inhibition indicative of an ordered bi-bi reaction. The rate equation describing this reaction mechanism is shown in table 7. Table 8 contains our values for the equilibrium constant and the eight kinetic constants determined under physiological conditions of ionic strength, pH, and temperature (7). In addition to these constants, solutions to the rate equation require values for the cellular [NAD^+],* [NADH], and [acetaldehyde] when the liver is presented with a specified [ethanol]. We have previously shown that when isolated hepatocytes are metabolizing ethanol at rates such as those seen in vivo, [acetaldehyde] is about 1 μM (9), and we assume that a similar concentration is characteristic of the liver in vivo. Bücher (4) has calculated that free cytosolic [NAD^+] in rat liver is 0.5 mM; and, with that value, the free cytosolic [NADH] can be obtained from lactate and pyruvate measurements and the equilibrium expression for the lactate dehydrogenase reaction (53).

*Brackets denote concentrations.

Table 7. Steady-State Rate Equation for an Ordered Bi-Bi Reaction

$$v = \frac{V_f V_r (AB - PQ/K_{eq})}{V_r K_{ia} K_b + V_r K_b A + V_r K_a B + V_r AB + \frac{V_f K_q P}{K_{eq}} + \frac{V_f K_p P}{K_{eq}} + \frac{V_f K_q AP}{K_{eq} K_{ia}} + \frac{V_f PQ}{K_{eq}} + \frac{V_r K_a BQ}{K_{iq}} + \frac{V_r ABP}{K_{ip}} + \frac{V_f BPQ}{K_{ib} K_{eq}}}$$

Definitions:

V_f and V_r are, respectively, the maximal velocities in the forward and reverse directions. A = [NAD^+]. B = [Ethanol]. P = [Acetaldehyde]. Q = [NADH]. K's are limiting Michaelis constants and K_i's are dissociation or inhibition constants. K_{eq} is the equilibrium constant. For discussions of these definitions and the mechanism of the alcohol dehydrogenase reaction, see references (5, 54).

Table 8. Equilibrium Constant and Kinetic Constants for Horse and Rat Liver Alcohol Dehydrogenase

Kinetic Constants	Value (mM) for Enzyme From	
	Horse Liver[a]	Rat Liver[b]
K_a	0.017	0.150 ± 0.019
K_{ia}	0.268	0.265 ± 0.007
K_b	0.55	1.07 ± 0.075
K_{ib}	19.4	20.3 ± 3.5
K_p	0.24	0.049 ± 0.007
K_{ip}	0.087	0.134 ± 0.007
K_q	0.027	0.0053 ± 0.0019
K_{iq}	0.015	0.0023 ± 0.0009
Equilibrium constant (K_{eq})[c]	1.94×10^{-4}	

[a] Values from reference (54), determined at pH 7.15, 25°.
[b] Determined at pH 7.3, 38°, I = 0.25.
[c] Determined with crystalline yeast alcohol dehydrogenase at 38°, pH 7, and I = 0.25. Value given is for pH = 7.0.

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In this way, we have obtained solutions to the rate equation at various $[NAD^+]/[NADH]$ ratios and acetaldehyde concentrations. In the range of 1 μM to 10 μM acetaldehyde and cytosolic free $[NAD^+]/[NADH]$ = 200 to 400 (values commonly found when the liver is metabolizing ethanol), it can be seen (table 9) that the rate of ethanol oxidation in the ADH reaction is very close to the average rate of ethanol metabolism in vivo. This result supports our suggestion that ADH is a major rate-determining factor for ethanol oxidation, and it also provides kinetic confirmation of the value for free cytosolic $[NAD^+]$ that Bücher (4) calculated from near-equilibrium considerations.

In this analysis we have given no consideration to the possible contribution of non-ADH systems; but, for two reasons, we believe that ethanol oxidation by those systems is less than 10 percent of the total. First, with isolated hepatocytes incubated under conditions where the control rates are similar to those in vivo, ethanol oxidation is inhibited by 92 to 95 percent by 4-methylpyrazole (9) or 4-pentylpyrazole (6). Second, isotope studies with liver slices from monkeys and from naive or ethanol-treated rats also indicate that 90 percent or more of ethanol oxidation occurs via ADH (18).

As a test of our analysis, we have calculated the rate of ethanol oxidation predicted from the kinetics of ADH and compared this value with rates in vivo measured in studies where, at minimum, [lactate]/[pyruvate] ratios and [ethanol] were also available. For the first six studies shown in table 10, the average difference between the predicted and measured rates is ±15 percent, which is remarkably good considering that those studies were done in

Table 9. Effect of [Acetaldehyde] and $[NAD^+]/[NADH]$ on Calculated Alcohol Dehydrogenase Activity

Acetaldehyde (mM)	Cytosolic $[NAD^+]/[NADH]$				
	500	400	300	200	100
	(rates of ethanol oxidation μmol/min/g liver)				
0.001	3.58	3.50	3.38	3.16	2.64
0.005	3.47	3.39	3.27	3.05	2.52
0.010	3.34	3.26	3.14	2.91	2.39
0.020	3.10	3.02	2.90	2.67	2.14
0.050	2.53	2.45	2.32	2.09	1.52

Calculations were made with free cytosolic $[NAD^+]$ = 0.5 mM and [ethanol] = 10 mM. For comparison, the average rate of ethanol metabolism in vivo = 3.3 μmol/min/g liver.

Table 10. Comparison of Rates of Ethanol Metabolism Measured In Vivo With Those Predicted From the Kinetics of ADH

Reference	Condition	Concentration (mM)			Rates (μmol/min/g)		
		Ethanol	Acetaldehyde	$\frac{[NAD^+]}{[NADH]}$	Predicted	Measured	$\frac{Predicted}{Measured}$
19	Control	10		405	3.51	3.59	0.98
	Clofibrate	10		366	3.47	3.71	0.94
17	1.7 g/kg	24.1		139	3.10	2.28	1.36
	3.0 g/kg	51.3		200	3.47	2.49	1.39
13	Control	22	0.015	192	3.09	3.6	0.85
	Nicotinamide	22	0.010	164	3.49	3.6	0.97
47	5 min	9.0		132	2.83		
	10 min	7.6		223	3.12	4.0	0.75
	15 min	6.7		285	3.18		
	30 min	4.5		211	2.81		
41	Control	7		299	3.23	3.33	0.97
	Clofibrate (14 days)	7		329	3.27	3.43	0.95
39	Control	6		500	3.33	3.33	1.00
	Aminooxyacetate	10		122	2.81	1.44	1.55
12	ANA	29.5	0.217	230	1.32(3.50)	3.58	(0.98)
	AA	28.7	0.117	230	1.93(3.49)	3.42	(1.02)
14	rr	15	0.173	196	1.12(3.27)	3.43	(0.95)
	RR	15	0.136	83	0.65(2.56)	3.17	(0.81)

Predicted rates are calculated from the rate equation in table 7, constants in table 8 with free cytosolic $[NAD^+]$ = 0.5 mM and [acetaldehyde] = 1 μM in those instances where no value was reported. For the nicotinamide group, free cytosolic $[NAD^+]$ was taken as 2.0 mM.

five different laboratories. For the last two studies, the agreement is poor because of the high acetaldehyde values—which are close to the levels expected at equilibrium. These two studies were done in a laboratory that has more recently (15) emphasized the problems of binding and artefactual production of acetaldehyde in rat tissues. If we use their data (12,14) to calculate a predicted rate of ethanol metabolism, but assume that most of the reported acetaldehyde is bound or otherwise not accessible to ADH, the agreement with the measured rate becomes very close.

A second test of the predictive value of ADH kinetics was made in our collaborative study with Kulkosky (see Discussion), where it was found that ethanol drinking could be predicted with a correlation coefficient = 0.92. A third test is suggested by the observations of Bosron and Li (see Discussion) that, when expressed on the basis of total liver weight, the content of ADH is decreased by starvation. After hearing these observations we confirmed them, and we estimate that ethanol oxidation per g body wt in starved rats should be 30 to 40 percent lower than in fed rats.

Implications for Ethanol Metabolism in Humans

Our studies have been concerned with ethanol metabolism in the rat, and hepatic ADH is unusual in this organism in that it appears to have no isozymes (22). In contrast, there are multiple isozymes of human liver ADH, plus an atypical form that possesses unique kinetic properties (50,51). When ADH activity is measured in biopsied or autopsied liver samples from individuals with the atypical enzyme, V_{max} for ethanol oxidation is 3 to 5 times greater than with samples from normal individuals. One argument raised against the conclusion that the level of ADH is rate determining in vivo is that the rate of ethanol elimination is not increased in people with the atypical enzyme. It should be noted, however, that the K_m's for ethanol and NAD^+ are also 3 times higher for the atypical enzyme (50). If there are no other differences, a kinetic analysis such as we have conducted for rat liver ADH indicates that, when V_{max} and the two K_m's are 3 times normal, ethanol oxidation via ADH would be about 30 percent faster. In two out of three instances (17,51) where ethanol elimination in vivo has been measured for individuals with the atypical enzyme, the rate was found to be 30 to 50 percent greater than for individuals with normal ADH. Thus, although it would be very difficult for human ADH, we take as a working hypothesis that a complete kinetic analysis would show that ethanol elimination

in humans could, as with the rat, be explained by the activity of ADH and no other considerations.

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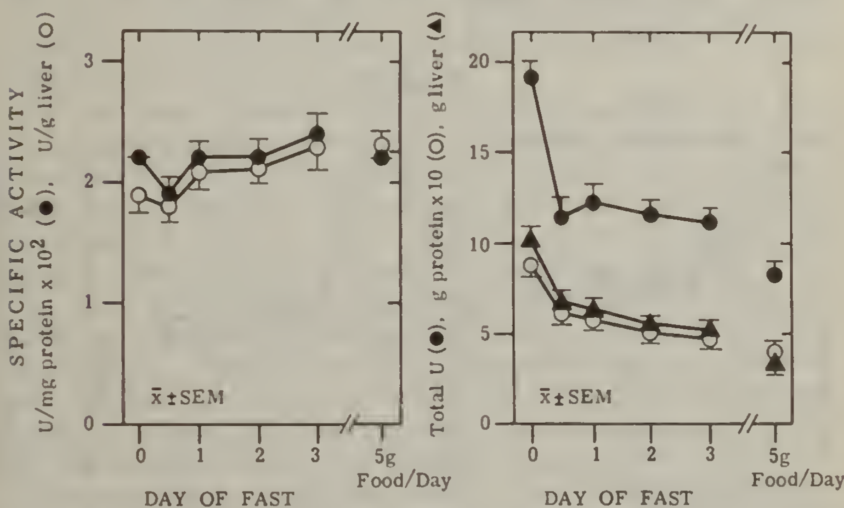
Discussion of Paper by Cornell et al.

Dr. Bosron: One factor that we think may be particularly pertinent to what Dr. Cornell has discussed is the effect of the nutritional state in the rat on alcohol dehydrogenase activity. We have examined alcohol dehydrogenase activity measured by the methods of Crow, Cornell, and Veech, except that it was done at 25°. In figure 1 (panel on the left), we looked at specific activity expressed in terms of u/mg of cytosolic protein and u/g of liver, as a function of the day of fast as well with a calorically restricted diet. The specific activity was relatively constant.

However, if one looks at the total activity of alcohol dehydrogenase in rat livers, that is, the solid circles (panel on the right), one can see that within about one-half day of fast, the total activity dropped about 40 percent. Note also that the total cytosolic protein and the grams of liver also dropped similarly, thereby accounting for the constancy in specific activity on the left.

Figure 1



In figure 2, we looked at the content of DNA in rat liver, also as a function of nutritional state; and, as you can see on the left, it remained relatively constant. Hence, we believed that a reasonable way to express alcohol dehydrogenase activity is in terms of specific activities expressed as u/mg of DNA, the open circles—or as total activity, the closed circles. If one does it in this fashion, alcohol dehydrogenase activity is clearly affected by the nutritional state of the rat, as much as 50 percent.

Dr. Kulkosky: I would like to present a brief description of the results of an effort at a rat model of alcoholism; these results appear compatible with a limiting role for liver alcohol dehydrogenase activity in the regulation of ethanol intake. We attempted to induce excessive ethanol intake in the rat through a polydipsia technique. Rats were given free access to rat chow, water, and a 0.125-percent saccharin + 3.0-percent glucose (+1.0-percent NaCl) solution (which rodents are known to consume daily in rather large amounts) (1). After establishment of large daily solution intakes, ethanol was gradually added to the saccharin + glucose (+NaCl) solutions, from 0.5 to 10.0 percent w/v. Very large mean intakes of ethanol resulted, as depicted in figure 3.

Ethanol intake at first increases with ethanol concentration of the solution but then becomes relatively constant. With each additional increase in ethanol concentration, the rats decreased their solution intakes proportionally and increased their water intakes, such that a relatively constant ethanol intake resulted,

Figure 2

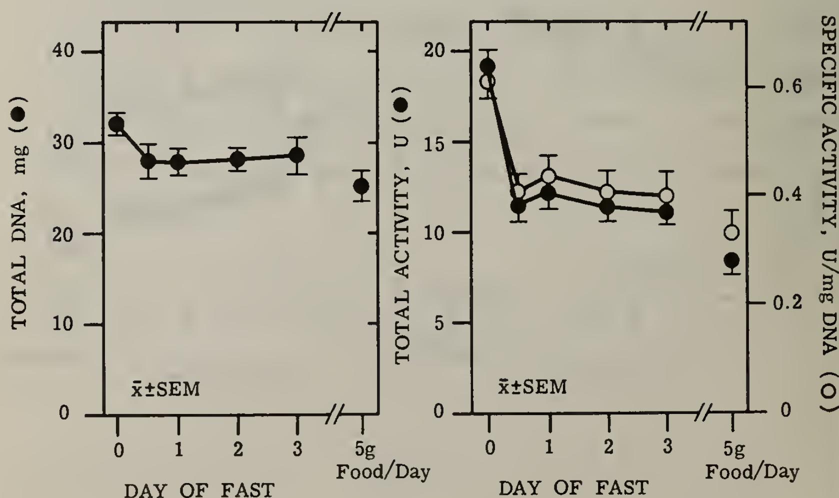
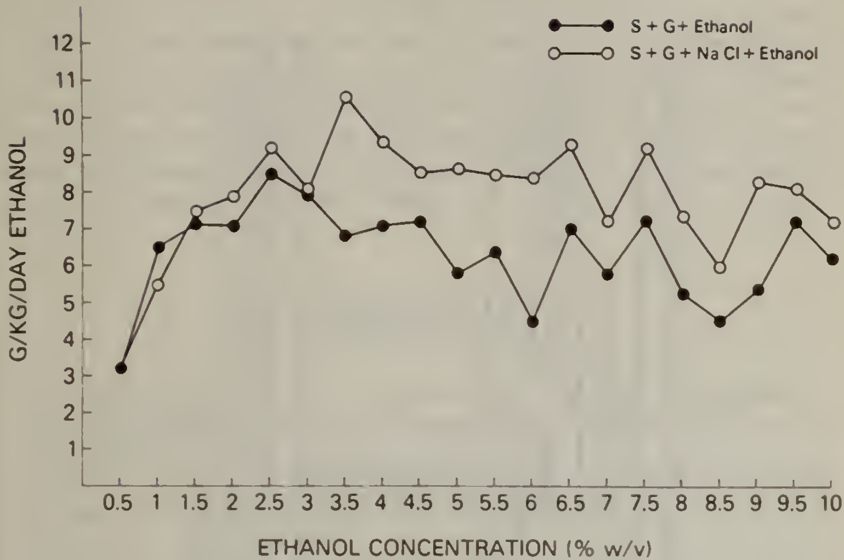


Figure 3



Mean g/k of body wt/d intakes of absolute ethanol of rats receiving saccharin + glucose + ethanol (solid circles) or saccharin + glucose + NaCl + ethanol (open circles) as a function of ethanol concentration (in % w/v).

at a mean of approximately 7 to 8 g/kg/d. The model was not successful in producing physical dependence on ethanol; no gross withdrawal symptoms resulted (2).

At that time, I became aware of the determination by Drs. Cornell, Crow, and Veech of ethanol metabolic rates in vitro and in isolated hepatocytes (3). Using the same strain of rats, they had obtained a measure of mean ethanol metabolic capacity of approximately 8 g/kg/d, a value quite close to the mean maximal intake we were able to induce with our polydipsia technique.

In order to test the postulate that the limitation of ethanol intake was related to the maximal daily ethanol metabolic rate, we employed the polydipsia technique in another group of rats and then sacrificed the animals. Drs. Cornell and Crow determined individual ethanol metabolic rates from liver homogenates in vitro. The results of this experiment are summarized in table 1.

The correlation of observed intakes and predicted metabolic rates was quite high, with a correlation coefficient of .93 ($p < .01$). Rats receiving ethanol had a 12.4-percent mean higher ethanol metabolic rate compared to rats drinking only the saccharin + glucose + NaCl solution. This rate was statistically nonsignificant.

Table 1. Comparison of Daily Ethanol Consumption With Predicted Metabolic Capacity

Rat	Ethanol Consumption (g/day)	Liver Wt (grams)	Rates (μmol/min/g liver)			
			V_{max} for ADH	Ethanol Consumption	Calculated Ethanol Metabolism	
					10 mM	5 mM
1	2.98	13.56	5.81	3.33	3.30	
3	2.54	14.65	4.90	2.62	2.76	
4	1.85	13.19	4.66	2.12	2.63	2.37
7	1.64	14.65	4.42	1.69	2.51	2.26
8	3.32	18.79	4.76	2.66	2.70	
11	3.25	15.74	5.35	3.12	3.04	

Male, inbred Wistar rats (360 to 460 g body wt) were kept for 1 month on a regime that optimizes alcohol consumption. A daily record of net ethanol consumption was kept. The calculated metabolic rates are based on the steady-state equation for an ordered bi-bi reaction, kinetic constants for rat liver ADH, free $[NAD^+] = 0.5$ mM, $[NAD^+]/[NADH] = 250$, and [acetaldehyde] = 1 μM.

In summary, the observed data indicate that this free-choice polydipsia technique does not successfully produce physical dependence on ethanol in the rat, in spite of high daily ethanol intakes. It appears that, because of the rat's intake regulation ability, gross intoxication and withdrawal are not produced. Rats do not exceed their daily ethanol metabolic capacity for a sustained period when they have free choice of ethanol solution, food, and water. However, the technique allows for examination of the effects of maximal volitional consumption of ethanol by rats.

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Multiple Molecular Forms of Human Liver Alcohol Dehydrogenase: Isolation and Properties of Π-ADH

William F. Bosron, Ting-Kai Li, Werner P. Dafeldecker, and Bert L. Vallee

It is generally accepted that the pharmacological, addictive, and pathological consequences of alcohol consumption are directly related to the chemical properties of ethanol and/or its metabolic products. Hence, knowledge about the enzymes responsible for its elimination is fundamental to our understanding of the etiology and underlying mechanisms of alcoholism. These enzymes have not been available in suitable purity and quantity from human tissue until recently, so such knowledge has had to be extrapolated from studies in other species, primarily the rat and horse. Moreover, a complex interrelationship, which has been difficult to unravel, exists between psychosocial and biological factors in alcoholism. Perhaps owing to these shortcomings, a biochemical basis for this disorder has remained obscure. Nevertheless, there has appeared, in recent years, increasingly convincing evidence indicating a genetic predisposition, not only for alcohol-metabolizing capacity, but also for both alcohol drinking behavior and alcoholism in some individuals. In this context, the study of the genetic variability of liver alcohol dehydrogenase (ADH) is of particular interest because it is the principal enzyme responsible for the oxidative metabolism of ethanol.

The initial attempts to isolate ADH from human liver by Vallee and coworkers by ion exchange chromatography indicated that there are multiple molecular forms of the enzyme (1). Subsequent work in several laboratories confirmed this observation and demonstrated by starch gel electrophoresis that as many as 6 to 10 ADH molecular forms are present in some liver homogenates (2,3, 4). Interestingly, the number and the amount of the individual molecular forms vary from liver to liver as do total and specific enzymatic activities. To account for this variability and multiplicity of ADH forms, a genetic model for their formation as isozymes was proposed by Smith et al. (3,5). However, because the

model was based entirely on studies that employed crude homogenates of postmortem tissue without regard to cause of death or the relative stability of different ADH forms, its validity could not be accepted with certainty. Therefore, studies were initiated to compare the activities and electrophoretic patterns of livers obtained at biopsy with those obtained within 12 hours of death from individuals dying from different causes (4).

The specific activity of ADH was examined in 6 biopsy samples, 33 autopsy samples from individuals who died from physical trauma, and 36 autopsy samples from hospitalized patients who died of cancer and other chronic illnesses (table 1). The mean specific activity of liver samples from apparently healthy individuals was significantly higher than that from the hospitalized patients. Thus, it was concluded that the health of an individual before death is a major determinant of ADH activity in liver. Moreover, storage of high-activity liver specimens at temperatures above −20° C for extended periods of time also resulted in significant decreases in ADH activity (4).

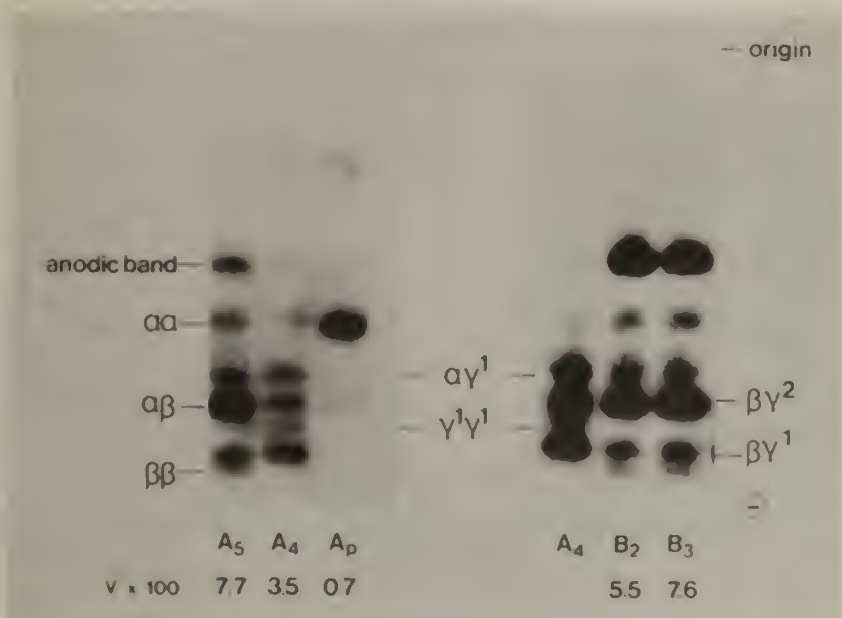
Not only did the biopsy and the traumatic death-related autopsy samples exhibit high specific activity, but most also contained a previously uncharacterized molecular form of ADH (4), in addition to those isozymes previously described by Smith et al. (5). As shown in figure 1, this new ADH form was particularly prominent in autopsy sample A_5 and biopsy samples B_2 and B_3. It was designated the "anodic band" because its electrophoretic mobility on starch gels was less than all previously described isozymes (including the $\alpha\alpha$ form), readily identified here in a liver specimen obtained from a premature infant, sample A_P.

Table 1. Specific Activity and Π-ADH Content of Biopsy and Autopsy Specimens

Source	Number of Samples	Specific Activity	Π-ADH
		(μmol/min/mg)	(% of total)
Biopsy	6	0.082 ± 0.020	N.D.
Autopsy, sudden traumatic deaths	33	0.070 ± 0.041	15 ± 9
Autopsy, disease-related deaths	36	0.027 ± 0.017	7 ± 6

0.1 M Glycine-NaOH, pH 10.5, 33 mM ethanol, 2.4 mM NAD^+, 25° C

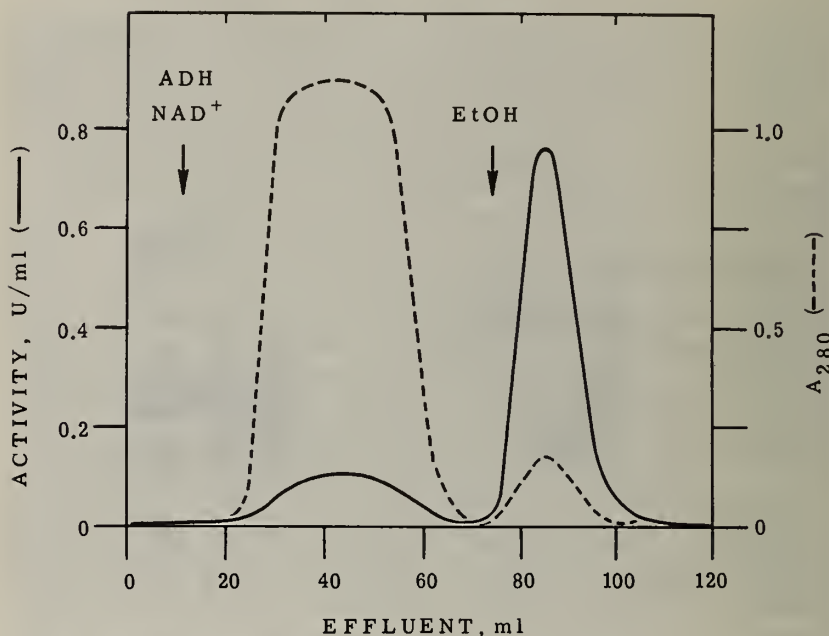
Figure 1. Starch Gel Electrophoresis of Human Liver ADH Obtained from Autopsy (A) and Biopsy (B) Specimens



Starch gels were electrophoresced at pH 7.7 and stained at pH 8.5 with 100 mM ethanol. The specific enzymatic activity (v) of the samples is expressed as μmol/min/mg protein. Specimen Ap was from the autopsy of a 3.5-pound premature infant who died 1 day after birth. Specimen A_4 is phenotype $ADH_3 1$ and specimens A_5, B_2, and B_3 are $ADH_3 2{\cdot}1$. (Reproduced with permission of John Wiley and Sons, Inc. [13]).

The relationship of the anodic band to high specific ADH activity suggested that this form may contribute significantly to total liver ADH activity. In order to characterize the physical-chemical and kinetic properties of this new ADH form, a procedure to isolate large (milligrams) quantities of enzyme was devised. The material was separated from the other molecular forms by means of an affinity chromatography procedure using the 4-substituted pyrazole derivative, 4-(3-[N-6-aminocaproyl] amino-propyl)-pyrazole (here abbreviated CapGapp), as the affinity ligand immobilized on cyanogen bromide-activated sepharose (6). Such pyrazole compounds have been shown to specifically bind and inhibit all mammalian alcohol dehydrogenases thus far studied (6,9,10).

Figure 2. CapGapp-Sepharose Affinity Chromatography of Human Liver ADH

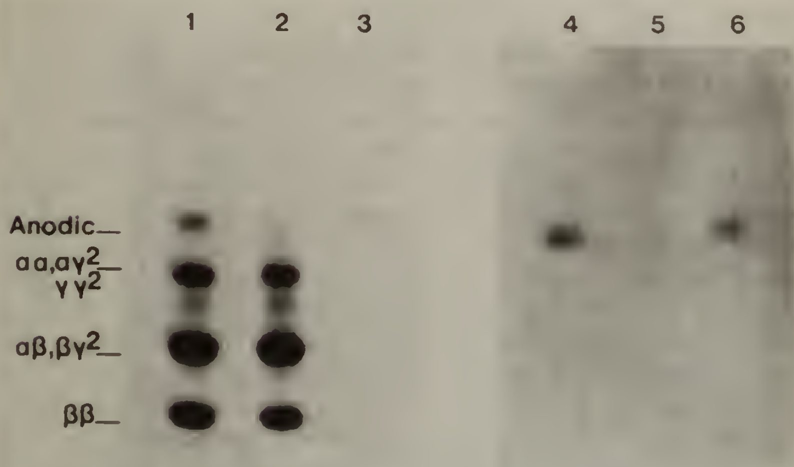


A liver homogenate-supernatant was purified on DEAE-cellulose (6,8) and applied to a 0.9 × 35 cm column of CapGapp-sepharose in 50 mM Na Pi, 3 mM NAD^+ at pH 7.5. ADH activity was determined with 33 mM ethanol, 2.4 mM NAD^+ in 0.1 M glycine-NaOH at pH 10.0 in the absence or presence of 33 μM 4-methylpyrazole. Π-ADH eluted in the column void, 20 to 70 ml, and the pyrazole-sensitive forms were eluted with 0.5 M ethanol after 75 ml of effluent (6).

Human liver homogenate supernatants were first partially purified on DEAE-cellulose and then applied to CapGapp-sepharose in the presence of NAD^+ (8). For the purification shown in figure 2, a significant portion of the activity did not bind to the affinity resin but eluted first in the void fraction from the column with the bulk of the protein. The remainder of the activity was eluted with 0.5 M ethanol. The physical-chemical and kinetic properties of this bound fraction of ADH have been described previously by Lange et al. (7).

The failure of a part of ADH to bind to CapGapp-sepharose suggested a differential sensitivity to inhibition by pyrazole compounds. Therefore, the starch gel electrophoretic patterns of the homogenate (samples 1 and 4, figure 3), the fractions that bound

Figure 3. Identification of the Human ADH Molecular Forms in Liver Homogenate and the Enzyme Fractions Separated by CapGapp-Sepharose



Starch gel electrophoresis of the homogenate supernatant (samples 1 and 4), the enzyme fraction that bound (samples 2 and 5), and did not bind (samples 3 and 6) to the affinity resin were performed at pH 7.7. Gels were stained in the absence (samples 1–3), or presence (samples 4–6) of 2 mM 4-methylpyrazole. (Reproduced with permission of the National Academy of Sciences, U.S.A. [12]).

to CapGapp-sepharose (samples 2 and 5), and the fractions that did not bind (samples 3 and 6) were examined by staining for ethanol-oxidizing activity in the absence and presence of 4-methylpyrazole. The band with the least electrophoretic mobility was identified as the anodic band. The remaining bands corresponded to those isozymes characteristic of phenotype $ADH_3 2$ (5) and comprised the fraction of ADH that bound to CapGapp-sepharose. As expected, all of them were inhibited by 4-methylpyrazole. By contrast, the anodic band did not bind to CapGapp-sepharose and was not inhibited by 4-methylpyrazole, as shown in figure 3. Thus the relative insensitivity to inhibition by pyrazole compounds of this ADH form, hereafter designated Π-ADH, accounts for its ease of separation by affinity chromatography on CapGapp-sepharose.

Π-ADH was purified by affinity chromatography on AMP-Agarose. Virtually all of the activity bound to the resin and was subsequently eluted with a linear gradient of NADH (figure 4).

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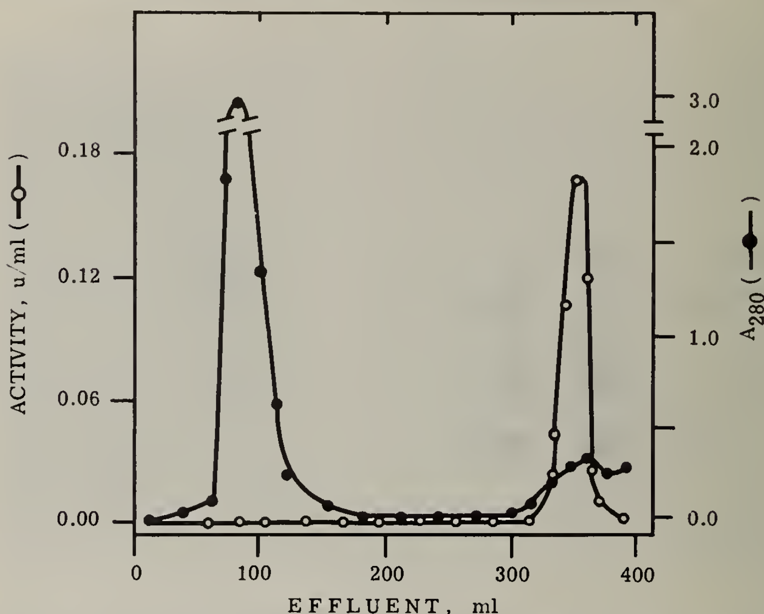
Figure 4. AMP-Agarose Affinity Chromatography of Π-ADH



ADH that did not bind to CapGapp-sepharose was precipitated with 75-percent ammonium sulfate and gel filtered on Bio-Gel P-6 in order to remove NAD^+. Enzyme was chromatographed on a 2.5 × 30 cm column of agarose-hexane-N, 6-AMP (PL Biochemicals, Milwaukee, Wis) in 0.1 M Tris-Cl, pH 8.6 at 4°C. Π-ADH was eluted with a linear gradient of 0 to 7 × 10^{-5} M NADH beginning at 300 ml of effluent. Π-ADH activity was determined as described in figure 2.

Purified in this manner, Π-ADH was homogeneous, as evidenced by SDS-gel electrophoresis and analytical ultracentrifugation (8).

The inability to detect Π-ADH in certain autopsy liver specimens, as opposed to those obtained at biopsy, suggested that this molecular form of the enzyme is more labile than the others in vivo (4). Consequently, the stability of purified Π-ADH was examined in vitro. Approximately 50 percent of its activity was lost within 24 hours when it was stored at pH 7.5 and 4° C. However, addition of 10^{-2} M ethanol effectively stabilized enzymatic activity for up to 2 weeks (8). Therefore, the extreme lability of Π-ADH, relative to the other molecular forms, readily accounts for the failure to detect its presence in previous work using postmortem livers (2,3,6).

Π-ADH was found to be remarkably similar with respect to molecular weight, subunit composition, and zinc content to the pyrazole-sensitive ADH forms described previously from human liver (7). It does, however, exhibit certain kinetic properties that are strikingly different from those described for previous preparations of human ADH. The K_m of ethanol for Π-ADH at pH 7.5 is approximately 20 mM (8), a value as much as 50 times those values reported previously for other preparations of ADH (2,9, 10). Moreover, 4-methylpyrazole is known to specifically inhibit horse and human liver ADH competitively with respect to ethanol with a K_I of less than 10^{-6} M (9,11). As demonstrated both by spectrophotometric assay and by activity staining of starch gels, similar concentrations of 4-methylpyrazole do not inhibit Π-ADH. However, if the 4-methylpyrazole is increased approximately 1,000 times to 0.5 and 1.5 mM, Π-ADH is eventually inhibited (12). The mode of inhibition is, under these circumstances, no longer competitive with respect to ethanol in the oxidative direction, but it is now competitive with respect to acetaldehyde in the reductive reaction (figure 5). The K_I calculated from the increase in slope of this reciprocal plot is approximately 1.4 mM.

These distinctive kinetic properties of Π-ADH, i.e., its insensitivity to inhibition by 4-methylpyrazole and high K_m for ethanol, have enabled the elucidation of its relative content in human liver samples and its potential role in hepatic ethanol oxidation. Specific ADH activities in liver homogenate supernatants were measured in the absence and presence of 33 μM 4-methylpyrazole at pH 10.5 (table 1). Π-ADH was expressed as the percentage of residual activity in the presence of inhibitor. The traumatic death-related autopsy samples with high specific activity had significantly more Π-ADH than the disease-related, low-activity samples, 15 versus 7 percent (table 1), again corroborating the predominance of anodic band in the first groups.

The contribution of Π-ADH to ethanol oxidation was examined in greater detail at pH 7.5 in a homogenate supernatant as a function of alcohol concentration (figure 6). In the absence of 4-methylpyrazole, activity increases progressively over the range of 0.3 to 100 mM ethanol. However, in the presence of 0.2 mM 4-methylpyrazole, activity appears only when ethanol concentration exceeds 3 mM and increases thereafter in parallel with that observed in the absence of 4-methylpyrazole. Therefore, Π-ADH contributes substantially to total activity at high ethanol concentrations in accord with its high K_m for ethanol (8). Importantly, at concentrations of ethanol that produce moderate to severe intoxication, i.e., 30 to 100 mM, Π-ADH represented as much as

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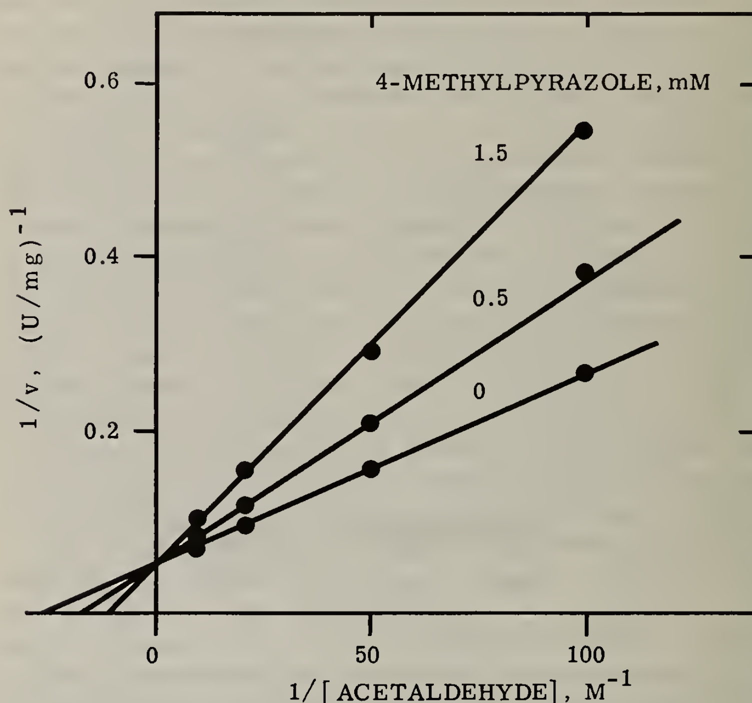
Figure 5. 4-Methylpyrazole Inhibition of Acetaldehyde Reduction by Π-ADH

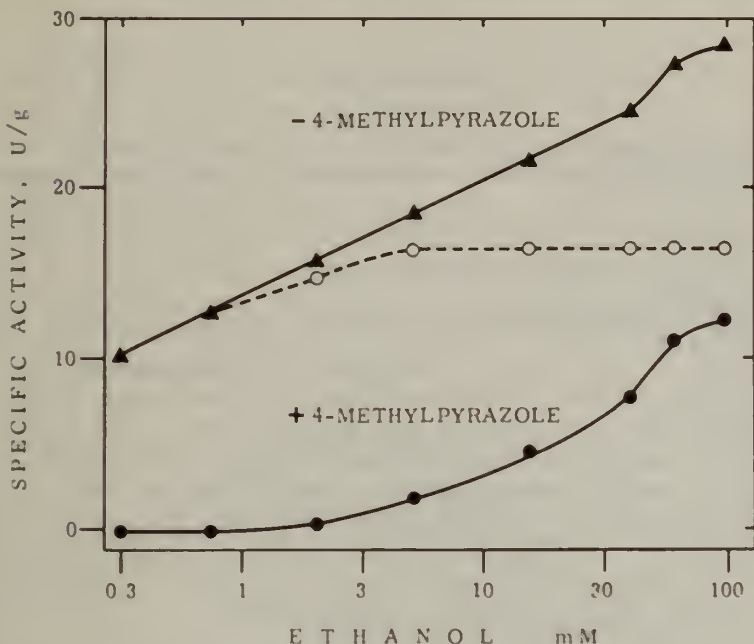


Enzymatic activity was determined with 0.2 mM NADH in 0.1 M Na Pi at pH 7.5 and 25°C in the absence and presence of 4-methylpyrazole. The data were analyzed by plotting the reciprocals of the velocity versus acetaldehyde concentration.

40 percent of the total alcohol-oxidizing activity. Moreover, in 10 homogenate supernatants, the contribution of Π-ADH determined with 60 mM alcohol ranged from 17 to 39 percent, with an average of 27 percent (12). This degree of variation suggests that, as with total ADH activity, there may exist an inherent biologic variation in Π-ADH activity. However, to what extent this may have been modified by postmortem change or other environmental factors is presently unknown.

The discovery of Π-ADH as a functionally distinct enzyme form should bear importantly on our understanding of normal human alcohol metabolism and its pathological derangement. For example, it is widely believed that ethanol elimination rates become maximal when ethanol concentrations exceed 5 mM. The studies

Figure 6. Contribution of Pyrazole-Resistant and Pyrazole-Sensitive Activity to Total LADH Activity in a Liver Homogenate



Alcohol dehydrogenase activity in the homogenate supernatant was determined at 0.3 to 100 mM ethanol with 2.4 mM NAD^+ in 0.1 M Na Pi pH 7.5. Pyrazole-resistant activity was determined in the presence of 0.2 mM 4-methylpyrazole. The difference between total and pyrazole-resistant activity is calculated to be the pyrazole-sensitive activity, dashed line. (Reproduced with permission of the National Academy of Sciences, U.S.A. [12]).

here, however, indicate that oxidation rates in vivo should increase when blood ethanol concentrations rise to intoxicating levels in some individuals. The failure of pyrazole compounds to inhibit ethanol oxidation is commonly viewed as functional evidence for non-ADH-mediated pathways of ethanol metabolism. The pyrazole insensitivity of Π-ADH indicates that such alternate pathways, or their lack in humans, cannot be inferred exclusively from the effects of these compounds.

As already noted, both the molecular heterogeneity of liver ADH and alcoholism in some individuals appear to be under genetic control. These considerations raise the provocative question whether the presence or absence of Π-ADH, or of any of the

other enzyme molecular forms, may prove to be biochemical links to alcoholism. Whether chronic alcohol consumption or malnutrition alters the relative distribution and amount of Π-ADH and other enzyme forms remains as another pertinent question.

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Discussion of Paper by Bosron et al.

Dr. Weiner: Inasmuch as the mechanism that Branden shows by X-ray crystallography suggests that the pyrazole would be binding to the zinc, did you find any other zinc inhibitor, such as o-phenanthroline, that would inhibit the enzyme? The second question, what about isobutyramide or fatty acids, the classical horse liver inhibitors? Did these affect the Π-enzyme?

Dr. Bosron: We can get some inhibition with isobutyramide, but we have not determined the inhibition patterns, whether they are competitive with acetaldehyde or not, as has been predicted. In terms of metal chelators, yes, o-phenanthroline did inhibit the Π-human alcohol dehydrogenase.

Dr. Lieber: This is a fascinating discovery, and I am particularly interested in your statement that it might contribute to alcohol metabolism in humans at intoxicating levels. I just wonder whether you have further evidence to support that statement. Does it actually contribute beyond what the low K_m ADH does to alcohol metabolism at intoxicating levels in humans?

Dr. Bosron: I think in cytosol, the slide that Dr. Li showed, that clearly if you increase ethanol concentration, the rate which went up in total activity was entirely due to the 4-methylpyrazole insensitive form, which is called the Π-ADH.

Dr. Lieber: Well, if we accept for the moment the study presented in Dr. Cornell's paper, that the low K_m ADH with normal nutrition is present in excess by perhaps 100 percent, the question is "What is the evidence that, in that situation, the high K_m ADH plays an additional role (particularly, if the rate-limiting factor is the reoxidation of NAD^+)?"

Dr. Li: Charles, the K_m for NAD^+ of this enzyme form and the others is about the same. Therefore, if there is any rate limitation on the low K_m form of ADH, the same rate limitation would pertain to the high K_m form. Now, we have also looked at the activities of ADH, maximal activities of human liver ADH, in biopsy specimens at pH 7.5 and I=0.15, and we find that the maximal activities are about that of the in vivo rates measured with 5 mM ethanol. Then, when you measure with 60 mM ethanol, you get an increase in rate that is variable in accord with what we have found for Π-ADH.

Dr. Vallee: I would just like to add a word to it, which I think is generated by Dr. Lieber's question. I do not think the implication is that this excludes additional mechanisms for ethanol oxidation such as you have been interested in. I do not believe that was the intent in making the statement. It should not be misunderstood. What it does state is that there is a mechanism for ethanol oxidation of major proportions, which is that by the normal enzyme and by a species which, oddly enough, functions optimally when the going gets roughest.

Nonlinearity of Blood Ethanol Elimination: Its Exaggeration After Chronic Alcohol Consumption and Its Relationship to a Non-ADH Pathway*

Mikko P. Salaspuro and Charles S. Lieber

Abstract

For more than 50 years, the prevailing concept has been that alcohol disappears linearly from the blood over a wide range of ethanol concentrations. In experiments with alcohol-fed baboons, it has now been demonstrated that the ethanol elimination curve is nonlinear and that the nonlinearity is exaggerated after chronic ethanol consumption. These findings are in favor of the existence (and induction) of a non-ADH pathway for ethanol, which is fully saturated only at high ethanol concentrations. This concept is further supported by the demonstration that 4-methylpyrazole (an alcohol dehydrogenase inhibitor) slows ethanol elimination rate significantly less in alcoholics with inadequate nutrition than in alcoholics with adequate nutrition. Because of the competitive mechanism of the inhibition, the proportional contribution of ADH pathway to total ethanol elimination is expected to be smaller in these alcoholics.

It is also generally accepted that, in normal individuals, the rate of ethanol oxidation by the ADH pathway is limited by the rate of NADH reoxidation. Only in some situations (such as protein deficiency) can hepatic ADH activity become rate limiting. We now report that chronic ethanol consumption itself results in an acceleration of the removal of cytosolic free NADH; as a consequence, ADH activity may become a rate-limiting step in the ADH pathway. In this situation, the reducing equivalents in hepatic cytosol no longer accumulate, and the acute effects of ethanol, mediated normally by the change in hepatic redox state, disappear after chronic ethanol consumption.

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It is well known that regular drinkers tolerate large amounts of alcoholic beverages, mainly because of central nervous system adaptation. In addition, alcoholics develop increased rates of blood ethanol clearance (Kater et al., 1969; Ugarte et al., 1972), so-called metabolic tolerance, which has been verified also by experimental alcohol administration (Lieber and DeCarli, 1970; Tobon and Mezey, 1971; Misra et al., 1971). Only a few enzymes are known to metabolize alcohol in the liver, but the quantitative contribution of the different pathways of ethanol oxidation to the metabolic tolerance has not been clarified. The purpose of this paper is to review the different mechanisms involved in accelerated ethanol metabolism after chronic ethanol consumption. Because of some recently observed results, special emphasis is given to the nonlinearity of blood ethanol disappearance and its relationship to a non-ADH pathway of alcohol metabolism. Associated adaptive metabolic changes, such as altered hepatic redox state during alcohol oxidation, are also discussed in light of these recent findings.

Alcohol Dehydrogenase (ADH) Pathway as Related to Accelerated Ethanol Metabolism

The main hepatic pathway for ethanol disposition involves alcohol dehydrogenase, a zinc-containing enzyme of cytosol. It catalyzes the conversion of ethanol to acetaldehyde in a reaction that requires NAD as a cofactor.

$$\text{Ethanol} + \text{NAD} \rightarrow \text{Acetaldehyde} + \text{NADH} + \text{H}^+$$

As a net result, ethanol oxidation generates an excess of reducing equivalents as free NADH in hepatic cytosol, primarily because the metabolic systems involved in NADH removal are not normally capable of fully preventing accumulation of NADH.

It is commonly accepted that hepatic ADH activity is not a rate-limiting step in ethanol metabolism. There are numerous examples (Lieber, 1977) of the lack of correlation between rates of ethanol oxidation and hepatic ADH activity. For instance, the existence of an atypical ADH with much higher in vitro activity is not associated with enhanced ethanol metabolism in vivo (Edwards and Price Evans, 1972). From the kinetic results of Theorell and Chance (1951), it can be calculated that when the NADH produced in ADH reaction can no longer be removed, its concentration increases with subsequent inhibition of ADH reaction. On this

basis, ethanol oxidation rate is normally regulated by the speed of the reoxidation of the ADH-NADH complex.

NADH reoxidation. The main pathways by which NADH produced in hepatic cytosol is reoxidized to NAD follow:

1. Reduction of cytosolic metabolites;
2. Synthesis of fatty acids and triglycerides;
3. Mitochondrial oxidation;
4. Microsomal NADPH utilization.

1. NADH may, at least partly, be reoxidized by the reduction of cytosolic intermediary metabolites. These metabolites may either leave the liver as the metabolic product of pyruvate reduction (lactic acid) or they may accumulate in hepatic cytosol, as in the case of glycerol phosphate. The quantitative contribution of these reductive reactions to total NADH reoxidation, however, is rather small.

2. The increased synthesis and deposition of fatty acids and triglycerides may be quantitatively more important. The NADPH needed for fatty acid synthesis is most probably derived from the oxalo-acetate-malate cycle, which enables the formation of NADPH from NADH (Thieden et al., 1972; Lieber, 1968).

3. The major pathway for NADH removal is its mitochondrial reoxidation. The mitochondrial membrane, however, is impermeable to NADH, and the reducing equivalents of NADH are transferred to the mitochondrial respiratory chain via shuttle mechanisms such as malate cycle, fatty acid elongation cycle, and α-glycerophosphate cycle (Hassinen, 1967; Chappel, 1968; Grunnet, 1970).

4. Another mechanism for NADH reoxidation is microsomal oxidation, including the microsomal ethanol-oxidizing system (MEOS), which has been suggested to consume NADPH derived from NADH via the malate-pyruvate-oxaloacetate cycle (Lieber and DeCarli, 1972). The role of this system in enhanced ethanol oxidation after chronic consumption will be discussed subsequently.

As to the ADH pathway, the main theory concerning the mechanism of accelerated ethanol metabolism after chronic consumption is through increased mitochondrial reoxidation of NADH. Actually there are sufficient data to support the concept that, at least artificially, ethanol oxidation can be stimulated by increasing NADH removal. ADP formation from ATP can be enhanced with fructose (Lundquist and Wolthers, 1958; Thieden et al., 1972), gluconeogenic precursors (Crow et al., 1977), uncoupling agents (Videla and Israel, 1970), and electron acceptors (Madison et al., 1967). Increased ADP formation leads to an increased electron flux in the mitochondrial respiratory chain and consequently to an enhanced reoxidation of NADH.

The effect of chronic consumption has been related to a kind of "hypermetabolic state" in the liver. The basis of this theory has been an increased oxygen consumption in the livers of animals chronically treated with ethanol, mimicking the effects of thyroxine (Israel et al., 1973; Bernstein et al., 1973; Thurman et al., 1976). The increased oxygen consumption has been attributed to an increased utilization of ATP by the NA^+-K^+-activated ATPase after chronic ethanol feeding (Israel et al., 1975). Controversial results as to the enhanced oxygen consumption and increased ATPase activity after chronic alcohol treatment have, however, been reported by others (Gordon, 1977; Cederbaum et al., 1977). On this basis, the theory that chronic ethanol consumption engenders a hypermetabolic state akin to hyperthyroidism, although interesting, cannot be considered as fully explaining all changes involved in enhanced ethanol metabolism.

There are situations in which hepatic ADH activity may become a rate-limiting step in the ADH pathway. In rat liver perfusion experiments, the administration of ethanol to the perfusion medium increases the lactate/pyruvate ratio of the perfusion medium from 10 to 80 (Forsander et al., 1965). This change is associated with almost total abolition of CO_2 production; i.e., the citric acid cycle (the main source of CO_2) is inhibited by ethanol. However, in perfusion experiments with protein-deficient livers, ethanol had no effect on lactate/pyruvate ratio and, furthermore, citric acid cycle was not inhibited by ethanol (Salaspuro and Mäenpää, 1966). This finding indicates that in protein-deficient livers there was no accumulation of cytosolic-free NADH; it can be explained by assuming that, instead of the rate of NADH reoxidation, it is hepatic ADH activity that limits the ethanol oxidation rate in protein-deficient livers. A decreased ethanol elimination rate associated with diminished hepatic redox changes and hepatic ADH activity caused by protein-deficient diet have been experimentally demonstrated also in humans (Bode et al., 1971).

Microsomal Ethanol-Oxidizing System as Related to Accelerated Ethanol Metabolism

MEOS has been differentiated from alcohol dehydrogenase by its subcellular localization (microsomes versus cytosol), pH optimum (7.4 versus 9 to 11), cofactor requirements (NADPH versus NAD), and relative lack of sensitivity to pyrazole (Lieber, 1977; Lieber and DeCarli, 1970; Teschke et al., 1974, 1976). From catalase, MEOS has been differentiated by its relative

insensitivity to catalase inhibitors such as azide and cyanide, and by the inability of a H_2O_2-generating system (glucose-glucose oxidase) to sustain ethanol oxidation in purified microsomal preparations (Teschke et al., 1974). Furthermore, isolated MEOS metabolizes higher aliphatic alcohols such as propanol and butanol, which are not substrates for catalase (Teschke et al., 1975).

Ethanol-oxidizing activity has recently been reconstituted with three microsomal components P-450, NADPH cytochrome c reductase, and lecithin (Ohnishi and Lieber, 1976). The K_m of the reconstituted MEOS for ethanol was 10 mM, which is similar to the K_m measured in crude microsomes and the MEOS fraction isolated by column chromatography (Lieber and DeCarli, 1968, 1970; Teschke et al., 1974). This reconstituted system required NADPH as a cofactor, did not react to an H_2O_2-generating system, and was insensitive to catalase inhibitors. Most probably, MEOS activity involves a mechanism akin to that of the cytochrome P-450-dependent drug detoxification in microsomes, possibly using a different type of cytochrome P-450 (Hasumura et al., 1975).

Although the existence of MEOS is very well documented, as it is for the ADH pathway, its contribution to accelerated ethanol metabolism after chronic consumption has not been clarified. There is, however, evidence indicating that chronic ethanol consumption produces an increase in its activity (Lieber and DeCarli, 1970), which has also been demonstrated in experiments with isolated hepatocytes in vitro (Teschke et al., 1977).

Concentration Dependence of Ethanol Metabolism

The linearity of the ethanol elimination curve over a wide range of blood alcohol concentrations has been the prevailing concept, based on early studies of Mellanby (1919) and Widmark (1932). Later it was shown that at very low blood ethanol concentrations, less than 2 mM or about 9 mg%, ethanol disappearance becomes exponential (Marshall and Fritz, 1953). Finally it was demonstrated that the low serum alcohol concentrations in humans follow Michaelis-Menten kinetics (Lundquist and Wolthers, 1958). However, there are some sporadic observations that indicate that ethanol disappearance may be more rapid after larger intravenous doses (Lereboullet et al., 1976; Haggard and Greenberg, 1934; Wilkinson et al., 1976). The variability in the interpretation of the results is most probably due to the different routes of ethanol administration used by investigators, to differences at blood ethanol

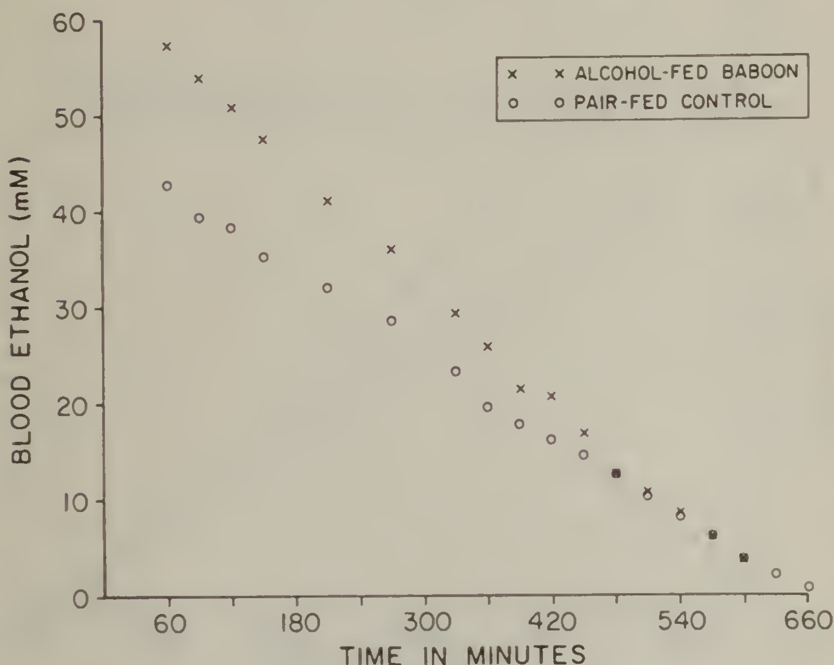
concentrations in which measurements have been taken, and, finally, to the relatively small number of blood ethanol determinations over a wide range of ethanol concentrations.

If it is assumed that ethanol is oxidized only by the classic K_m alcohol dehydrogenase, the ethanol elimination curve should be virtually linear at all ethanol concentrations above 5 mmol/l. By contrast, if other pathways with a higher K_m value for ethanol play a role, they should shift the linear curve to a nonlinear one.

We have recently tested the linearity of the blood ethanol elimination curve in our baboon model for alcoholic liver (Salaspuro and Lieber, 1977). The experiments have been extended to verify the effect of chronic alcohol consumption on the possible concentration dependence of ethanol metabolism in the same animal model. Blood ethanol clearance in alcohol-fed baboons, as well as in their pair-fed controls, was determined after an intravenous infusion of ethanol in an amount to reach a blood concentration of from 40 to 50 mM. Blood ethanol concentrations were determined by gas chromatography at 30-minute intervals; ethanol elimination rates were measured separately between 45 to 20 mmol/l and 15 to 5 mmol/l. These concentration ranges were selected both for empirical reasons and also because the K_m value of microsomal ethanol-oxidizing system is known to be about 10 mM (Lieber and DeCarli, 1968, 1970; Teschke et al., 1974). To avoid effects of fasting, dextrose was continuously infused to meet the average caloric demands of the animals. The other details of the experiment will be published separately. We have gathered preliminary results from studies carried out before and after 2 months and 24 months of alcohol feeding, as well as from animals pair-fed the isocaloric control diet.

The results of a typical experiment in a baboon-pair are shown in figures 1a–d. On superficial inspection, the points seem to form a straight line (a), and a linear analysis may seem warranted (b). However, many points are either above or below the line. On the other hand, if the linear regression of the curve is calculated separately at high (45 to 20 mM) and low (15 to 5 mM) ethanol concentrations, the points fit the line much better (c). The final regression lines are shown in (d). Blood ethanol clearance is clearly faster at high ethanol concentrations than at low ones, especially in the alcohol-fed animal. On this basis, ethanol elimination rates in subsequent experiments have been calculated separately for both ranges of ethanol concentrations. Our preliminary results show that, in naive animals, ethanol elimination rates at high ethanol concentrations (HE) are about 10 percent higher than those at low ethanol concentrations (LE). After 2 months of alcohol

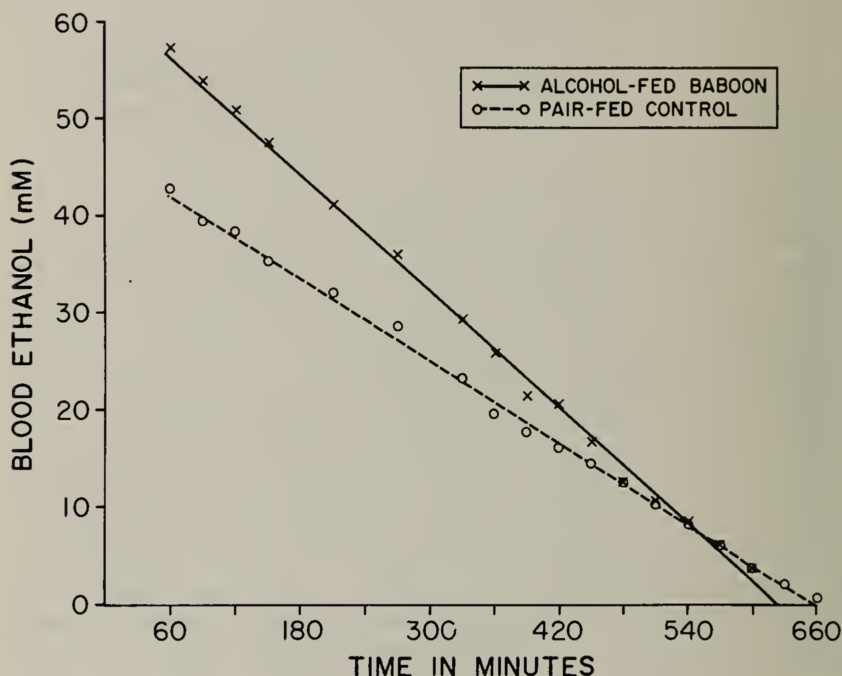
Figure 1a. Blood Ethanol Clearance in a Baboon Fed Alcohol 2 Years and in its Pair-Fed Control



feeding, HE was 14 percent higher than LE, and after 24 months of alcohol, the corresponding difference was about 30 percent. It should be emphasized that, although ethanol elimination rates increased at both ethanol concentration ranges, the increase at high ethanol concentration was significantly greater. The finding indicates that the contribution of the ethanol metabolizing system that is fully saturated only at high ethanol concentrations is greater after chronic alcohol consumption than before it.

Recently these findings from baboon experiments have been confirmed in a human volunteer studied under metabolic ward conditions (figure 2). It can be seen that certainly after 4 weeks of alcohol (2 to 4g/kg/day), the blood ethanol elimination curve is nonlinear and that the increase in ethanol elimination rate occurs especially at high ethanol concentrations. The results are comparable to the ones reported recently from experiments in rats and human alcoholics (Feinman et al., in press). The existence of the new labile anodic alcohol dehydrogenase-isoenzyme with a higher

Figure 1b. Blood Ethanol Clearance in an Alcohol-Fed Baboon and in its Pair-Fed Control



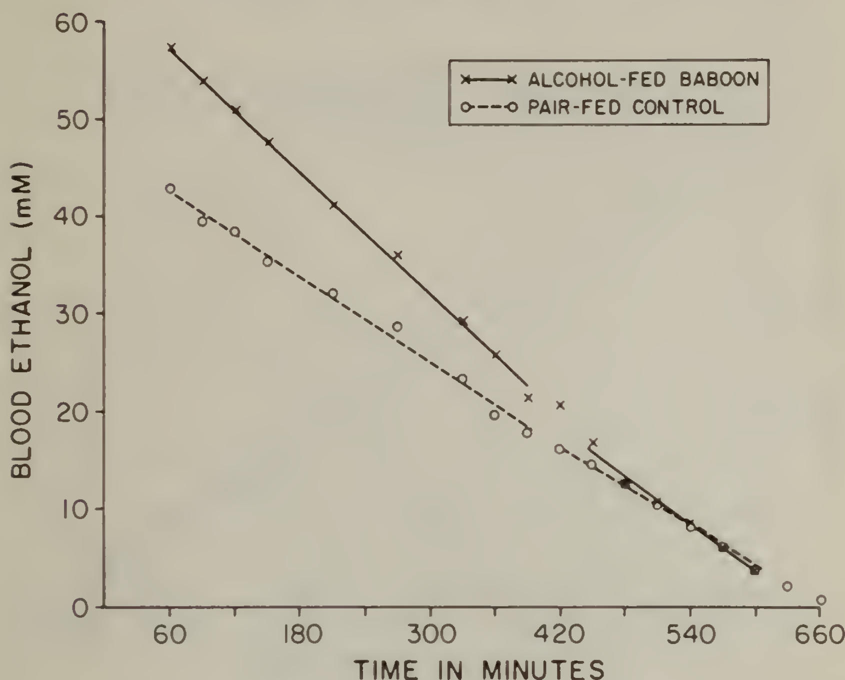
Linear regression lines are calculated from all blood ethanol values in figure 1a.

K_m for ethanol* (Li and Magnes, 1975; Bosron et al., 1977) could, in fact, contribute to the nonlinear ethanol clearance in our human volunteer; but in baboons, so far, we have not been able to demonstrate increased ADH activity in vitro with increasing ethanol concentrations from 5 to 50 mmol/l. The measurements have been done in fresh 100,000G supernatants in pH 7.4 from three baboons, one of which had been fed alcohol for 2 years and actually had a nonlinear ethanol elimination curve. None of these baboons showed evidence of a high K_m ADH.

In addition to the nonlinearity of the ethanol elimination curve, there is other recent in vivo evidence to support the increasing

*We are grateful to Dr. T. -K. Li for his helpful advice for the determination of an anodic high K_m alcohol dehydrogenase.

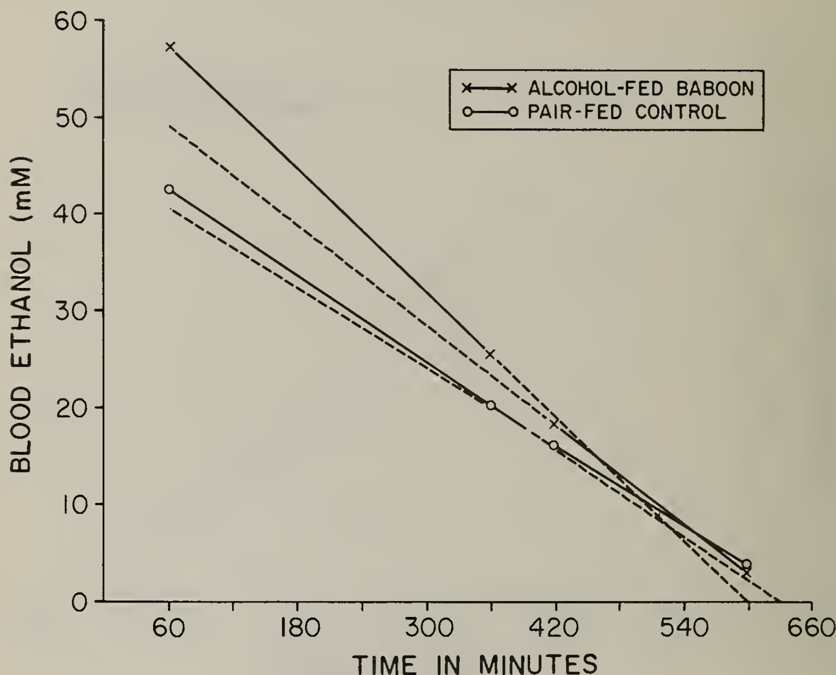
Figure 1c. Blood Ethanol Clearance in an Alcohol-Fed Baboon and in its Pair-Fed Control



Linear regression lines are calculated separately at high and at low ethanol concentrations.

contribution of a non-ADH pathway to total ethanol elimination in chronic alcoholics. It has been demonstrated that 4-methylpyrazole (4-MP, an alcohol dehydrogenase inhibitor) inhibits ethanol elimination rate significantly less in alcoholics with inadequate nutrition than in alcoholics with adequate nutrition (Salaspuro et al., 1975, 1978 in press). Because of the competitive mechanism of the inhibition, the proportional contribution of ADH pathway to total ethanol elimination must have been smaller in these alcoholics. Furthermore, although these alcoholics with inadequate nutrition apparently had decreased hepatic ADH activity due to protein deficiency, and although ADH was further decreased by 4-MP, the ethanol elimination rates were still higher than those in controls. This difference in elimination rates favors the existence of a non-ADH pathway for ethanol metabolism.

Figure 1d. Blood Ethanol Clearance in an Alcohol-Fed Baboon and in its Pair-Fed Control

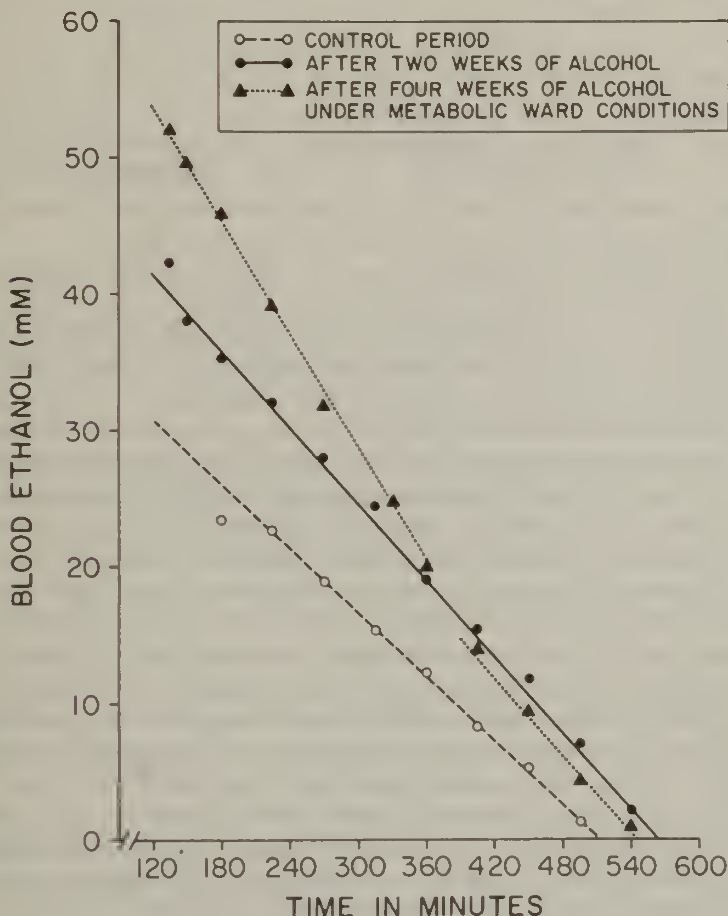


Final linear regression lines from Figure 1c. Dotted lines represent the extrapolations of solid lines.

These findings cannot be explained completely by the existence of the anodic form of ADH with a higher K_m and with a lower 4-MP sensitivity. The ethanol elimination rates were measured at rather low blood ethanol concentrations (<20 mM), whereas the K_m for anodic ADH has been reported to be about 20 mM (Bosron et al., 1977). Furthermore, the contribution of this isoenzyme, with higher activity to ethanol oxidation, should have resulted in a higher production of NADH and, consequently, in a greater inhibition of galactose elimination—which was not the case.

The adaptive increase in ethanol elimination rate (far beyond the generally accepted level of 7 g/hr), as well as the apparent nonlinearity of ethanol elimination curve, may have some important clinical implications. At present, a constant rate of ethanol

Figure 2. Blood Ethanol Clearance in a Human Volunteer Studied under Metabolic Ward Conditions



elimination and also the linearity of the elimination curve are widely assumed for various medicolegal purposes, in order to determine retrospectively the blood ethanol concentration at a given time: In view of the above, calculations based on linearity and constant elimination rate should be interpreted with caution.

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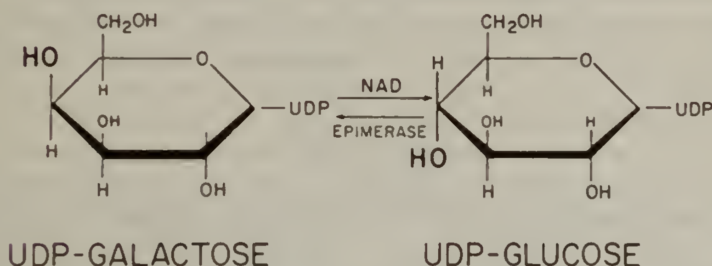
Adaptation of Hepatic Redox Changes During Chronic Alcohol Consumption

An important change in liver metabolism caused by hepatic ethanol oxidation is the shift in the cytosolic and mitochondrial redox potentials to a more reduced state (Forsander et al., 1958). This activity is reflected by several secondary changes in hepatic metabolism, such as the inhibition of tricarboxylic acid cycle (Lundsgaard, 1938; Forsander et al., 1965), with associated decrease in fatty acid oxidation (Lieber and Schmid 1961; Lieber et al., 1967); the inhibition of hepatic gluconeogenesis from various precursors (Krebs et al., 1969); and changes in redox pairs such as lactate and pyruvate (Forsander et al., 1965).

Especially in in vitro experiments, the changes in lactate/pyruvate ratio are widely used to reflect hepatic redox alterations (Forsander et al., 1965; Salaspuro and Mäenpää, 1966). However, in in vivo situations, the changes of this ratio in the peripheral blood are not reliable, because of the contribution of the peripheral tissues to the formation of lactate and pyruvate. In fact, it has been reported that alcoholics may have higher blood lactate and pyruvate levels than nonalcoholics, especially after exercise (Chalmers et al., 1977). Galactose is metabolized mainly in the liver, and in the reaction in which UDP-galactose is epimerized to UDP-glucose, NAD functions as a coenzyme and NADH formed during ethanol oxidation has been shown to be a potent inhibitor of the reaction (Isselbacher and McCarthy, 1959). The epimerization step, however, does not result in a net production of NADH, because the reaction involves only the internal NAD-coupled epimerization of hydroxyl and hydrogen molecules (figure 3). For this reason, galactose metabolism itself does not have an effect on the redox state of the liver and the rate of galactose elimination in vivo can be used as a reflector of hepatic NADH/NAD ratio both in rat (Salaspuro and Salaspuro, 1968) and in humans (Salaspuro and Kesäniemi, 1973).

As stated before, it is generally accepted that in normal individuals the hepatic ADH activity is not a rate-limiting step in ethanol oxidation and that the ethanol oxidation rate is mainly regulated by mitochondrial NADH reoxidation. If, during chronic alcohol consumption, NADH removal from hepatic cytosol is increased—either by mitochondrial reoxidation or by the microsomal ethanol-oxidizing system via NADPH—the expected consequence is that cytosolic NADH tends to decrease, as has been demonstrated in rats fed alcohol chronically (Domschke et al.,

Figure 3. Epimerase Reaction, Which Catalyzes the Formation of UDP-Glucose From UDP-Galactose



1974). More NADH, however, is immediately formed by the ADH reaction because of the excess hepatic ADH; as the end result, there is still enough NADH to prevent, for instance, galactose epimerization. On the other hand, if hepatic ADH activity is decreased by protein deficiency (or by 4-methylpyrazole), the prevailing hepatic ADH may become a rate-limiting step in the ADH pathway. Consequently, the formation of NADH via alcohol dehydrogenase cannot increase, and cytosolic-free NADH starts to decrease, which is reflected as a lack of the expected change in the lactate/pyruvate ratio and as a failing inhibition of citric acid cycle function by ethanol in perfused rat livers (Salaspuro and Mäenpää, 1966). In in vivo experiments, similar changes in hepatic metabolism can be detected as a lack of the normal inhibition of galactose elimination by ethanol (Salaspuro and Salaspuro, 1968; Salaspuro and Kesäniemi, 1973; Salaspuro et al., 1975, 1978 in press). In addition to the effect of protein deficiency and 4-methylpyrazole, the diminished inhibitory effect of ethanol on galactose elimination has also been demonstrated in alcoholics with apparent adequate nutrition, as well as in patients with thyrotoxicosis (Salaspuro and Kesäniemi, 1973). These findings indicate that even chronic ethanol consumption alone, without associated protein deficiency, and also the increased mitochondrial reoxidation of NADH (in thyrotoxicosis) may result in a metabolic adaptation. The end result is that hepatic ADH activity, rather than NADH reoxidation, is a rate-limiting step in the ADH pathway.

In order to further elucidate this hypothesis, the effect of ethanol on galactose elimination rate has been measured in our

baboon model of alcoholic liver injury. Nutritional factors can be controlled and the concomitant effect of protein deficiency excluded. For this purpose, galactose was infused intravenously at the dose of 500 mg/kg body wt. After infusion, blood galactose concentration was determined enzymatically at 5-minute intervals during the subsequent 60 minutes. The amount of galactose excreted in urine was also analyzed, and galactose elimination rate was calculated according to Tygstrup (1964). To determine the effect of ethanol on galactose elimination, ethanol was infused intravenously 1 hour before galactose in an amount to achieve a blood concentration of about 40 mM. Our preliminary results show that, in control animals, alcohol inhibits galactose elimination rate by 46 percent. After 2 to 3 months of alcohol feeding, the ethanol-induced inhibition was not yet significantly decreased (40 percent), although ethanol elimination rate was already increased by 21 percent. But after 24 months of alcohol feeding, when ethanol elimination was accelerated by 64 percent, the inhibition of galactose elimination by ethanol was only 12 percent. As stated before, the lack of inhibition was not due to the decrease in hepatic ADH activity.

It can be concluded that chronic ethanol consumption itself results in an acceleration of the removal of cytosolic-free NADH. This adaptation leads to the change in the regulation of ethanol oxidation rate in the ADH pathway. Instead of the rate of NADH removal, it is hepatic ADH activity that finally limits ADH pathway in alcohol-fed animals. In this step, there is no longer an accumulation of NADH in hepatic cytosol; consequently, the metabolic effects of acute ethanol administration may be completely different from the ones in naive animals. However, at least in baboons, this adaptation seems to be rather slow. After 3 months of alcohol, the regulation of the rate of ADH pathway is qualitatively the same as in naive animals; i.e., ethanol has the same acute effect on hepatic redox state, both in alcohol-fed and control animals. The implications of these metabolic adaptations are obvious.

1. The rate-limiting factor of ethanol oxidation may vary in alcoholics or in experimental animals fed alcohol chronically. Instead of the rate of NADH reoxidation, hepatic ADH activity becomes a rate-limiting step in the ADH pathway, when either hepatic ADH activity decreases (as in protein deficiency) or when the capacity to remove cytosolic-reducing equivalents is sufficiently induced by chronic ethanol consumption.

2. Depending on the limiting step in the ADH pathway, there may or may not be an accumulation of reducing equivalents in hepatic cytosol after the acute administration of alcohol.

3. Consequently, the acute effects of ethanol on hepatic intermediary metabolism may be completely different in alcoholics, as well as in animals fed alcohol chronically, when compared to those in controls.

4. On the basis of these recent findings, one must question whether any of the acute metabolic effects of alcohol still occur in the chronic situation; this should be borne in mind when interpreting the results of metabolic experiments performed in alcoholics or alcohol-fed animals.

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Section VI: Other Effects of Alcohol on Nutrition

The Fetal Alcohol Syndrome: Possible Implications of Nutrient Deficiencies

Lucille S. Hurley

Historical Aspects

Alcoholic women have a high risk of giving birth to children with growth retardation, congenital malformations, and mental deficiency. Knowledge of the deleterious effects of parental alcohol consumption on the offspring dates back to classical times (Warner and Rosett, 1975). In parts of the ancient world, for example, a newly married couple was forbidden to drink wine on their wedding night for fear that a child might be conceived in drunkenness. More recently, during England's "Gin Epidemic" of the 18th century, the writer and social reformer Henry Fielding asked, "What must become of an infant who is conceived in gin, with the poisonous distillation of which it is nourished, both in the womb and at the breast?"

The artist Hogarth illustrated some of these problems in his "Gin Alley" (Coffey, 1966). And in 1899, Dr. Sullivan, physician to a Liverpool prison, published a careful study showing that the frequency of stillbirths and neonatal deaths was higher among women who were alcoholic than it was in a matched control group. He further found that these frequencies declined when the women were abstemious, as they were obliged to be during a prison sentence.

Thus, although the widespread and often excessive use of alcohol was not generally disapproved of, for hundreds of years, consumption of large amounts of alcoholic beverages by pregnant women was believed to have harmful effects on their infants. During Prohibition in the United States, however, interest in the effects of alcohol during pregnancy declined, as was evidenced in the English language medical literature. By the end of Prohibition, the acceptance of a connection between alcoholism and what had been called in Hogarth's time "weak, feeble and distempered children" (Coffey, 1966) was largely forgotten. By the 1940's, the

earlier writings, frequently in the moralistic and unscientific vein encountered in 19th-century medical literature, were completely rejected (Warner and Rosett, 1975).

Recent Characterization

The recent rediscovery of the deleterious effects of alcoholism during pregnancy on the development of the offspring was made independently by Lemoine and his coworkers (1968) in Nantes, France, and by Jones and Smith and their coworkers (1973) of Seattle, Wash. The close similarity between the reports, described independently by two research groups in different parts of the world, strongly suggests the validity of the observations.

The Seattle workers called the condition the fetal alcohol syndrome (FAS). Since then, many case reports of FAS have been published from around the world (NIAAA, 1977). The major features of the syndrome are growth retardation; small head size; anomalies of the face, eyes, heart, joints, and external genitalia; and mental deficiency. Other anomalies include micrognathia; hypoplastic midfacial structures including epicanthic folds, broad nasal ridge, upturned nares, and long upper lip. There are also abnormalities of the ears, and the palpebral fissures are small (Mulvihill and Yeager, 1976; Mulvihill et al., 1976). It is thought that this abnormality may actually be secondary to microphthalmia. Other ocular defects common in FAS are ptosis and strabismus. A wide mouth, prominent ears, and a narrow bifrontal diameter are also common features of the syndrome (Hanson et al., 1976). Other malformations that occur include cleft palate, visceral anomalies, and small hemangiomas.

The growth failure in these children occurs both prenatally and postnatally. At the time of birth, the deficit in body length is often greater than the deficit in body weight. Postnatally, FAS babies usually gain weight poorly, and body weight is more affected than is body height.

There is no catchup in growth during infancy and early childhood. The persisting deficiency of growth appears not to be the consequence of the postnatal environment; affected babies raised in foster care from early infancy generally show no better growth or performance than those raised by the alcoholic mothers. Even infants hospitalized for failure to thrive have not shown catchup growth.

The perinatal mortality of infants with FAS is high, and those who survive show manifestations of neurological difficulties.

During the neonatal period, there is often tremulousness, hyperactivity, and irritability. Although some of these symptoms might result from alcohol withdrawal after birth, the tremulousness frequently persists for a long time, even years, and the fine motor dysfunction and developmental delay may be permanent. Mental deficiency of varying severity also occurs in these children and, in the only recorded necropsy study, considerable malformation of the central nervous system was found. Thus it seems that much of the abnormal performance in the early period, as well as the persistent mental deficiency, is secondary to alterations in brain development and function resulting from the prenatal effect on morphogenesis of the central nervous system (Hanson et al., 1976; Jones et al., 1974).

The incidence of fetal alcohol syndrome in children of chronically alcoholic women appears from present evidence to be in the range of 30 to 50 percent. Prenatal and postnatal growth failure, developmental delay, and microcephaly all occur in about 90 to 95 percent of the infants with FAS. Ocular defects occur in 40 to 60 percent. Anomalies of the jaw and ear are seen in half of the cases; receding chin occurs in 30 percent; and cleft palate in 15 percent. The frequency of other defects ranges from 38 to 73 percent (Mulvihill and Yeager, 1976).

It should be emphasized that the syndrome, as I have described it so far, is based on the outcome of pregnancy of chronically and severely alcoholic women. Very little is known at the present time about the risk and possible consequences of lower intakes of alcohol. Neither are the critical factors involved understood. For example, in relation to alcohol consumption, is it the continuous alcohol level in the maternal blood that is important, or maximum concentrations during binge drinking? The effect of lower levels of alcohol intake on fetal development, perhaps producing only part of the syndrome, is unexplored.

Congenital abnormalities have also been produced in experimental animals given alcohol during pregnancy, and an animal model of FAS has been developed in mice (Tze and Lee, 1975; Chernoff, 1977).

Possible Mechanisms

What are the possible mechanisms that could bring about the abnormal development resulting in fetal alcohol syndrome? First, there may be a direct effect of alcohol itself on the developing embryo. We know that alcohol taken by the mother is transported

across the placenta. In sheep, a positive correlation was found between maternal and fetal blood alcohol concentration during a 1- or 2-hour infusion of alcohol into the pregnant ewe (Mann et al., 1975).

There could also be an indirect effect of chronic alcoholism that might alter the maternal metabolism in such a way as to produce teratogenic effects on the child. I propose that nutritional deficiencies may possibly be involved in the development of the fetal alcohol syndrome. It should be remembered, however, that there are several confounding factors involved in cases of chronic alcoholism among pregnant women. Heavy drinking is often associated with other risk factors for congenital abnormalities; these factors include heavy smoking, use of other drugs, emotional stress, injury from falls or violence, and poor prenatal medical care.

Teratogenic Aspects of Nutrient Deficiencies

Although many nutritional deficiencies are teratogenic, I am going to limit my discussion to three nutrients; folate, magnesium, and zinc, which I think are most likely to be involved in FAS. All three of these nutrients have been recognized as problems in the nutritional status of alcoholics. Furthermore, deficiencies of these substances are highly teratogenic in experimental animals, and there is at least some evidence of similar effects in humans.

Magnesium

Magnesium deficiency is teratogenic in rats. When rats were given a diet severely deficient in magnesium (0.2 mg/100 g) throughout pregnancy, no fetuses were carried to term. When the deficient diet was given from day 6 to day 14 of gestation, the incidence of resorptions was very high and many of the surviving fetuses were malformed. Malformations included cleft lip; short tongue; hydrocephalus; micrognathia or agnathia; club feet; fused digits; polydactyly; syndactyly; short or curly tail; herniations; and heart, lung, and urogenital anomalies (Hurley et al., 1976). Even milder deficiencies caused congenital abnormalities (Cosens et al., 1977), high neonatal mortality, and abnormal histology of the brain (Wang et al., 1971; Gunther et al., 1973).

There is also some indirect evidence that magnesium deficiency may be a problem in human pregnancy (Hurley, 1971).

Folic Acid

Experimental Animals. The importance of folic acid during pregnancy in mammals has been studied extensively, primarily with the use of folic acid antagonists (for review, see Hurley, 1977a). However, even without an antagonist in the diet, folate deficiency causes congenital malformations in rats. With a relatively mild deficiency, a variety of malformations was found, in cluding cleft lip, hydrocephaly, failure of closure of thoracic and abdominal walls, and eye defects (Giroud and Lefebvres-Boisselet, 1951).

Nelson and her colleagues (1952; 1955; 1956; Asling, 1961) extensively studied the wide variety of congenital malformations produced by folate deficiency in the pregnant rat, using an antagonist, x-methyl pterolyglutamic acid (x-methyl PGA), in combination with a folate-deficient diet. There was a high incidence of resorptions and multiple congenital defects. The frequency of the various types of defects depended on the timing. The young showed marked edema and anemia; cleft palate; numerous anomalies of the face; syndactyly; a wide variety of sketetal malformations; and defects of the lungs, eyes, and urogenital and cardiovascular systems. Even short-term, transitory deficiency, in combination with the folate antagonist, caused high incidences of multiple malformations. Similar malformations have also been produced in the mouse and the cat with the same antagonist (Tuchmann-Duplessis et al., 1959).

Biochemical Effects. Although some investigators have attempted to determine the mechanism by which folate deficiency produces teratogenic effects, little progress has been made. There is, nonetheless, some evidence that the effect of folate antagonists in inhibiting nucleic acid synthesis may play a role (Hurley, 1977b).

Embryos from folate-deficient rats also showed abnormal enzymic differentiation. There were alterations in isozyme patterns for a number of enzymes. In addition, the specific activity of phosphomonoesterases in tissues from experimental fetuses did not parallel that of controls for any of the fetal ages studied. The changes in enzyme patterns were correlated with abnormal chondrogenesis and osteogenesis.

Humans. Folic acid is clearly essential for the developing human embryo, as is evident from studies using folate antagonists as abortifacient agents. When these compounds were given during the first trimester of pregnancy, fetal death, followed by spontaneous abortion, resulted. However, if the antagonist was given in

insufficient quantities or was given too late during the pregnancy, the result was not abortion but development of a malformed fetus (Thiersch, 1952; Goetsch, 1962).

In less extreme conditions, however, the possible relationship of folate deficiency in pregnant women to malformations or other abnormalities of pregnancy is not clear. In an early retrospective study of 17 pregnant women with megaloblastic anemia (indicating folate deficiency), 5 of the women gave birth to infants with congenital malformations (Fraser and Watt, 1964). In another retrospective study, Hibbard and coworkers used the urinary excretion of formiminoglutamic acid (FIGLU) after the ingestion of histidine as an indication of folate deficiency. Sixty-two percent of the mothers of malformed infants had a positive response (indicating folate deficiency), as compared with 17 percent of mothers of normal infants. The relationship of positive FIGLU excretion tests and malformations was even more striking when only central system malformations were considered. There also seemed to be a relationship between folic acid deficiency in pregnant women and the occurrence of placental abruption and spontaneous abortions. However, 73 percent of women with defective folate metabolism, as measured by the FIGLU test in a previous pregnancy, developed the same condition in the next pregnancy; this finding suggests that a genetic factor involving folate metabolism may be involved.

A recent prospective study of more than 800 women corroborates these findings. Folate levels of erythrocytes were measured in early pregnancy. In women with low erythrocyte folate, the incidence of small-for-date infants and of malformations was higher than in those with normal folate; the difference was highly significant (Hibbard, 1975).

Smithells and coworkers (1975) have also reported, in a large prospective study, that significantly lower blood levels of red cell folate were found in mothers subsequently giving birth to infants with neural tube defects than those found in controls. Finally, Gandy and Jacobson (1977) have amassed impressive evidence suggesting that an inadequate level of maternal serum folate brings about a depression of fetal growth rate that may persist into the first year of life. In addition, Gross et al. (1974) have shown that children whose mothers were severely folate-deficient showed abnormal or delayed behavioral development.

Although there is now considerable evidence of a high correlation between the incidence of folic acid deficiency and various complications of pregnancy, conflicting results have been reported. In some studies, little correlation was found between

folate status, as measured by plasma folate levels and by the morphology of the red cells, and problems associated with abnormal development (for reviews see Scott et al., 1970; Hall, 1972).

Zinc

Experimental Animals. A deficiency of zinc in the maternal diet rapidly produces a wide variety of developmental defects in experimental animals (for reviews see Hurley, 1975; 1977a; 1977b). When normal female rats, fed a complete diet before mating, were given a zinc-deficient diet during pregnancy (days 0 to 21), about one-half the implantation sites were resorbed. The full-term young weighed about one-half that of controls, and 90 to 100 percent of fetuses showed gross congenital malformations. Food-intake controls had normal young.

Shorter periods of deficiency were also teratogenic. When the dietary zinc deficiency was imposed from days 6 to 14 of gestation, about one-half the young were abnormal. Even when the deficiency lasted for only the first 10 days of pregnancy, 22 percent of the full-term fetuses were malformed. Many investigators have confirmed the teratogenic effects of zinc deficiency in rats.

The congenital malformations produced by lack of zinc in rats are varied; they affect every organ system and occur in high incidence. A large number of skeletal malformations as well as soft tissue anomalies are seen. Malformations include cleft palate; cleft lip; short or missing mandible; curvature of the spine; club feet; syndactyly; curly or stubby tail; various brain anomalies such as hydrocephaly, anencephaly, and exencephaly; microphthalmia or anophthalmia; herniations; spina bifida; and heart, lung, and urogenital abnormalities. Frequencies ranged in one experiment from 13 to 83 percent of living young at term.

The malformations of the nervous system were especially noteworthy. In full-term fetuses of rats given the zinc-deficient diet from the beginning of pregnancy to term, 47 percent of the full-term fetuses had brain anomalies; 3 percent had spina bifida; and 42 percent had microphthalmia or anophthalmia. In addition, the spinal cord and olfactory tract also showed anomalies. Prenatal zinc deficiency affected many derivatives of the primitive neural tube.

Mild or Marginal Zinc Deficiency. Relatively mild states of zinc deficiency are probably more relevant to human problems than the extreme zinc deficiency described so far.

Marginal zinc deficiency has also been studied in pregnant rats. One approach was to correlate the level of zinc in the diet with the

incidence of malformations. With diets containing less than 9 ppm zinc during pregnancy, there was a high incidence of fetal death and malformation. Both total litter weight and fetal weight at term correlated with the level of dietary zinc up to 14 ppm, but there was no correlation between the incidence of malformations and the fetal zinc content or the maternal plasma zinc level at term. However, maternal plasma zinc during the second week of pregnancy was correlated with frequency of malformations.

In another experiment, the rats were fed the marginally deficient diet (9 ppm zinc) during pregnancy; this diet did not cause gross malformations at birth. At parturition, the rats were given a normal diet. The survival of offspring was significantly lower than in animals fed the normal diet throughout. Eighty-one percent of the living young born to stock-fed females survived to weaning, but only 46 percent of living young born to females fed the marginally deficient diet survived to this age.

Cross-fostering studies were carried out with appropriate controls; that is, offspring of females that received the marginally deficient diet during pregnancy were suckled by females normally fed during pregnancy, and pups of females normally fed during pregnancy were suckled by females fed the marginally deficient diet during pregnancy.

The survival of pups from females marginally deficient during pregnancy was the same whether they were suckled by their own mothers or by foster mothers fed normally during pregnancy. Conversely, the pups of females normally fed during pregnancy showed depression of postnatal survival when they were fed by females given the marginally deficient diet during pregnancy.

Thus, the maternal diet during pregnancy affected both the development of the offspring and the ability of the female to suckle her young. The offspring suffered irreversible effects of prenatal zinc deficiency; and, at the same time, the female's ability to suckle and/or care for her young was diminished by the deficiency during pregnancy. Marginal zinc deficiency during prenatal life thus caused an irreversible change which subsequently affected postnatal development.

A transitory deficiency of zinc during prenatal life was also used as a means of examining the effects of mild rather than severe zinc deficiency. When normal pregnant rats were given a zinc-deficient diet from day 6 to day 14 of gestation, maternal plasma zinc levels fell rapidly; but they quickly returned to original values after zinc was refed. Young born to these females showed a high rate of stillbirths, a high incidence of congenital malformations, low birth weight, and very poor neonatal survival.

Concentration of zinc in postpartum maternal plasma and milk (as well as in plasma of the pups) was normal, suggesting that postnatal zinc nutriture of the young was adequate. A short period of zinc deficiency during prenatal life thus caused an irreversible change that subsequently affected postnatal development.

Mechanism. Prenatal zinc deficiency affects many derivatives of the neural tube as well as those of other systems. The nature of these malformations, as well as their diverse origins, suggests that the action of zinc is on fundamental rather than secondary processes. Zinc deficiency in pregnant animals, as well as in nonpregnant and juvenile animals, primarily affects rapidly proliferating tissues—the embryo, the gonads, and the skin, where cell division is occurring at a rapid rate.

Present evidence suggests that congenital malformations in zinc-deficient embryos, as well as lesions of the gonads and skin, are brought about by impaired synthesis of nucleic acids. We think that the effect of zinc deficiency on nucleic acid synthesis produces an asynchrony of mitotic rhythms. This characteristic is manifested by the large numbers of cells observed in mitotic arrest, which then produce asynchronous growth patterns.

Prolongation of the mitotic interval and reduction in the number of specific cells early in development could combine to produce a wide range of abnormalities. Asynchrony in histogenesis and organogenesis could therefore result from alterations in differential rates of growth.

Experiments with a number of systems, in vitro as well as in vivo, and with various species, have shown a requirement for zinc in DNA synthesis. In zinc-deficient rat embryos at 12 days of gestation, incorporation of tritiated thymidine into DNA was much lower than normal, suggesting that DNA synthesis was depressed. The head region was more vulnerable than the body, but both could be brought back to normal levels by injection of zinc into the pregnant female prior to delivery.

The activity of thymidine kinase was also depressed in zinc-deficient embryos. The thymidine kinase pathway for the production of thymidine nucleotides is not prominent in normal adult cells but becomes important for DNA synthesis in tissues undergoing rapid cell division. Inasmuch as the effect of zinc deficiency on cell division has been found to be most extreme in rapidly proliferating tissues, the relationship of zinc to thymidine kinase in the developing embryo might be of critical importance.

Another enzyme involved in DNA synthesis is also depressed in zinc-deficient embryos. DNA-polymerase activity was lower in embryos from dams given a zinc-deficient diet than that in

controls. The normal increase with embryonic age of both of these enzymes did not occur in the zinc-deficient rats. Thus, decreased activity in thymidine kinase and DNA polymerase may lead to depression of nucleic acid synthesis in zinc deficiency. However, recent work with the microorganism *Euglena gracilis* (Falchuk et al., 1975) indicates that zinc may be required at every stage of the cell cycle; thus, thymidine kinase and DNA polymerase are probably not the only factors involved.

Zinc Deficiency in Humans. The occurrence of zinc deficiency in humans, resulting in hypogonadal dwarfism, in the Middle East is now well established. More recently, zinc deficiency has also been found in children in the United States (Hambidge et al., 1972).

There is now evidence that zinc deficiency is also teratogenic for humans. Epidemiological data may support a relationship between zinc deficiency and malformations of the central nervous system in humans: the two countries, Egypt and Iran, in which zinc deficiency was first found in people, both have high rates of such malformations (Sever and Emanuel, 1973; Sever, 1975).

Another type of evidence comes from women with the disease acrodermatitis enteropathica, a genetic disorder of zinc metabolism. Until it was learned (in the early 1970's) that the signs and symptoms of the condition could be cured with oral zinc therapy, patients were treated with a drug that permitted survival and growth, but they were not able to maintain plasma zinc at normal levels. Thus, women with the genetic trait who became pregnant had low concentrations of zinc in their blood plasma. The outcomes of these pregnancies were extremely poor. The numbers of miscarriages and of infants with malformations were very much higher than those in normal population. Out of seven pregnancies, there were two infants with major congenital malformations and one spontaneous abortion (Hambidge, 1975).

In Sweden, a correlation was found between low zinc in pregnant women and various complications of pregnancy. Women who gave birth to malformed infants, or to post-term infants, or who had abnormal deliveries, had significantly lower serum zinc levels than women whose deliveries and infants were normal or whose infants were of low birth weight (Jameson, 1976).

Conclusion

I have proposed that a deficiency of one or more specific nutrients in the maternal plasma may be involved in producing the

fetal alcohol syndrome. The nutrients that are most likely to be implicated are folate, zinc, and magnesium. Maternal dietary deficiencies of these essential factors are teratogenic in experimental animals, and there is also evidence in humans of deleterious effects on the offspring.

This hypothesis should be investigated by seeking correlations between maternal plasma levels of zinc, magnesium, and folate and erythrocyte folate (or other measures of folate status) and occurrence of FAS. These measurements should also be carried out in the infants themselves. Experimental animal models should be studied in relation to these nutrients.

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Discussion of Paper by Hurley

Dr. Schenker: I would like to share with you some unpublished data from our laboratory that bear on the questions you have raised. Dr. Henderson in our unit has developed an animal model of the fetal alcohol syndrome similar to that which has been described previously in mice and in other animal species. We have a very, very high rate of resorption, in the order of 50-fold, as well as a very low weight of these babies and a high mortality rate.

We were obviously interested in the same types of questions you have raised, and you might be interested to know that these animals are strictly pair-fed, so there is absolutely no difference in weight, which, of course, does not rule out the possibility of zinc deficiency.

What is against zinc deficiency, an important consideration, is the fact that the DNA levels in every tissue in the body are normal, and so is DNA synthesis. DNA synthesis, measured with thymidine and using the same approach you have reported, appears to be normal.

Now I was hoping to have the zinc levels today, but unfortunately they will not be ready until sometime next week; so we have to remain somewhat dubious as to what the final outcome will be. That is the reason I am asking which tissue one should really look at, because I was hoping for some help in that area.

But at the present time, at least in this animal model, and of course we do not know how relevant it is to humans, we would not think, in our unit, that zinc deficiency is a critical component of the fetal alcohol syndrome.

Dr. Hurley: Well, I think that until you actually have the data on the zinc content of the fetuses you really cannot say whether it is or not. The total DNA content could be normal and still have an abnormal synthesis.

Dr. Falchuk: I think it is important, in view of the fact that thymidine incorporation has been used in this particular type of experimentation and in the data, that you suggested DNA content itself is used. There are differences in terms of interpretation when one uses a label to indicate about DNA synthesis. And as

you saw, in even the *Euglena*, where we know for a fact that there is zinc deficiency, the DNA content also does not determine what is going on in terms of the metabolism. But I think it further indicates the issue that you raised about zinc content itself.

From Dr. Hurley's data, you saw that during the time when the mother's serum zinc level fell down to about 40 percent of the normal, between 6 and 14 days, if you measured the serum zinc in the mother, you would say she is depleted of zinc. Yet there is no evidence in the mother that she has any problems with zinc metabolism. And yet, at the same time, the fetus, obviously, is undergoing some remarkable changes in terms of its metabolism of zinc.

So I think what Dr. Vallee was trying to say is that it is difficult to make an assessment of zinc nutriture and its relationship to metabolic events in cell division from a measurement (such as zinc content, DNA content, or thymidine incorporation), unless you actually know everything else that is going on.

In terms of these types of experiments, it is clear that it is not the maternal zinc content that is a problem, even though that is low in one tissue; but, from Dr. Hurley's data, the liver zinc content is normal, the bone content is normal, so the mother is perfectly all right. Yet, depending on what tissue you biopsy and measure, the zinc content can vary. So I think it is just a matter of principle in terms of what a number means metabolically. And the truth of the matter is, I am totally confused about how to answer that question (which is asked all the time whenever I talk any place), because everyone wants to know the answer. Well, I would like to give it, and I think each of you would also like to give it.

Dr. Hurley: I certainly do agree, Dr. Falchuk.

Hypothesis Concerning the Effects of Dietary Nonsteroidal Estrogen on the Feminization of Male Alcoholics*

Roger Lester, David H. Van Thiel, Patricia K. Eagon, A. Forrest Imhoff, and Stanley E. Fisher

Hardcore, chronic alcoholic men commonly are feminized. Changes in their sexual function and appearance include impotence, sterility, testicular atrophy, altered hair distribution, gynecomastia, and cutaneous vascular changes. These changes can be ascribed to two basic mechanisms: hypoandrogenization and hyperestrogenization (1).

An initial characterization of the pathogenesis of the hypoandrogenization of the male alcoholic has been provided (1,2,3,4, 5,6). It is now abundantly clear that this phenomenon is due to the direct effect of alcohol on the testis, to coexistent hypothalamic-pituitary suppression by alcohol and thus to diminished central stimulation of the testis, and to changes in hormonal metabolism produced by alcohol-induced changes in the liver. The net result of these changes is to decrease the amount of testosterone available to target tissues and to diminish and ultimately to eliminate spermatogenesis (2).

The pathogenesis of the hyperestrogenization of the male alcoholic has remained in greater doubt. Despite unequivocal evidence of symptomatic and biochemical evidence of severe hyperestrogenization, most alcoholic men maintain normal plasma concentrations of estradiol (E_2), the principal female sex hormone (2,7,8,9,10,11,12). It has been suggested that normal E_2 levels in the presence of diminished testosterone levels might produce hyperestrogenization (11), but this concept is not universally accepted. In one study of patients with liver disease, total plasma E_2 was normal, but unbound E_2 was increased (11). Unfortunately, this attractive hypothesis has not been confirmed in all other studies. Presumably as the result of increased peripheral conversion

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of adrenocortical steroidal precursors, plasma estrone (E_1) concentrations are elevated (4,8). On the other hand, E_1 is a weak estrogen with only one-fifth the potency of E_2 when measured by standard bioassay techniques, and the E_1 elevations observed in chronic alcoholics have been of a modest degree.

We have entertained an alternative hypothesis. Nature abounds in nonsteroidal substances that are weakly estrogenic (13). These substances are found in a variety of plant species that might form the basis for preparations of food or drink. There are animal models of the effect of excessive ingestion of plants high in nonsteroidal estrogens; hyperestrogenization of both quail and sheep have been shown to occur as the result of grazing on certain species of plants known to contain certain nonsteroidal estrogens (14,15). Presumably, the amount of nonsteroidal estrogen in the normal human diet is insufficient to produce estrogenic effects in the human. In the presence of an abnormal diet, or, more likely, if insufficient metabolism of nonsteroidal estrogenic substances were to occur because of the presence of advanced liver disease, sufficient quantities might accumulate in the plasma to produce estrogenic effects.

The standard tests for plasma estrogens are radio immunoassays that are highly specific, and therefore nonsteroidal plasma estrogens would remain unmeasured, even in the presence of quantities sufficient to induce marked hyperestrogenization. Consequently we have searched for an alternative measure of estrogenicity sufficiently sensitive to detect the low concentrations of material anticipated to be present in plasma. This method must also discriminate between estrogens of a variety of chemical configurations and other substances with no estrogenic activity.

For this purpose, we have examined cytoplasmic hepatic estrogen receptors in male and female livers. In brief, estrogen receptors can be demonstrated in the $100{,}000 \times g$ supernate of both male and female liver homogenates. A high molecular weight protein, existing as the major E_2 binding protein in female supernatant and a minor E_2 binding protein in the male supernatant, is demonstrable. This protein specifically binds both steroidal and nonsteroidal estrogen; nonestrogenic hormonal steroids, bile acids, and endogenous and exogenous substrates transported and metabolized by the liver fail to interact with it.

The principal E_2 binding protein in male hepatic, cytoplasmic supernatant is a low molecular weight protein with somewhat different binding characteristics. This protein specifically binds steroidal estrogen but does not interact with such nonsteroidal estrogens as diethylstilbestrol. As with the high molecular weight

E_2 binding protein, no interactions are exhibited with other nonestrogenic steroidal hormones, bile acids, or liver-metabolized substrates.

The male and female E_2 binding proteins might thus serve as the basis for protein binding assay for nonsteroidal estrogen. ^3H-E_2 is incubated with 100,000 $\times$ g supernate of male and female liver homogenate. Standard displacement curves are generated by adding varying quantities of steroidal and nonsteroidal estrogens. The results are expressed as a ratio of ^3H-E_2 bound after displacement/^3H-E_2 bound with no added estrogen and are plotted against the logarithm of the molar amount of estrogen added. The data are subjected to logit transformation to produce straightline standard curves. Plasma samples are then extracted in ether, and three concentrations of the extract are used to test for displacement of the tracer ^3H-E_2. The results are then expressed as equivalents of steroidal or nonsteroidal estrogen. Nonsteroidal estrogens should produce displacement of tracer ^3H-E_2 from the predominant female, but not from the predominant male, estrogen binding protein.

The results are in much too formative a stage at this time to report definitive data. However, in a brief survey of 15 sera from hardcore male alcoholics with liver disease, 3 sera were found that appeared to behave as if nonsteroidal estrogen were present. Displacement was observed at an order of magnitude greater than could be accounted for by the known concentrations of steroidal estrogen measured by radioimmunoassay in the samples. ^3H-E_2 was displaced from female, but not from male, rat liver supernate. These preliminary data may represent the first demonstration of nonsteroidal estrogen accumulating in the plasma in association with liver disease.

In summary, it is difficult to explain the marked hyperestrogenization of alcoholic men in terms of measurable levels of plasma steroidal estrogen. It is possible that the observed hyperestrogenization is due to the accumulation of dietary nonsteroidal estrogen as the result of liver disease. One means to approach this problem is through the use of a protein-binding assay, using the discriminatory characteristics of the binding proteins in male and female rat liver cytosol. Studies are now in progress to explore this hypothesis.

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Discussion of Paper by Lester et al.

Dr. Schenker: I think one of the most interesting parts is the indication that the cellular receptor that binds estradiol differs between the male and female, because as you set it up against all these different estrogenic substances, you see a different pattern. I wonder if you could elaborate on that. Are there other indications from other types of study about this? And what is the mechanism of this difference in the estrogen receptors? Does it have anything to do with the determination of secondary sex characteristics? How does it fit into endocrinology?

Dr. Lester: Of course, the receptor that we are talking about is the receptor in the liver cell. We have no idea whether this sort of difference may exist elsewhere, and we were most surprised to find this. We had studied the male almost as an afterthought and therefore were very surprised to find the difference in biologic activity. I might say that the difference in activity that I have shown is not absolute; one can doctor the conditions of one's assay and make the male receptor behave like the female receptor. That is, it will react under certain in vitro conditions with things like diethylstilbestrol, nonsteroidal estrogens. So we wondered whether this was some very bizarre form of artefact we had introduced. But in addition to this difference in activity, at least under certain in vitro conditions, there are indeed other differences between the male and female estrogen receptor in the liver. The molecular weight of the two receptors (as determined at least in a crude way) on sucrose gradient is distinct. Female receptor seems to weigh approximately 200,000 daltons. The male receptor appears to weigh less than half that.

Dr. Lieber: I wonder what additional evidence you might have to show that you are dealing with dietary substances.

Dr. Lester: It is a perfectly reasonable question to ask, and I might say that, in the course of talking about dietary substances, I intend to include the possibility that some of these substances are in alcoholic beverages. That is why I was really so fascinated with the results discussed earlier that suggested maybe there were activities in the dealcoholized residuum of wine. In certain species that eat an excess of certain foods, hyperestrogenization, in fact, occurs. It occurs specifically in a kind of quail that eats an

excess of certain seeds containing an excess of estrogenic materials, and it occurs in sheep that consume huge amounts of clover, which happens to contain an excess of certain estrogenic materials. So the precedent is there in nature; and the interaction of liver disease with even a moderate intake might conceivably produce the changes that I am talking about.

I should warn you that, in the usual sense, these are very weak estrogenic substances we are talking about, with the strength of about 1:10,000 to 1:100,000 of the strength of estradiol. On the other hand, in the presence of liver disease, with the inability to metabolize them, it is perfectly conceivable that they might accumulate in excess and produce biologic effects in a human.

Dr. Lumeng: You isolated the estrogen receptor from rats, is that correct?

Dr. Lester: The source of the receptor was rats.

Dr. Lumeng: Do you have evidence that a similar receptor exists in human liver?

Dr. Lester: No, none.

Dr. Lumeng: Are you pursuing studies to confirm that these receptors are also present in human liver? Otherwise, as I understand your goal, you merely use these receptors from rat liver as an assay tool.

Dr. Lester: Yes, that is absolutely right. Our intention is not to worry about the interaction of estrogen with the liver receptor, but rather to use the receptor, as I say, as kind of an assay procedure. We did not really care whether it was a human source of serum and rat source as a receptor. Our only intention was to use it as a source of an assay, just as one might use rabbit antibody to do a radioimmunoassay on the plasma of human material. The disconnection between the source is not important to us. But yes, we are interested in knowing how wide a species variation there is in this line. We have looked at frogs; as it turned out, the estrogen receptor in frogs is different in a variety of ways from that in rats.

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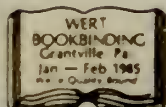

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