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EFFECTS OF LONG-TERM INGESTION OF ETHANOL ON SELECTED ASPECTS OF ETHANOL METABOLISM AND LIPOGENESIS IN MOUSE LIVER

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EFFECTS OF LONG-TERM INGESTION OF ETHANOL ON SELECTED ASPECTS OF ETHANOL METABOLISM AND LIPOGENESIS IN MOUSE LIVER

by

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Abstract

EFFECTS OF LONG-TERM INGESTION OF ETHANOL IN SELECTED ASPECTS OF ETHANOL METABOLISM AND LIPOGENESIS ON MOUSE LIVER

by

ZEV STERN

Adviser: Professor George H. Fried

Mice of the C57Bl/6J strain were fed a liquid diet in which 36% of the calories consisted of ethanol. Control mice were pair-fed a diet in which ethanol was isocalorically replaced by carbohydrate. Ethanol-fed mice developed fatty livers. Control, but not ethanol-fed mice, gained weight throughout the experiment. Ethanol-fed mice were depleted of hepatic glycogen. Activity of the microsomal ethanoloxidizing system (MEOS) tended to rise in ethanol-fed mice, but this increase did not reach the 5% level of significance. There was a highly significant decrease in activity of malic enzyme, an enzyme involved in the synthesis of fatty acids, in ethanol-fed mice. Activity of lactate dehydrogenase also declined in ethanol-fed mice, while activities of glucose-6-phosphate dehydrogenase and alphaglycerophosphate dehydrogenase did not change. It is suggested that the very low carbohydrate content, and the relatively high fat content, of the ethanol-containing diet, as well as ethanol itself, are involved in these metabolic alterations. It appears that, under these conditions,

livers of ethanol-fed mice synthesize very little fatty acids de novo. This situation is contrasted with genetic obesity, which lipogenic enzyme activities are elevated in livers that are accumulating lipid.

DEDICATION

This is dedicated to my beloved wife Madelyn, without whose help and support during my long and sometimes tortuous studies I would certainly have accomplished nothing, and also to my son Nehemiah Akiva and daughter Sarah Aliza.

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I wish to express my thank and appreciation to my sponsor, Prof. George Fried, who placed at my disposal his laboratory and equipment, helped me navigate through the bureaucracy whenever supplies were needed, and was of invaluable assistance in innumerable other ways during the course of my studies. He will always be regarded as not merely a sponsor and mentor but a friend.

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Last but not least, thanks are due to Ms. Lenore De Carli at the laboratory of Dr. Charles S. Lieber at Bronx Veterans Hospital, for her helpful suggestions concerning the MEOS assay, and to Ms. Pamela Slavin for typing the manuscript.

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INTRODUCTION

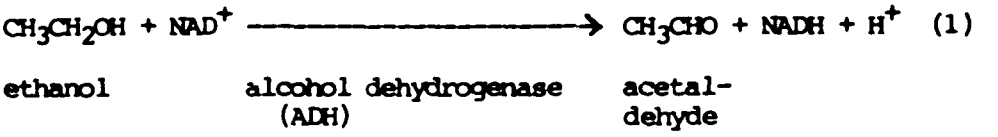
Alcoholism is a major social and public-health problem in the United States. The contribution of ethanol (C_2H_5OH) to traffic accidents is well publicized, as are ethanol's a deleterious effects on mental functioning. These effects are seen in normally sober individuals following an occasional drinking binge, while the disease of alcoholism is characterized by long-term heavy ingestion of the substance leading to a fairly constant blood alcohol level. Aside from the cerebral effects and motor impairments mentioned above, the chronic disease also has profound somatic effects, especially on the liver, the principal site of ethanol metabolism (Lieber 1975b). The course of liver injury typically begins with fatty liver, reversible if consumption of ethanol is discontinued, and progresses to alcoholic hepatitis and finally cirrhosis. The increasing incidence of alcoholism in urban areas of the United States has led to a parallel increase in the death rate from cirrhosis, though not all alcoholics develop cirrhosis.

It has been uncertain whether these effects are the result of ethanol per se, of congeners present in alcoholic beverages, or of malnutrition. Alcoholics tend to be malnourished due to a number of factors. Ethanol supplies 7.1 kilocalories per gram. Thus, 500 ml of 86 proof whiskey, a typical daily intake for an alcoholic, supplies about 1200 kcal, roughly half the individual's caloric needs, but supplies no protein, vitamins, minerals or fiber. Ethanol also irritates the gastrointestinal tract, leading

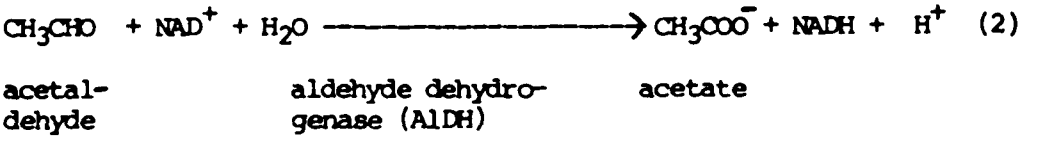
to intestinal malabsorption and decreased appetite; ethanol, a very small molecule, is absorbed readily. In addition, socio-economic factors may interfere with an alcoholic's ability to obtain wholesome food.

A rat model was developed to separate the effects of ethanol from those of malnutrition (De Carli and Lieber 1967). Experimental animals consume, as their sole source of food and drink, a liquid diet of which 35% of the calories are supplied by ethanol. In control animals ethanol is replaced by a calorically equivalent amount of carbohydrate (malto-dextrins). In both diets fat (olive oil, corn oil, and ethyl linoleate) contributes 35% of the calories and protein (mostly casein) supplies 18%, with the balance consisting of carbohydrate. Adequate vitamins and minerals are added. Both diets are made up to a density of one kilocalorie per ml. Thus, the ethanol-containing diet contains less than 6.5% ethanol by volume, a concentration unlikely to cause gastrointestinal irritation. Under this regimen both ethanol-fed rate and pair-fed controls maintained or increased their weights and appeared healthy. However, after 24 days of ethanol feeding rats developed fatty livers. Alcoholic hepatitis and cirrhosis never developed, presumably because the rats' life span is too short. It appeared from this work that the metabolic effects of ethanol are genuine, and not mere artifacts of malnutrition.

About 90% of ingested ethanol is metabolized in the liver. When ethanol intake is light, essentially all is metabolized by the following pathway:

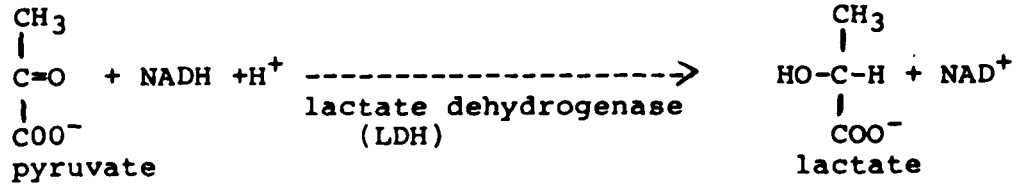


(NAD = Nicotinamide Adenine Dinucleotide)



These two reactions result in an accumulation of NADH, which can be reoxidized by the following mechanisms:

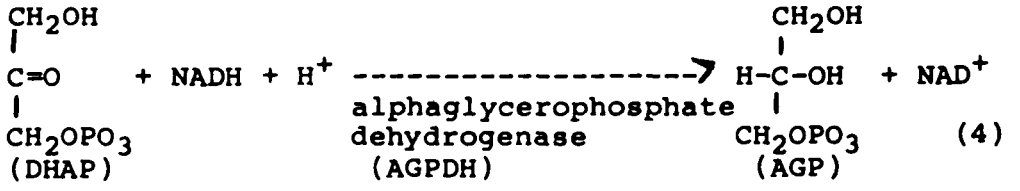
1) An increase in the ratio of lactate to pyruvate:



The consequent buildup of lactate can lead to metabolic acidosis. It also interferes with excretion of uric acid by the kidney, which may explain the observation that gout is often aggravated by ethanol abuse (Newcombe 1972). Gluconeogenesis is inhibited by the reduced availability of pyruvate; this can precipitate acute hypoglycemia in alcoholics who are glycogen-depleted (Lieber 1975a).

2) Electron flow from NADH to molecular oxygen, with phosphorylation of adenosine diphosphate (ADP) to form adenosine triphosphate (ATP). This is the usual metabolic pathway for extracting energy from nutrients; thus, ethanol is displacing dietary fat and carbohydrate. High concentration of NADH and ATP inhibit the Krebs cycle; Krebs cycle substrates may then contribute to the appearance of fatty liver (Lundquist, 1975).

3) Enhanced lipogenesis. NADH reduces dihydroxyacetone phosphate (DHAP) to alphaglycerophosphate (AGP):



(4)

Simultaneously, through transhydrogenation to nicotinamide adenine dinucleotide phosphate (NADP) to produce NADPH, NADH derived from ethanol provides reducing power for the synthesis of fatty acids (Lundquist 1975, Lieber 1975a). These are esterified to glycerophosphate to form triacylglycerols (triglycerides).

While the first two pathways are limited, respectively, by the supply of pyruvate and the demand for ATP, synthesis of glycerophosphate and fatty acids is limited in principle only by the liver's capacity to synthesize, secrete and ultimately accumulate triacylglycerols and related lipids.

Excess fatty acids can also be metabolized to ketones, precipitating alcoholic ketosis (Lieber 1975a).

Lieber and De Carli (1970a) found that cytosolic alcohol dehydrogenase activity did not increase in rats after long-term feeding with ethanol. However, Sze (1975) obtained opposite results, i.e. ADH was induced as long as the adrenals were intact or glucocorticoids were administered.

In rats subjected to long-term feeding of ethanol, as well as in human alcoholics, a fundamentally different metabolic pathway comes into play. This is known as the microsomal ethanol oxidizing system (MEOS). It is associated with the smooth endoplasmic reticulum, unlike ADH, which is located in the cytosol, but similar to other hepatic drug detoxifying systems. This mixed-function oxidase utilizes as a cofactor the reduced compound NADPH. Molecular oxygen serves as the hydrogen acceptor in the following reaction:

$$CH_3CH_2OH + NADPH + H^+ + O_2 \xrightarrow[(MEOS)]{} CH_3CHO + NADP^+ + 2H_2O \quad (5)$$

This pathway can be thought of as another metabolic strategy to dispose of excess reducing power generated by the oxidation of ethanol. However, unlike the pathways outlined above, this one does not conserve energy either as ATP or as fat. Energy from the ethanol-derived calories is dissipated as heat; thus these calories are not fully available to

support the energy and anabolic requirements of the organism. This was further demonstrated by experiments on detoxified alcoholics hospitalized in a metabolic ward (Pirola and Lieber 1972). In the first experiment, subjects were given a diet on which they maintained their body weight. When carbohydrate was gradually and isocalorically replaced by ethanol until the latter provided 50% of the calories, the subjects lost weight. In the second experiment, 2000 kilocalories per day were added to the maintenance diet as either ethanol or chocolate. Chocolate resulted in more and steadier weight gain than did ethanol.

The nature of the microsomal system has been a subject of debate, with some investigators speculating on a role for catalase:

$$H_2O_2 + CH_3CH_2OH \xrightarrow[\text{catalase}]{} CH_3CHO + 2H_2O \qquad (6)$$

(Thurman et al. 1972). However, MEOS and catalase have been separated by column chromatography (Teschke et al. 1972, 1974). The MEOS fraction oxidized ethanol in the presence of NADPH or a NADPH-generating system. There was no activity with an H_2O_2-generating system unless exogenous catalase was supplied, in which case activity was about half that promoted by NADPH. Moreover, Ohnishi and Lieber (1977) separated MEOS into three components: cytochrome P450, NADPH: cytochrome c reductase, and phospholipid. Reconstitution experiments showed that the increase in activity observed when rats are subjected to long-term

feeding with ethanol can be attributed to cytochrome P450. Also, electrophoresis reveals qualitative changes in cytochrome P450 from ethanol-fed rats. These reconstituted systems showed no alcohol dehydrogenase or catalase activity, and were insensitive to inhibitors of both these enzymes.

The involvement of cytochrome P450 is a feature that MEOS shares with other hepatic microsomal drug detoxifying systems (Kato 1966); so is an oxidative pathway that wastes energy. It is suggested that this similarity partially explains the observed cross-tolerance and synergism of ethanol with other drugs, e.g. barbiturates. If ethanol and other substances compete for the same detoxifying enzymes, ethanol would be expected to slow metabolism of these substances, which is, in fact, observed in alcoholics (Rubin et al 1970). However, enhanced detoxifying activity in alcoholics should accelerate the clearance of other drugs when ethanol is absent. This also agrees with observations (Kater at al. 1969, Misra et al. 1971). Some exogenous substances, including many carcinogens, are activated instead of detoxified by microsomal oxidases. Accordingly, long-term treatment with ethanol enhances the toxicity of carbon tetrachloride (Hasumura et al. 1974), and alcoholics are more sensitive to carbon tetrachloride than are non-alcoholics (Moon 1950). The hepatotoxicity of an overdose of acetaminophen is similarly increased by prior long-term feeding of ethanol (Sato et al. 1981, Altomare et al. 1984, Tredger et al. 1985).

In a newly discovered metabolic pathway operating in humans, ethanol is esterified to long-chain fatty acids to form fatty acid ethyl esters (Laposata and Lange 1986):

$$CH_3CH_2OH + R\text{-}\overset{\overset{O}{\|}}{C}\text{-}OH \longrightarrow R\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}CH_2CH_3, \qquad (7)$$

where R is a hydrocarbon chain.

This pathway operates in several organs, including the liver, in alcoholics and non-alcoholics. Its contribution to total ethanol metabolism appears to be small. Whether this pathway exists in nonhuman species and, if so, how great a role it plays, has not been ascertained.

The metabolic alterations produced by long-term treatment with ethanol are accompanied by structural alterations in liver cells. Fat droplets accumulate in the cytoplasm, mitochondria are distorted and the smooth endoplasmic reticulum (SER) proliferates (Iseri et al. 1966). Proliferation of SER is also seen in response to various other drugs (Meldolesi 1967). When smooth and rough endoplasmic reticulum were separated by ultra-centrifugation, MEOS and cytochrome P450 were increased in the smooth fraction, and catalase was actually decreased (Ishii et al. 1973).

Most of the work to date on long-term feeding with ethanol has been done with rats. However, there are

Most of the work to date on long-term feeding with ethanol has been done with rats. However, there are advantages in studying this problem in smaller rodents which are easier and less expensive to maintain. More is known about the genetics of mice than rats (Green 1975) and a greater number of inbred strains are available. Of particular interest is the obese (ob) mutation (Ingalls et al. 1950). Mice homozygous for this allele become markedly obese at about one month of age, and are characterized by hyperphagia, inactivity, moderate hyperglycemia, hyperinsulinism and insulin resistance. Enhanced lipogenesis is reflected in increased activities of lipogenic enzymes in the liver (Fried and Antopol 1966). Since ethanol also promotes disordered lipid metabolism, it would be interesting to compare the two conditions and to study possible interactions.

Aspects of ethanol metabolism are under genetic control. Some humans appear to inherit a predisposition to alcoholism, and individual humans, as well as racial groups, vary in their rates of ethanol metabolism. Human alcohol dehydrogenase was shown to be polymorphic; the relative amounts of the various forms in a given liver depend, in part, on the genetic background of the donor (Li et al. 1977, Bosron et al. 1980). Genetic differences in the inducibility of MEOS in mice have also been found (Hjelle et al. 1981). Hence, the genetic background of experimental animals used in experiments on ethanol metabolism must be specified; one cannot assume that findings in one strain

will hold in another. This study used the inbred strain C57Bl/6J, developed at Jackson Laboratories. This is the strain in which the ob mutation was discovered and which is maintained by Jackson. Lean mice of this strain were employed to determine:

1) Whether long-term treatment with ethanol can produce a fatty liver in mice of this strain.

2) The possible inducibility by ethanol of lipogenic enzymes, alcohol dehydrogenase and the microsomal ethanol oxidizing system.

3) Effects of ethanol treatment on hepatic glycogen levels, which might involve an aspect of the influence of ethanol on carbohydrate metabolism.

4) Structural alterations in the liver that might be produced by ethanol treatment. The endoplasmic reticulum and mitochondria are major sites where alterations might be expected.

If the ethanol-fed mouse is to be considered comparable to the ethanol-fed rat as a model of the human disease, than ethanol feeding must, at a minimum, produce a fatty liver. If fatty livers do not develop, that would be significant in itself and would warrant further study of ethanol metabolism in this species. If a fatty liver is produced, it could represent either a physiologic adaptation to protracted ingestion of ethanol or a pathologic change premonitory to the liver injury seen in longer-lived species, including

humans, as a consequence of ethanol abuse.

The enzyme studies reported in this research can help distinguish between these possibilities. As increase in lipogenic enzyme activities would indicate a liver that, like those of genetically obese mice, is highly competent and adapts well to the metabolic challenge. A decrease in these activities would suggest fatty degeneration rather than adaptive hyperlipogenesis. This impression would be reinforced if histological examination reveals ultra-structural derangements.

Since mice used in these studies consumed isocaloic diets, ethanol has to replace another dietary component or components. As in the studies performed by other investigators on rats, carbohydrate was chosen as the nutrient to be replaced. Thus, the ethanol-fed mice consumed very little carbohydrate. This may be a significant metabolic stress, independent of ethanol. Inadequate carbohydrate intake would be expected to cause depletion of hepatic glycogen reserves; if livers of ethanol-fed and control mice are similar in glycogen content it would be difficult to attribute other findings to low dietary carbohydrate.

It should be noted that ethanol is relatively new to the human diet and is almost entirely absent from the diet of rodents in nature. It is therefore unlikely that enzymes of ethanol metabolism evolved as such. One may speculate on the identity of the "natural substrates" of these enzyme

systems. Cholesterol, a metabolite of cholesterol, or some similar hydroxylated compound may be candidates. In view of the role of P450-based systems in detoxifying xenobiotics (see above, p. 7), the natural function of MEOS may be to detoxify any of a variety of toxicants naturally present in the plant foods that rodents in nature eat (Ames 1983). The inducibility or non-inducibility of ethanol-metabolizing enzymes by ethanol may raise questions for future research into the evolution of these enzyme systems.

MATERIALS AND METHODS

Animal maintenance and breeding

C57Bl/6J mice were purchased at about five weeks of age from Jackson laboratories or derived from brother-sister matings in our colony. Those derived from our colony were weaned at about three weeks of age. Mice were housed in a temperature-controlled (20-25°) room with twelve hours of light and twelve hours of darkness per day. No more than four mice of the same sex were housed together, and breeding cages consisted of one or two females with one male. Cages were of the wire-bottom type, set directly on aluminum trays with heat-treated wood chips for bedding. Mice were given free access to water and Purina Mouse Chow while awaiting experiments. Males were switched to Purina Rodent Laboratory Chow on attaining a weight of 25g, unless they were part of a mating group, in which case they and the females were kept on Mouse Chow. Mouse Chow has about twice the fat content of the more widely used Rodent Laboratory Chow; it was found that the latter, while adequate as a maintenance diet, did not provide satisfactory growth and reproduction with the C57Bl/6J strain.

Experimental diet

For ethanol-feeding experiments, male mice at least ten weeks of age were fed, as their sole source of food and drink, a liquid diet based on that of Lieber and his colleagues (De Carli and Lieber 1967). The diet was purchased from BioServ (Frenchtown, NJ) and reconstituted

purchased from BioServ (Frenchtown, NJ) and reconstituted with water, ethanol, and if necessary, malto-dextrins to the desired ethanol and carbohydrate content. Dry diet was kept refrigerated and used within four months of purchase. Ethanol was introduced very gradually, from 7% of total calories to 35% in increments of 7% at intervals of no less than two days. Control mice were pair-fed the identical diet with carbohydrate (malto-dextrins) isocalorically substituted for ethanol. Spontaneous food intake was monitored for the first few days; thereafter the animal that consumed more (almost always the control) was given the volume consumed by the paired mouse on the previous day. Fat (corn oil, olive oil, and ethyl linoleate) comprised 35% of the calories and protein (casein with supplemental methionine) contributed 19%. When reconstituted the diets contained one kilocalorie per ml. Diets were given in graduated drinking tubes (purchased from BioServ) and renewed at least once daily. Mice were housed in individual cages while receiving liquid diets, and were sacrificed when they had been receiving 35% of their calories as ethanol for at least three weeks.

Extraction and quantitation of hepatic lipids

Mice were weighed, than sacrificed by decapitation and exsanguinated. The abdominal cavity was opened, the gall bladder removed and the liver quickly excised, rinsed in ice-cold 0.9% NaCl, blotted dry and weighed. Lipids were

extracted using a modification of the Folch procedure (Folch et al. 1957). Pieces of liver weighing about 300 mg were weighed and homogenized in 20 volumes of chloroform-methanol (2:1) with a motor-driven pestle. The final volume of homogenate was noted and homogenates were transferred to conical centrifuge tubes, covered with aluminum foil and refrigerated for several hours or overnight. Any solvent lost to evaporation was then replaced and the tubes were mixed and centrifuged for 15 minutes in a table-top clinical centrifuge. Two-ml aliquots were taken from the supernatants, mixed with 0.2 volume of water and centrifuged again for 15 minutes. The upper (polar) phase was aspirated and discarded and the walls of the tubes were washed three times with pure upper-phase solvent. Enough methanol was added to make one phase of the lower (nonpolar) phase and any remaining rinse fluid. Aliquots of 0.1-0.2 ml were taken in triplicate from each sample, transferred to large test tubes and evaporated to a whitish emulsion under a stream of nitrogen. Lipid in the emulsion was measured using a modification of the method of Amenta (1964). 2.0 ml of 0.25% potassium dichromate in concentrated sulfuric acid were added to each tube, and the tubes were covered with marbles and placed in a boiling water bath for 30-40 minutes. The tubes were then cooled, and aliquots were diluted 40-fold with water. Absorbance at 350 nm was measured with a Gilford 240 spectrophotometer against a blank of water. Standards were prepared with 0.1% tripalmitin in chloroform-methanol, from which aliquots

containing up to 250 µg tripalmitin were treated as above, except that evaporation proceeded to dryness. Included in the standards was a "zero control" consisting of solvent only. Since many substances reduce dichromate, care was taken to keep glassware scrupulously clean and measurements of standards and samples were made in triplicate. Absorbance was plotted against quantity of tripalmitin; this plot had a negative slope since absorbance (by dichromate) and quantity of lipid were inversely related. The lipid content of the samples was determined from the standard plot.

Extraction and quantitation of hepatic glycogen

Glycogen was extracted from livers and measured by modifications of the methods of Good et al. (1933) and Seifter et al. (1950). Centrifuge tubes were charged with 2-3 ml of 30% KOH, covered with marbles and weighed. A small piece of liver weighing 0.2-0.3 g was added to each tube. The tubes were weighed again and the exact weight of the tissue calculated by difference. The tubes were heated in a boiling water bath, shaking every five minutes, for approximately 15 minutes or until homogeneous suspensions were obtained upon mixing. The suspensions were cooled in an ice bath. 0.5 ml of saturated Na_2SO_4 and 1.1 volumes of 95% ethanol were added, making a final ethanol concentration of 50%. The tubes were mixed, kept on ice for one hour, mixed again, heated to just below boiling, cooled on ice and

mixed. The mixtures, now containing white precipitates of glycogen, were spun down in a clinical centrifuge. The supernatants were discarded and the tubes drained by inversion over paper towels. Precipitates were redissolved in 2 ml of water and reprecipitated with 1.1 volumes of 95% ethanol. The washed precipitates were dissolved in up to 5 ml of water and an aliquot of each was diluted 50-to-100-fold, keeping the final volume at 5 ml. These final dilutions were transferred to large test tubes. Each tube was placed in a beaker of ice-cold water kept in an ice bath, and 10 ml of 0.2% anthrone in concentrated H_2SO_4 ("anthrone reagent") were added from a fast-flowing burette. The tubes were then cooled in the ice bath, covered with marbles and heated in a boiling water bath for ten minutes. This procedure hydrolyzes the glycogen and converts the resulting glucose to a chemical species that absorbs light at 620 nm. The tubes are then cooled again in an ice bath and allowed to come to room temperature. Absorbance at 620 nm is then determined spectrophoto-metrically. Standards consisted of 20-100 µg of glucose dissolved in 5 ml of water, to which anthrone reagent was added and which were then handled like the glycogen solutions. Absorbance was plotted against quantity of glucose. Glucose values for the precipitated tissue extracts, determined from the standard curve, were divided by 1.11 to derive glycogen values.

Anthrone reagent was prepared fresh as soon as possible before use. Glycogen solutions in water were frozen overnight when convenient. Preliminary studies showed that this freezing does not affect the results.

Enzyme assays

Preparation of tissues

Mice were sacrificed and livers obtained and weighed as previously described. If mitochondria were desired for assay of cytochrome oxidase, the liver was homogenized in nine volumes of a mitochondrial isolation medium consisting of 0.25 M sucrose, 20 mM Tris-HCl pH 7.4, and 10 mM EDTA, using a motor-driven pestle. The homogenate was centrifuged at 1000 x g and 2° for 10 minutes in a Sorvall RC-2B refrigerated centrifuge. The nuclear pellet was discarded and the supernatant was centrifuged for 20 minutes at 2° and 17,000 x g. The resulting mitochondrial pellet was washed twice by resuspending in isolation medium and centrifuging for five minutes at 2° and 17,000 x g. The washed mitochondria were resuspended in 5 ml of isolation medium and kept on ice; cytochrome oxidase was assayed the same day. If microsomes were desired they were isolated from the supernatant (see below); otherwise the supernatants were saved for assay of cytosolic enzymes.

If mitochondria were not desired, livers were homogenized in nine volumes of 0.15 M KCl. Homogenates were centrifuged at 9000 x g if microsomes were desired, otherwise at 12,000 x g. Centrifugations were carried out at 2° for 30 minutes; the pellets were discarded and the supernatants recentrifued under the same conditions for 10 minutes. If necessary, fat cakes were aspirated with a Pasteur pipette. Supernatants from which microsomes were to be isolated were centrifuged for 60 minutes at 2° and 100,000 x g in a Beckman Model L-3 ultracentrifuge with the #50-Ti rotor. The pellets were washed by resuspending in 0.15 M KCl and centrifuging for 30 minutes under the same conditions. Washed microsomes were resuspended in 0.15 M KCl such that 1.0 ml of suspension represented the microsomes from 400 mg of liver (wet weight). Assay of MEOS was performed the same day. Supernatants remaining from isolation of mitochondria and microsomes, as well as 12,000 x g supernatants, were saved for assay of cytosolic enzymes. Alphaglycerophosphate dehydrogenase was assayed on the day of preparation; samples were frozen at -20° until assay of ADH, glucose-6-phosphate dehydrogenase (G6PD), malic enzyme and LDH.

Livers could be rinsed in 0.9% NaCl and frozen for as long as two months for assay of the cytosolic enzymes, cytochrome oxidase and MEOS; glycogen and lipids were isolated from fresh livers only.

Assay procedures

Cytochrome c was reduced and cytochrome oxidase assayed using a modification of the method of Wharton and Tzagoloff (1967). A 1% solution of cytochrome c was prepared in 50 mM Tris-HCl pH 8.0. The solution was reduced with sodium hydrosulfite powder, not sodium dithionite as Wharton and Tzagoloff used. A small quantity of reductant was slowly added to and dissolved in the cytochrome solution, resulting in a color change from deep red to orange red. Addition of hydrosulfite was stopped when the color change was complete. The flask was stoppered and shaken vigorously to oxidize excess hydrosulfite. Nitrogen gas was then bubbled through the solution until it no longer smelled of H_2S. The solution's absorbance was measured spectrophotometrically at 565 nm and 550nm, and the proportion of reduced cytochrome was calculated from the formula:

$$\frac{A_{550}}{A_{565}} = \frac{19x + 9}{-4.5x + 7.5} \qquad (8)$$

where x is the fraction of reduced cytochrome c. If this fraction was appreciably different from unity, the procedure was repeated. Once prepared, reduced cytochrome c was stable for at least one month at -20°, but was rechecked spectrophotometrically before each use.

The assay of cytochrome oxidase was performed in a buffer of 50 mM sodium or potassium phosphate pH 7.1, containing 0.7 ml of 1% reduced cytochrome c and 0.01 or 0.02 ml of mitochondrial suspension in a total volume of 2.0 ml. Controls contained, in addition to mitochondria, 0.02 ml of 0.1M potassium ferricyanide to oxidize the cytochrome. The reaction was started by adding the enzyme (mitochondria), and absorbance at 550 nm was followed for several minutes in a recording spectrophotometer. Results were expressed as the change in A_{550} per minute.

The microsomal ethanoloxidizing system (MEOS) was assayed using a modification of the method of Lieber and De Carli (1970a). Erlenmeyer flasks (50 ml) equipped with center wells were used, with 0.6 ml of 0.015 M semicarbazide HCl in 0.16 M potassium phosphate pH 7.0 placed in each center well. The reaction took place in the main compartments in an incubation medium consisting of 0.8 M phosphate buffer pH 7.4, 50 mM $MgCl_2$, 50 mM ethanol, 0.1 mM NADPH and 0.25 ml microsomal suspension in a total volume of 3.0 ml. The flasks were stoppered with serum caps and the reactions were started by injecting NADPH into the flasks through the caps. The flasks were then incubated at 37° in either a shaking water bath or a non-shaking water bath with intermittent hand swirling. Reactions were stopped after 5, 10 or 15 minutes by injecting 0.5 ml of 70% perchloric acid (PCA) through the serum cap into the main compartment. The flasks were allowed to stand at room temperature several

hours or overnight to allow acetaldehyde produced in the main compartments to diffuse into the center wells and react with the semicarbazide. Controls containing ethanol alone and ethanol with microsomes but no NADPH were included, as were "zero-time" controls to which PCA was added before starting the reaction. After the diffusion period, 0.2 ml of the contents of each center well was diluted with water to 1.0 ml and absorbance at 224 nm was measured. The quantity of acetaldehyde formed was determined from a standard curve generated in each experiment by incubating flasks containing known amounts of acetaldehyde (10-30 ug) with the experimental flasks. MEOS activity was expressed as nanomoles of acetaldehyde formed per minute.

It was found that stock laboratory ethanol yielded erratic results, apparently due to contamination with acetaldehyde or other substances that react with semicarbazide. Therefore, refrigerated absolute ethanol was used to prepare a 0.5 M working solution, which was heated to 40° to eliminate volatile contaminants including acetaldehyde immediately before the assay. This procedure was found to enhance the reliability of the assay.

Alcohol dehydrogenase was measured using the method of Bonnischen and Brink (1955). The reaction mixture contained 0.45mM NAD, 0.5M ethanol, 0.1M glycine-NaOH pH 9.6 and 0.05 or 0.10 ml tissue supernatant in a volume of 3.0 ml. The reaction was started by adding ethanol after pre-incubating

for ten minutes; this relatively long pre-incubation was found to be necessary to eliminate endogenous activity. Absorbance at 340 nm was followed for several minutes in a recording spectrophotometer. Activity was expressed as micromoles of NADH formed per minute.

Other pyridine-linked dehydrogenases were assayed using the methods of Kaplan and Fried (1973). Reaction mixtures contained a total volume of 3.0 ml; appearance or degradation of reduced coenzyme was followed with a recording spectrophotometer at 340 nm. Assay conditions were as follows:

1) Alphaglycerophosphate dehydrogenase -- 0.1 mM NADH, 60 mM potassium phosphate pH7.4, 23 mM dihydroxyacetone phosphate (DHAP), 0.02 or 0.04 ml tissue supernatant

2) Lactate dehydrogenase -- 0.1 mM NADH, 0.33 mM pyruvate, 0.1 M potassium phosphate pH 7.4, 0.02 or 0.04 ml of 1.0% tissue supernatant (tenfold dilution of the stock tissue supernatant).

3) Malic enzyme -- 25 mM Tris HCl pH 7.4, 1.0 mM $MnCl_2$, 0.4 mM NADP, 0.6 mM L-malate, 0.05 or 0.10 ml tissue supernatant

4) Glucose-6-phosphate dehydrogenase -- 67 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1.2 mM NADP, 3.3 mM glucose-6-phosphate, 0.05 or 0.10 ml tissue supernatant.

All assays were performed with two levels of homogenate to assure proportionality. NADH and pyruvate were dissolved in 0.1 M potassium phosphate pH 7.4; NADH was used within several hours of preparation. Malate was dissolved in 0.1 M Tris-HCl pH 7.4. NADP, pyruvate and glucose-6-phosphate were dissolved in water. DHAP was prepared by hydrolysis of the dicyclohexylamine dimethyl ketal according to the manufacturer's instructions, and was stable for several weeks at -20°. Solutions of pyruvate, malate and glucose-6-phosphate were stable in the refrigerator for several months, and NADP was used within two days of preparation. Results were expressed as micromoles of reduced coenzyme formed or degraded per minute per gram (wet weight) of liver or per milligram of protein. Protein was determined by the method of Lowry (1951). Reagent B was preserved with 0.1% potassium iodide. The standard was crystalline bovine serum albumin (25-100 µg).

Light microscopy

Immediately after sacrifice, portions of liver were cut into pieces of ca. 5 mm on a side. The pieces were placed in a fixative consisting of 29% ethanol, 11% Formalin, and 19% acetic acid in water. Tissues were fixed for two days, then transferred to 50% ethanol for one hour, then to 70% ethanol and refrigerated for about one week. Dehydration was continued in 95% and 100% ethanol for two hours each.

Tissues were then cleared for one hour in xylol and infiltrated in paraffin for two changes of one hour each, followed by embedding.

10-μm sections were cut with a microtome and stained with either Harris hematoxylin (for nuclei) and eosin (cytoplasmic counterstain) or with periodic acid-Schiff reagent (PAS) for glycogen and Harris hematoxylin (nuclear counterstain).

Electron miscroscopy

As soon as possible after sacrifice, a portion of liver was placed in a pool of glutaraldehyde fixative (3% glutaraldehyde in 0.1 M sodium cacodylate pH 7.2) on a strip of Parafilm and minced into pieces about 1 mm on a side. The pieces were transferred to a vial containing additional fixative and refrigerated overnight. The fixative was renewed the next day and the tissue kept refrigerated until further processing about one week later.

The fixative was decanted and the tissue was rinsed with cacodylate buffer, washed ten minutes with fresh buffer, postfixed for three hours with cacdylate-buffered 1% osmium tetroxide and washed again in cacodylate buffer. The tissue was then dehydrated in 50% ethanol for ten minutes, then in 70% ethanol three hours or overnight in the refrigerator. Dehydration continued in 95% ethanol for ten minutes and in 100% ethanol for three changes of 10 minutes each. Ethanol was cleared with two five-minute changes of

propylene oxide prior to infiltration with and embedding in Spurr.

Tissues were incubated for 1-3 hours in a mixture of equal volumes of propylene oxide and Spurr, then left overnight at room temperature in pure Spurr. The remaining Spurr was stored overnight at -20° in a covered beaker, to be used for embedding the next day. It was allowed to come to room temperature before the cover was removed, to minimize deliquescence. Tissues were embedded in Spurr in BEEM cap-sules and cured overnight at 60°.

Tissues were sectioned on a Porter-BlumMT-2B ultramicrotome with freshly cut glass knives or a diamond knife. The sections were captured on copper grids and stained for 20 minutes with 2% aqueous uranyl acetate and for thirty seconds with lead citrate (Reynolds 1963). Sections were examined on either a Philips EM 300 or a Zeiss EM S-2 electron microscope.

RESULTS

Weight and general health

As shown in Table 1, ethanol-fed mice generally maintained their body weight, while pair-fed controls gained significant amounts of weight, shown upon dissection to consist primarily of adipose mass. Liver weight did not vary significantly between ethanol-treated and control mice. Thus, hepatosomatic index [HSI = (weight of liver)/(body weight) x 100] was significantly higher in ethanol-treated animals.

Mice in both groups appeared healthy and behaved normally, except for ataxia and slight nervousness in a few ethanol-treated animals toward the end of the feeding period. In a few instances, ethanol-treated mice that appeared healthy, were behaving normally and whose weight and food consumption were typical of the group would suddenly die. This was not totally unexpected (Petersen, D.R., personal communication).

Hepatic lipids

Lipids were extracted from liver samples with chloroform - methanol (2:1) and quantitated by dichromate reduction. Ethanol treatment nearly doubled hepatic lipids, whether these were expressed on a weight basis or a total organ basis (Table 2).

Hepatic glycogen

Glycogen was extracted from the livers of ethanol-fed and control mice. Control livers contained more than eight times the glycogen of livers from ethanol-fed mice when expressed on a weight basis. On a total organ basis, the difference was a factor of six (Table 3). It can be concluded that ethanol feeding under these conditions depletes hepatic glycogen.

Cytochrome oxidase

Cytochrome oxidase was assayed as a mitochondrial marker enzyme. Activity of this enzyme did not differ in livers of control and ethanol-treated mice, whether expressed in terms of liver protein, liver weight, animal weight or total liver (Table 4).

Enzymes of ethanol metabolism

There were no significant differences between ethanol-fed and control mice in activity of cytosolic NAD-linked alcohol dehydrogenase (Table 5) or MEOS (Table 6). This is true regardless of how the results are expressed.

Other pyridine-linked dehydrogenases

There were no significant differences in activity of glucose-6-phosphate dehydrogenase between livers of ethanol-fed and control mice (Table 7). The same was found for alphaglycerophosphate dehydrogenase (Table 8). In contrast,

ethanol feeding depressed activity of malic enzyme to half the control levels (Table 9). Lactate dehydrogenase levels were also significantly depressed in ethanol-fed mice (Table 10).

Histology

Hematoxylin-and-eosin staining of paraffin sections from livers of ethanol-fed mice showed no necrosis or other gross pathology. There was marked vacuolation, consistent with accumulations of cytoplasmic fat, particularly in regions removed from central veins. In sections from control mice there was much less vacuolation and the vacuoles were evenly distributed throughout the lobule. Liver cells from control mice were uniform in size. In ethanol-fed mice, the heavily vacuolated cells were enlarged and the centrilobular cells with few vacuoles were smaller than cells from control mice (Figs. 1-4).

PAS staining showed essentially uniform distribution of stain in sections from control mice, but very marked zonation in sections from ethanol-fed mice. There was heavy deposition of PAS-positive material near central veins, with peripheral areas nearly free of stain (Figs. 5-8). These findings are consistent with the results of biochemical measurement of hepatic lipid and glycogen (see above, p. 27-28) showing increased fat and decreased glycogen in livers of ethanol-fed mice.

Electron micrographs of liver cells of ethanol-fed mice showed large numbers of fat droplets, up to 5 μm in diameter. Sections from controls showed many fewer droplets. Mitochondria from ethanol-fed mice tended to be elongated or dumbbell-shaped, whereas mitochondria from controls tended toward the usual spherical or ovoid conformations. Sections from ethanol-fed mice contained small electron-lucent vesicles of unknown function that appeared to be undergoing fusion. These vesicles were not found in sections from control mice. Sections from both control and ethanol-fed mice contained normal-appearing rough endoplasmic reticulum; however, accumulations of smooth endoplasmic reticulum, such as were reported by others in ethanol-fed rats (Iseri et al. 1966) were not found in ethanol-fed mice in this study. Cell nuclei appeared normal in both control and ethanol-fed mice. Other aspects of cellular fine structure appeared unremarkable and were not suggestive of gross pathology (Figs. 9-13).

DISCUSSION

Overview

The metabolic consequences of long-term feeding of ethanol have been extensively studied in rats and baboons, as well as in humans (Lieber 1975a). Comparatively little work, however, has been done in mice. Each animal species has its advantages and disadvantages as a model of human alcoholism, and none is entirely satisfactory. The drug-seeking behavior that typifies long-term consumers of ethanol has so far not been satisfactorily reproduced in any non-human species. Animals will avoid consuming ethanol if possible. Even when the substance was added to the drinking water of rats, they failed to ingest enough ethanol to produce significant metabolic effects (Lieber and De Carli 1982). This difficulty prompted Lieber's group to devise a totally liquid diet that incorporates ethanol as the animals' single source of food and liquid (Lieber et al. 1965). With this technique, they were able to provide rats with a nutritionally adequate diet in which ethanol constituted 36% of the calories; in baboons, the percentage can be increased to 50% (Lieber and De Carli 1974). It should be noted that, in rats, ethanol must be introduced slowly to be tolerated; Lieber's group introduced ethanol in three incremental steps over five days. In this study, it was found that in mice, ethanol should be introduced even more slowly, using five increments over at least nine days (see Materials and Methods, p. 14).

Of the three animal models enumerated above, only baboons are primates and only they live long enough to develop alcoholic hepatitis and cirrhosis. Hence, that model is most relevant to the later stages of alcoholism in humans. However, baboons are expensive and difficult to breed and maintain. Rats are smaller and reproduce prolifically, but their livers are still large enough to allow multiple analyses on the same organ. This was not always the case in mice. Another advantage of rats over mice is that rats are large enough to force-feed by nasogastric tube. This allows a comparison of the effects of a single large dose of ethanol ("acute") with those of smaller doses over a long period of time ("chronic"). Nevertheless, mice were chosen in the present studies because the genetics of these mammals are better known than those of rats. One of many mutations available for study in the mouse is the obese (ob) mutation in the C57Bl/6J strain. Mice that are homozygous for this mutation become markedly obese beginning at about one month of age. Relative to lean mice of the same strain, they overeat and are inactive, moderately hyperglycemic, hyperinsulinemic and insulin resistant (see Introduction, p. 9). Their livers actively accumulate fat and glycogen, and activities of enzymes involved in the biosynthesis of lipids are elevated. Such livers can be described as highly competent physiologically; the accumulation of fat is not pathological but adaptive. These studies were designed to determine whether long-term

feeding of ethanol would cause fatty livers in mice and, if so, whether the fatty livers are competent, as in obese mice, or whether they are an indication of liver pathology. Knowing the effects of ethanol on lean mice of the strain in which the obese mutation is maintained would allow a meaningful study of the effects of ethanol on the obese mice themselves.

These studies clearly demonstrate that long-term feeding of ethanol produces a fatty liver in mice, just as it does in rats. The fatty liver was accompanied by two salient features that need to be explained:

1) Ethanol-fed mice, unlike controls, do not gain weight during the feeding period and, at the end of the experiment, they weigh significantly less than controls.

2) The accumulation of fat is accompanied by a paradoxical decline in activity of malic enzyme, an enzyme involved in the synthesis of fatty acids. Activities of two other lipogenic enzymes were unchanged.

A likely explanation for these findings appears to be that the livers of ethanol-fed mice in this study are under metabolic stress, unlike the situation in genetically obese mice. This stress appears to originate partly from ethanol itself and partly from the concomitant low dietary carbohydrate.

Morphological changes

Weight

As shown in Table 1, the weight of ethanol-fed mice remained stable during the experiment, while controls gained weight. This resulted in ethanol-fed mice weighing significantly less than controls when they were sacrificed. On dissection, ethanol-fed mice were found to be markedly depleted of adipose tissue; dissection findings were otherwise unremarkable in both groups. The livers of ethanol-fed mice were normal in color, and their weights did not differ from those of control livers. Hence, the hepatosomatic index (weight of liver as percentage of body weight) of ethanol-fed mice was higher than that of control mice.

The difference in weight between the two groups is consistent with several possibilities:

1) Ethanol is oxidized by MEOS. This mechanism was reported by others in ethanol-fed rats (Lieber 1975a, b; Lieber and DeCarli 1970a; Ohnishi and Lieber 1977). Since MEOS transfers electrons from ethanol and NADPH directly to oxygen, without producing ATP, organisms employing this pathway are metabolically less efficient than they would be otherwise. This would explain the failure of ethanol-fed mice to gain weight, despite ingesting the same number of calories as do controls.

This study did not demonstrate induction of MEOS by ethanol feeding, but the possibility of induction cannot be ruled out (see below, p. 44). It is also possible that MEOS is not rate limiting but constitutively present at high levels and ready to oxidize ethanol when ethanol is available. While this study did not demonstrate inducibility of MEOS it unequivocally demonstrated its presence, since high level of MEOS were found in all livers examined.

2) Ethanol is oxidized by catalase. This pathway is similar to MEOS, except that hydrogen peroxide, and not NADPH, is the co-reductant. Like MEOS, catalase is wasteful of energy because it transfers electrons directly to oxygen. The role of catalase in oxidizing ethanol in rat liver is a subject of debate (Thurman et al. 1972; Ohnishi and Lieber 1977). It is possible that catalase figures in ethanol metabolism more prominently than it does in rats. This study did not examine ethanol oxidation by catalase.

3) Ethanol-fed mice in this study suffered from malnuturition, possibly including vitamin deficiency. The fact that the ethanol and control diets are isocaloric and contain the same quantities of vitamins and minerals does not completely rule out this possibility. Ethanol can interfere with the absorption of vitamins and other nutrients by irritating the gastrointestinal tract (lieber 1975b). Toward the end

of the feeding period a number of ethanol-fed mice became ataxic and hyperexcitable, a finding consistent with vitamin deficiency adversely affecting nervous system function. Also toward the end of the experiment, three ethanol-fed mice of a total of 36 suddenly died. However, the mice that died were not always the ones that exhibited behavioral abnormalities. The concentration of ethanol used in this study (less than 6.5% v/v) is unlikely to interfere with absorption of nutrients, and a vitamin deficiency is not likely to strike apparently healthy mice at random and leave others unaffected. The observed behavioral abnormalities can be explained by direct cerebral effects of ethanol, and the sudden deaths may be attributed to hypoglycemia or ketoacidosis (Lieber 1975a; also see below p.). Thus, while it is possible that ethanol-fed mice suffered from vitamin deficiency, it appears unlikely.

4) Ethanol increases the basal metabolic rate of mice. Thus, ethanol-fed mice receiving the same number of calories as do controls actually require more. A similar effect is observed in response to physical training in rodents and humans; trained subjects require more calories to maintain their weight than untrained ones do (Bjorntorp 1978; Blair et al. 1981; Bray 1969; Epstien et al. 1985; Johnson et al. 1956; Mayer et al. 1954, 1956; Segal et al. 1985; Wood et al.

1982). Ethanol and physical training both result in increased production by the liver of highdensity lipoproteins (HDL) (Williams et al. 1985; Wood et al. 1982, 1983). Since ethanol-fed mice were no more active than controls, a similar mechanism for the observed increase in caloric requirements appears unlikely but several experimental approaches to test the hypothesis are possible (see below, p. 55).

Hepatic lipid

As shown in Table 2, the fat content of livers of ethanol-fed mice was nearly twice that of livers of control mice. Large numbers of fat droplets are evident in electron micrographs of livers of ethanol-fed mice, and many fewer such droplets were found in livers of controls (Fig. 9, 12, 13).

The elevation of hepatic lipids in ethanol-fed mice is expected, and agrees with findings reported by others in rats. The lipid could be derived from any or all of the following:

1) Mobilization of adipose reserves - This was reported in rats that received a single large dose of ethanol (Lieber 1974, Lieber et al. 1966).

2) Endogenously synthesized fat - The liver could utilize both reducing equivalents and acetate derived from ethanol. Ethanol shifts the NADH/NAD and NADPH/NADP redox couples toward reduction, and lipogensis would reoxidize the coenzymes. The capacity of the liver for secreting lipids is finite, however, so lipid accumulates in the cytoplasm of the hepatocyte. In humans and other primates, this accumulation eventually obstructs blood flow in the liver, leading to hepatitis and cirrhosis. This is not observed in rodents, presumably due to their shorter life span.

3) Dietary fat - The liver normally internalizes chylomicron remnants that include dietary triacylglycerols, as well as free and esterified cholesterol. These could be oxidized in the Krebs cycle, but since that cycle is inhibited by the hepatocyte's altered redox state, the lipids would tend to accumulate in the hepatocyte. The observed distribution of lipid within the liver is puzzling. In livers of ethanol-fed mice most of the vacuoles were near the periphery of the lobule, while cells closer to central veins had much less vacoulation (Fig. 1-4). This is opposite to what was reported in ethanol-fed rats, where most of the fat vacuoles were near central veins (Iseri et al. 1966). In untreated animals of both species, fat vacuoles are smaller than in ethanol-fed animals, and their distribution is panlobular. The pattern found in this

study may represent a species difference in the response to ethanol feeding. However,sustained dietary deficiencies sometimes result in preferential deposition of fat on the periphery (Bloom and Fawcett 1975). The ethanol diet used in this study contained very little carbohydrate. This was true in Iseri's study on rats as well, but the effect on mice might be more pronounced because of their smaller size.

Mitrochondrial structure and function

Hepatic mitochondria from ethanol-treated mice tended to be elongated or dumbbell-shaped (Fig. 10, 11). Such irregularly shaped mitochondria are often found in liver cells near central veins in untreated mice (Bloom and Fawcett 1975). It cannot be determined from these studies, in which ultra-thin sections for electron microscopy were cut at random, whether ethanol feeding distorts mitochondria of peripheral hepatocytes or whether sections from ethanol-fed mice happened to be taken from cells closer to central veins than the cells from control mice were.

As shown in Table 4, there was no significant difference in cytochrome oxidase activity between livers of ethanol-fed and control mice. This is consistent with normal function of the mitochondrial electron transport chain in ethanol-fed mice. Since the altered redox state in liver cells of these mice inhibits both glycolysis and the Krebs cycle, much of the energy required by the cells would

be realized by oxidation in the electron transport chain of NADH derived from ethanol.

Enzymes of the electron transport chain are located in the inner membrane, while those of the Krebs cycle are mostly in the matrix. Ethanol feeding appears to increase the function of the former and inhibit that of the latter. The irregular mitochondrial shapes observed in hepatocytes of ethanol-fed mice in this study tend to increase the membrane surface area. Thus, if the shape changes are the result of ethanol feeding, they would be consistent with the altered bioenergetics caused by ethanol.

Hepatic glycogen

As shown in Table 3, livers of ethanol-fed mice are markedly depleted of glycogen. Ethanol-fed mice in this study consumed little carbohydrate (11% of total calories) and, since their adipose mass was depleted and since they did not gain weight, they were probably in a state of negative energy balance. Their altered hepatic redox state would inhibit gluconeogenesis, since any pyruvate in the liver would be rapidly converted to lactate. In view of the foregoing, it is expected that hepatic glycogen would be hydrolyzed to maintain glucose homeostasis. Acute hypoglycemia occasionally causes death in human alcohols who are depleted of hepatic glycogen (Lieber 1975a); perhaps this is the cause of the sudden deaths noted above (p. 36) in experimental mice in this study.

While the glycogen depletion in ethanol-fed mice was expected, the distribution of glycogen within the liver, as revealed by PAS staining of paraffin sections, presented difficulties in interpretation. As expected, the distribution of PAS-positive material was uniform in livers of control mice. In ethanol-fed mice, PAS-positive material was densest in cells closest to central veins and very sparse in peripheral cells (Fig. 5-8). Usually, glycogen is deposited first and most heavily in cells at the periphery, and removed first from cells in the centrilobular region, generating a pattern opposite to what was observed in this study (Bloom and Fawcett 1975). The significance of this finding is not clear, at present, but may be elucidated by further study (see below, p. 58).

Endoplasmic reticulum

Proliferation of smooth or vesicular endoplasmic reticulum, reported by others in ethanol-fed rats (Iseri et al. 1966),was not found in this study. Normal rough endoplasmic reticulum was found in livers of both control and ethanol-fed mice, suggesting that protein synthesis was occurring in ethanol-fed rats. Small electron-lucent vesicles of unknown function were observed by electron microscopy in livers of ethanol-fed mice (Fig. 10, 11). They appeared to be undergoing fusion. They could be coalescing fat droplets, but their appearance was unlike that of other fat droplets in the same cell. Another

possibility is that they are of microsomal origin with membranes altered either as a processing artifact or by ethanol feeding. There is evidence in rats that long-term feeding of ethanol damages hepatic membranes. Changes in the shape of mitochondria are accompanied by changes in their membranes (Iseri et al. 1966). Hepatic and serum levels of gamma-glutamyltranspeptidase (GGTP) are elevated by ethanol feeding while other dietary manipulations, including low carbohydrate, elevate hepatic but not serum values (Yamada et al. 1985). The authors suggest that ethanol causes damage to the plasma membrane and, consequently, leakage of GGTP into the blood. Other studies suggest that ethanol feeding elevates levels of hepatic oxygen radicals, leading to lipid peroxidation and damage to membranes (Rosen et at. 1983; Lieber and Savolainen 1984).

In this context, the finding in this study that ethanol feeding depresses hepatic lactate dehydrogenase activity (Table 10) is interesting. LDH is thought to be a bidirectional, non-rate-limiting, "housekeeping" enzyme. If it is shown that depressed hepatic levels of this enzyme are accompanied by elevated serum levels, that would suggest that LDH was leaking into the blood and would provide further indication that long-term feeding of ethanol damages hepatic plasma membranes.

Pathways of ethanol metabolism

It will be recalled (see Introduction, pp. 3-6) that ethanol can be metabolized by oxidative mechanisms employing alcohol dehydrogenase, MEOS or catalase, and it can be esterified non-oxidatively to long-chain fatty acids. The alcohol dehydrogenase reaction appears to be the primary metabolic pathway at low to moderate levels of ethanol consumption in rats. At higher levels of consumption MEOS takes on a greater role (Lieber and De Carli 1970a). These studies found no significant elevation of alcohol dehydrogenase or MEOS in livers of ethanol-fed mice (Tables 5-6). With respect to ADH, these results agree with Lieber and De Carli (1970a); however, other investigators found that long-term feeding of ethanol does induce ADH in rat liver (Sze 1975). It was found that ADH in human liver is considerably polymorphic, with variant forms differing in such parameters as pH optimum and affinity for substrate (Li et al. 1977, Bosron et al. 1980). If this polymorphism is also present in rodents, the conflicting data on induction of ADH by ethanol might be explained. It should also be noted that ADH in rodents (and primates) probably did not evolve as an ethanol-metabolizing enzyme; thus it is not surprising that ADH activity would be insensitive to the presence or absence of ethanol.

The results for MEOS are more difficult to interpret. In each of the six pairs of mice studied, the MEOS activity of the ethanol-fed mouse was considerably higher than that of the pair-fed control. However, the variation within each group was so high that the difference between the means fell short of statistical significance, contrary to results reported in rats (Lieber and De Carli 1970a). Due to the small size of individual mouse livers and their paucity of microsomes, the MEOS assay was technically difficult, and this difficulty might have contributed to the high internal variation. Other investigators using a different method found that ethanol feeding does induce MEOS in livers of mice of the same strain used in this study (Petersen and Atkinson 1980, Petersen et al. 1982). P450-based systems have been implicated in the detoxification of other toxic substances, e.g. phenobarbital (Kato 1966) and Vitamin A (Sato and Lieber 1981). Induction of and competition for microsomal enzymes by ethanol may partially explain the observed synergism and cross-tolerance between ethanol and other substances in rodents and humans (Altomare et al. 1984, Kater et al. 1969, Misra et al. 1974). There is evidence that a P450-based microsomal system that oxidizes xenobiotics and is induced by them operates in teleost fish (Stegman et al. 1986). Since ethanol is not a significant component of rodent diets in nature, it is expected that rodents would metabolize it as a xenobiotic, or drug, in the microsomal system that evolved for this function. In view

of the evolutionary antiquity of this metabolic strategy, it would be surprising to find that ethanol induces a microsomal drug-detoxifying system in rats and humans but not in mice. However, the failure of this study to find proliferation of smooth endoplasmic reticulum, the presumed site of MEOS, in ethanol-fed mice is evidence against MEOS induction by ethanol. Resolution of this question will require more research (see below, p. 53).

It is possible that ethanol feeding in mice induces a system involving catalase despite contrary findings in rats (Ohnishi and Lieber 1977, Teschke et al. 1972). Another possibility involves the nonoxidative pathway recently discovered in humans (Laposata and Lange 1986). It is not yet known if this mechanism operates in mice. Even if it does, it does not appear likely to have a significant role in total ethanol metabolism. Chronic alcoholics who were not acutely intoxicated at the time of death were shown on autopsy to have high levels of fatty acid ethyl esters only in adipose tissue, while nonalcoholics who had high blood ethanol levels at death had high levels of these esters in several organs including the liver. This suggests that fatty acid ethyl esters are cleared rapidly from the liver and other sites of production and are stored in adipose tissue along with the more common triacylglycerols. The ethanol-fed mice in this study were depleted of adipose tissue; it therefore seems unlikely, though not impossible, that much ethanol is being sequestered in fatty acid esters.

Lipogenic enzymes

The synthesis of triacylglycerols involves two separate biosynthetic pathways. Long-chain fatty acids are synthesized from acetyl CoA, which is derived from the oxidation of carbohydrates and fats, and which is also a product of ethanol metabolism. Construction of long-chain fatty acids from two-carbon acetyl CoA moieties requires NADPH as a reductant. Fatty acids thus formed are esterified to glycerophosphate, which is produced by reduction of dihydroxyacetone phosphate, a glycolytic intermediate. This reduction requires NADH. Alternatively, free glycerol derived from dietary fat can be directly phosphorylated by the enzyme glycerol kinase, using ATP as a phosphate donor, and the resulting glycerophosphate esterified to fatty acids.

NADPH for fatty acid synthesis can originate from several sources including:

1) The pentose shunt. The initial step in this pathway involves transfer of electrons from glucose-6-phosphate to NADP in the presence of glucose-6-phosphate dehydrogenase.

2) Oxidative decarboxylation of malate. Electrons are transferred from malate, a Krebs cycle intermediate, to NADP. Carbon dioxide is released and pyruvate is generated. This reaction is catalyzed by malic enzyme

and is a reversal of an anaplerotic reaction that generates malate during heavy Krebs cycle activity (Lehninger 1975).

3) Transhydrogenation from NADH. This would be expected to predominate in hepatocytes of ethanol-fed animals, where oxidation of ethanol produces abundant NADH.

Table 9 shows that malic enzyme activity in livers of ethanol-fed mice is only half of the activity in controls. This result agrees with that reported by Fellenius et al. (1973) in rats. Malic enzyme can operate in either direction depending on the relative concentrations of substrates and cofactors. In the direction of NADPH formation malate is required. This compound is generated in the Krebs cycle, which is inhibited by long-term feeding of ethanol (Lieber 1975a). In the opposite direction pyruvate is required. Pyruvate is expected to be scarce in livers of ethanol-fed mice due both to its rapid conversion to lactate in the reducing environment and to the low carbohydrate content of the ethanol-containing diet. However, generation of NADPH by malic enzyme is a key step in a cycle that effects transhydrogenation from NADH to NADP (Lundquist 1975). Hence, the decline in activity of this enzyme in ethanol-fed mice suggests that, although livers of these mice accumulate fat, little de novo fatty acid synthesis occurs.

In the study cited above (Fellenius et al. 1973), where ethanol feeding reduced malic enzyme activity in rat liver, the rats' carbohydrate source was sucrose. Sucrose increases hepatic lipogenesis and lipogenic enzyme activities above the level produced by its monosaccharide constituents glucose and fructose (Michaelis and Szepesi 1973, 1974; Michaelis et al. 1975). It was therefore possible that Fellenius' control values for malic enzyme were abnormally elevated and that the decreases they observed in ethanol-fed rats, who consumed less sucrose, were spurious. The present study, in which malto-dextrins served as the carbohydrate source, provides evidence against this possibility.

Reducing power for fatty acid synthesis can also originate from the reaction catalyzed by G6PD. As shown in Table 7, activity of this enzyme did not differ in ethanol-fed and control mice. Unlike malic enzyme, G6PD does not function in a metabolic pathway that is inhibited by ethanol feeding. It is possible that G6PD activity in control mice is regulated at a basal level sufficient to provide the cell with pentoses required for nucleic acid synthesis, and that activity would not be depressed below that level.

In the synthesis of triacylglycerols, fatty acids are esterified to glycerol-3-phosphate, which is a product of the reaction catalyzed by AGPDH. This reaction would appear to be adaptive during ethanol feeding, since it

simultaneously produces glycerol phosphate required for lipogenesis and reoxidizes NADH formed in the alcohol dehydrogenase reaction. However, Table 8 shows that there is no significant difference between ethanol-fed and control mice in AGPDH activity. This may be because the reaction requires dihydroxyacetone phosphate, a glycolytic intermediate. While NADH is abundant in the hepatocyte of an ethanol-fed mouse, glycolysis is inhibited and glycolytic intermediates are scarce. The scarcity would be compounded by the low carbohydrate content of the ethanol-containing diet. However, AGPDH is also a component of a shuttle that transports reducing equivalents derived from cytosolic NADH into the mitochondria. Since ADH is a cytosolic enzyme, the livers of ethanol-fed mice produce large amounts of NADH in the cytosol. The results of this study suggest that either basal levels of AGPDH are sufficient to shuttle into the mitochondria the excess reducing power generated by ethanol feeding, or that ethanol feeding induces alternative shuttle systems. They also reinforce the supposition that ethanol feeding does not appreciably increase de novo synthesis of fatty acids under the conditions of the study, i.e., low dietary carbohydrate.

It should be noted that the fat in the ethanol-containing diet would generate free glycerol, which can be phosphorylated by the glycerol kinase reaction and made available for re-esterification to fatty acids that also are derived from dietary fat. Triacylglycerols thus formed,

originating from dietary fat, would then accumulate, causing the observed fatty liver. Activity of glycerol kinase was not examined in the present study.

Ethanol ingestion and obesity compared

The accumulation of hepatic lipid engendered by ethanol consumption is unlike that reported in genetically obese mice (Fried and Antopol 1966; Kaplan and Fried 1975). Mice in those studies were fed unrestricted quantities of a high-carbohydrate, low-fat standard laboratory chow. The caloric intake of obese mice exceeds their energy expenditure, and the excess is converted by the liver to fat. Lipogenic enzyme activities are elevated to meet the liver's increased need for lipogenesis. Such livers are in an adaptive anabolic state, highly competent and functional. The mere presence of higher than normal amounts of fat in the liver cannot be considered pathological. By contrast, livers of ethanol-fed mice under the conditions of this study suffer profound metabolic derangements. The redox state of the NAD/NADH, and probably also the NADP/NADPH couples, is shifted toward the reduced forms. Fatty acid and glucose oxidation in the Krebs cycle, which require oxidized pyridine nucleotides, appear to be inhibited by the altered redox state, and the livers seem to be meeting their energy needs by oxidizing NADH derived from ethanol. Depletion of adipose mass and hepatic glycogen suggest that the organism as a whole is unable to meet its caloric needs with the ethanol-containing diet. Extrahepatic tissues that cannot

metabolize ethanol, and whose redox state is normal, oxidize stored fat and dietary substrates, including ethanol-derived acetate released by the liver. The liver, like the extrahepatic tissues, appears to be in a catabolic state. The decrease in lipogenic enzyme activity suggests that little fat is synthesized in the liver. Instead, the accumulated fat appears to be of dietary origin.

The contrast between the fat accumulation in ethanol feeding and in genetic obesity is pointedly illustrated by studies in which genetically obese ("fatty") Zucker rats were fed a low-carbohydrate, ethanol-containing diet similar to the one used in this study. The obese rats accumulated less hepatic lipid, and had lower lipogenic enzyme activities, on the ethanol diet than on the control diet (Karsenty et al. 1985a, b). Ethanol apparently impairs the ability of the livers of obese rats to synthesize fat de novo. The authors speculate that the low carbohydrate content of the ethanol diet contributes to the impairment; the present study suggests that lipogenesis in livers of ethanol-fed non-obese mice is also impaired by low dietary carbohydrate.

One point of similarity between the obese and ethanol-fed states may be the liver's response to insulin. It was reported that healthy humans receiving a sub-intoxicating dose of ethanol have a lesser response to intravenous insulin than do control subjects ingesting saline instead of

ethanol (Yki-Jarvinen and Nikkila 1985). Genetically obese mice are known to be insulin resistant. Caution is required when extrapolating from acute to chronic intake of ethanol and from humans to rodents. However, it would be advantageous to be resistant to insulin's promotion of glucose uptake when glucose is scarce and fat readily available, as is the case in ethanol-fed mice in this study.

Suggestions for future research

Ethanol concentration in the diet

In this study, ethanol comprised 36% of total calories in the ethanol-containing diet. This concentration was chosen because it was used in Lieber's work with rats. However, food intake per unit of body weight is inversely proportional to body weight. Mice weigh about one-tenth as much as rats. Hence, mice receive a higher ethanol dose per unit of body weight per day as do rats, even though the concentration of ethanol in the rat and mouse diets are identical. The food intake of ethanol-fed mice in this study tended to fall when the concentration of ethanol in the diet was increased from 28% to 36% of total calories. This might have contributed to the decreased adiposity, failure to gain weight, and even to the sudden deaths occasionally observed in ethanol-fed mice (Petersen, personal communication). The concentration of ethanol should be reduced to 28-30% of total calories. If the ethanol-fed mice maintain their food intake and gain weight,

that would suggest that the failure of such animals to gain weight in the present study resulted at least partially from inadequate caloric intake, an inappropriately high dose of ethanol, or both. That a given caloric intake was inadequate for ethanol-fed mice and adequate for controls suggests either an energy-wasting metabolic pathway (e.g. MEOS) or an increased basal metabolic rate in ethanol-fed mice. It would be useful to monitor blood ethanol levels and compare them at different concentrations of ethanol in the diet. If the lower concentration yields a lower blood ethanol level and abolishes the behavioral abnormalities noted above (p. 27), it would suggest that the unusual behavior resulted from direct effects of ethanol rather than a vitamin deficiency.

The possibility of a vitamin deficiency, though remote, can be investigated by increasing the vitamin content of the diet, particularly that of Vitamin A, which was found to be depleted in livers of ethanol-fed rats (Sato and Lieber 1981). In determining the doses of vitamins it should be noted that excessive Vitamin A can be toxic (Goodhart and Shils 1973).

Elucidation of pathways for ethanol metabolism

The closeness to statistical significance of the results for MEOS in the present study warrants further investigation. Using more mice or measuring acetaldehyde production by gas-liquid chromatography, as was done by

other investigators (Petersen and Atkinson 1980, Petersen et al. 1982), might reduce the internal variability that characterized the results of this study.

Other predictions of the hypothesis that ethanol feeding induces MEOS may also be tested. Drugs that are detoxified by P450-based microsomal systems, such as barbiturates, should show enhanced toxicity and slower clearance from the body when administered concurrently with ethanol. Clearance time should be reduced and toxicity decreased (i.e., tolerance should develop) when the drug is administered to a mouse chronically fed ethanol but currently ethanol-free. Substances that are converted by microsomal systems to toxic metabolites (e.g. carbon tetrachloride and acetaminophen) should show reduced toxicity when administered concurrently with ethanol and enhanced toxicity when administered alone to a mouse previously habituated to ethanol.

It should be determined whether alcohol dehydrogenase is as polymorphic in mice as it appears to be in humans. If it is, then the effect of long-term feeding of ethanol on each major isozyme should be determined separately. This might help resolve conflicting data from different laboratories on whether ADH is induced by ethanol. The presence or absence in mice of the nonoxidative pathway recently discovered in humans should be established. If the the pathway exists in mice, its inducibility by ethanol feeding should be investigated. The extent to which

catalase is involved in ethanol metabolism during long-term consumption should also be determined.

Metabolic rate and endocrine control

The basal metabolic rate of ethanol-fed and control mice should be examined. If it is determined that ethanol feeding increases basal metabolic rate, then the failure of ethanol-fed mice to gain weight would be explained even if oxidation of ethanol by MEOS or catalase is not increased. Levels of hormones involved in mobilizing substrates to support increased metabolic rate should be examined. These hormones, which figure in the release of substrates to fuel the increased metabolic demands of exercise, include insulin, glucagon, catecholamines and somatotrophin (Galbo et al. 1975, 1976; Glick 1968; Gollnick 1967; Hartley et al. 1972). Even if hormone levels are unchanged as a result of ethanol feeding, secondary effects, such as on receptor binding, are possible and should be examined (Winder and Heninger 1971; York et al. 1978).

Sufficiency of dietary carbohydrate and source of hepatic lipid

The results of the present study suggest that insufficient dietary carbohydrate may contribute to some of the metabolic changes induced by ethanol feeding. This possibility can be investigated by altering the ethanol-containing diet to keep the carbohydrate content constant

and high, substituting ethanol for fat instead (Lieber and De Carli 1970b). It should be noted that the high fat content of the Lieber-De Carli diet was chosen to simulate the composition of the American diet. The modification suggested here would sacrifice some similarity to the human situation that the experimental animals are intended to model, but would help resolve possible confounding effects of low carbohydrate on the experimental results. It may also be closer to the diet to which humans are genetically best adapted (Eaton and Konner 1985). If a higher proportion of carbohydrate results in increased activity of lipogenic enzymes, or at least abolishes the decline in malic enzyme activity, that would support the hypothesis that, in the present study, insufficient carbohydrate precluded de novo synthesis of fatty acids. If increased dietary carbohydrate has no effect on lipogenic enzyme activities, then it could be tentatively concluded that ethanol itself profoundly suppresses intermediary metabolism irrespective of availability of substrates, and that the decline of lipogenic capacity is a degenerative change due to ethanol itself.

It is possible to ascertain directly whether the lipid that accumulates in the livers of ethanol-fed mice originates from dietary fat or is endogenously synthesized. If it is endogenously synthesized, the precursor substrates can be identified by isotopically labeling one or another component of the diet and determining if the label is

recovered in hepatic lipid. If the hepatic lipid is derived from dietary fat and not synthesized endogenously, then radioactivity would be found in hepatic lipid if fat is labeled but not if carbohydrate is labeled. To avoid feeding mice radioactive substances for several weeks, "atypical" fatty acids could be incorporated into the diet and searched for in hepatic lipid (Lieber et al. 1966). Inability to recover them there would suggest, but not prove, that the fat accumulating in the liver was endogenously synthesized. Conversely, substantial recovery of the marker fatty acid would suggest that the fat accumulation is derived primarily from dietary fat. Such studies can be performed both with the diet used in the present study and with a diet higher in carbohydrate and lower in fat. If the hepatic lipid is primarily of dietary or adipose origin with a low-carbohydrate diet, but endogenously synthesized when ethanol-fed mice ingest more carbohydrate, it would suggest that the apparent decline in lipogenic capacity observed in the present study is attributable to insufficient dietary carbohydrate. It should also be determined if ethanol feeding causes qualitative or quantitative changes in the low density lipoprotein (LDL) receptor, recently shown to be essential to uptake of circulating lipid by the hepatocyte (Brown and Goldstein 1984), and if such changes correlate with differences in the composition of the diet.

Studies similar to those performed by Karsenty et al. (1958a, b) on fatty rats should be performed on genetically obese mice. If ethanol-fed obese mice accumulate less hepatic fat than do obese mice on the control diet, that would be evidence that the fatty liver induced by ethanol is an indicator of metabolic stress and not an adaptive response by a competent organ, as is the case in chow-fed obese mice.

Histological study

The histological results of the present study are consistent with the biochemical measurements of hepatic lipid and glycogen. However, determining the validity and significance of the anomalous distributions of fat and glycogen would require more detailed study. PAS staining should be performed on tissue fixed in a more alcoholic fixative (e.g. Rossman's fluid) than the one used in the present study, since nonalcoholic aqueous fixatives tend to dissolve glycogen. Fat accumulations can be directly visualized, not merely inferred from excessive vacuolation, by staining frozen sections with a lipophilic stain such as Oil Red O.

If different patterns of distribution of glycogen or fat are found when the carbohydrate content of the diet is increased, specific patterns might be correlated with carbohydrate deficiency. It might be possible to detect movement of hepatic lipid or glycogen between liver cells by

radioactively labeling components of the diet for a brief time period or by injecting labeled precursors.

More cells should be examined by electron microscopy than was the case in the present study, and the location of those cells within the liver lobule should be ascertained. It will then be possible to determine whether the altered mitochondria observed in the present study are the result of ethanol feeding, low dietary carbohydrate, or a sampling artifact. An expanded ultrastructural study would also allow for definitive conclusions regarding effects of ethanol on smooth endoplasmic reticulum and other membranous structures. If smooth endoplasmic reticulum does not proliferate when mice are fed ethanol, that would suggest that a P450-linked microsomal system is not induced in this species. Extensive damage to membranes would indicate hepatic degeneration, unlike the adaptive changes observed in genetically obese mice.

The competence of livers of ethanol-fed mice can also be assessed by ascertaining if these livers secrete, in appropriate quantity, substances secreted by normal livers, such as albumin and fibrinogen. Conversely, degenerating livers would leak into the bloodstream substances such as GGTP or LDH that are normally confined to the liver; studies such as that performed by Yamada et al. (1985) on GGTP in rat liver should be undertaken in mice.

Concluding remarks

The present study demonstrated that long-term feeding of ethanol produces a fatty liver in mice. This parallels findings in rats, baboons and humans. Other features of the ethanol-fed mouse include relative metabolic inefficiency (indicated by failure to gain weight), reduced adiposity, decreased hepatic malic enzyme activity and drastically reduced hepatic glycogen content. These findings may represent a specific effect of ethanol, confounding effects of low dietary carbohydrate, or both. Resolution of this question, as well as determination of whether the observations reported in this study indicate frank hepatic degeneration, awaits further study.

"Competence" and "degeneration" are, of course, relative terms. Substances such as carbon tetrachloride and acetaminophen, as well as ethanol ingested over a long period of time by primates (including humans) produce marked hepatic degeneration. By contrast, livers of genetically obese animals process large amounts of lipid but are highly competent. It appears that the ethanol-fed mouse represents an intermediate state, and is thus an appropriate model for the beginning - and presumably reversible - stages of alcoholic liver injury in humans.

TABLE 1

WEIGHTS OF EXPERIMENTAL MICE

	Body weight (g) (initial)	Body weight (g) (final)	Liver weight (g)	HSI (%)
Control	25.6 ± .38 (18)	28.7 ± .85 (a) (18)	1.2 ± .3 (18)	4 ± .1 (18)
Ethanol-fed	25.8 ± .4 (18)	25.3 ± .5 (15)	1.3 ± .03 (15)	5 ± .1 (15)
P	N.S.	< .01	N.S.	< .001

Values are the means ± standard error of the mean for the number of observations in parentheses.
P is determined by Student's t-test. N.S. = Not Significant
(a) Significantly different from initial weight at P < .01 level.
HSI = Hepatosomatic index = (Liver weight/Body weight) X 100.
Three ethanol-fed mice died during the experiments.

TABLE 2

HEPATIC LIPIDS IN ETHANOL-FED AND CONTROL MICE

	Per g liver	Per liver	Per g Mouse
Control	46.6 ± 1.15 (6)	58.9 ± 1.48 (6)	1.94 ± .11 (6)
Ethanol-fed	83.0 ± 6.5 (5)	110.5 ± 10.5 (5)	4.40 ± .83 (5)
P	$< .001$	$< .001$	$< .02$

Values are reported as miligrams ± standard error of the mean of the number of observations in parentheses. P is determined by the Student's t-test.

TABLE 3

HEPATIC GLYCOGEN IN ETHANOL-FED AND CONTROL MICE

	Per g liver	Per liver	Per g Mouse
Control	24.34 ± 3.72 (6)	20.87 ± 2.63 (6)	0.85 ± .11 (6)
Ethanol-fed	2.82 ± .44 (4)	3.48 ± .58 (4)	0.15 ± .02 (4)
P	< .001	< .001	< .001

Values are reported as miligrams ± standard error of the mean of the number of observations in parentheses. P is determined by the Student's t-test.

TABLE 4

HEPATIC MITOCHONDRIAL CYTOCHROME OXIDASE IN ETHANOL-FED AND CONTROL MICE

	Per mg protein	Per g liver	Per liver	Per g mouse
Control	12.6 ± 4.3 (6)	97.2 ± 36.2 (6)	121 ± 40 (6)	4.19 ± 1.5 (6)
Ethanol-fed	8.25 ± 2.6 (6)	87.3 ± 32.2 (6)	107 ± 38 (6)	4.00 ± 1.4 (6)
P	N.S.	N.S.	N.S.	N.S.

P values are determined by the Student's t-test. N.S. = Not Significant

Values are reported as the change in A_{550} per minute ± standard error of the mean for the number of observations in parentheses.

TABLE 5

HEPATIC ALCOHOL DEHYDROGENASE IN ETHANOL-FED AND CONTROL MICE

	Per mg protein	Per g liver	Per liver	Per g mouse
Control	.014 ± .002 (6)	1.21 ± .07 (6)	1.56 ± .13 (6)	.051 ± .004 (6)
Ethanol-fed	.016 ± .001 (6)	1.45 ± .15 (6)	1.75 ± .15 (6)	.066 ± .006 (6)
P	N.S.	N.S.	N.S.	N.S.

Values are reported as micromoles of NADH formed per minutes ± standard error of the mean for the number of observations in parentheses.
P is determined by the Student's t-test. N.S. = Not Significant

TABLE 6

HEPATIC MICROSOMAL ETHANOL-OXIDIZING SYSTEM IN ETHANOL-FED AND CONTROL MICE

	Per mg protein	Per g liver	Per liver	Per g mouse
Control	38.3 ± 6.2 (6)	473 ± 74 (6)	614 ± 98 (6)	19.8 ± 2.9 (6)
Ethanol-fed	50.9 ± 8.2 (6)	733 ± 148 (6)	883 ± 156 (6)	33.1 ± 5.9 (6)
P	N.S.	N.S.	N.S.	N.S.

Values are reported as nanomoles of acetaldehyde formed per minute ± standard error of the mean for the number of observations in parentheses. P is determined by the Student's t-test. N.S. = Not Significant

TABLE 7

ACTIVITY OF HEPATIC GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN ETHANOL-FED AND CONTROL MICE

	Per mg protein	Per g liver	Per liver	Per mouse
Control	.014 ± .002 (6)	1.25 ± .18 (6)	1.60 ± .20 (6)	.053 ± .007 (6)
Ethanol-fed	.010 ± .002 (6)	0.93 ± .11 (6)	1.12 ± .12 (6)	.042 ± .005 (6)
P	N.S.	N.S.	N.S.	N.S.

Values are reported as micromoles of NADPH formed per minute ± standard error of the mean for the number of observations in parentheses.
P is determined by the Student's t-test. N.S. = Not Significant

TABLE 8

ACTIVITY OF HEPATIC ALPHAGLYCEROPHOSPHATE DEHYDROGENASE IN ETHANOL-FED AND CONTROL MICE

	Per mg protein	Per g liver	Per liver	Per g mouse
Control	.635 ± .056 (6)	55.1 ± 6.7 (6)	69.6 ± 6.3 (6)	2.31 ± .27 (6)
Ethanol-fed	.473 ± .07 (6)	43.7 ± 5.2 (6)	50.7 ± 5.9 (6)	1.90 ± .20 (6)
P	N.S.	N.S.	N.S.	N.S.

Values are reported as micromoles of NADH degraded per minute ± standard error of the mean for the number of observations in parentheses.
P is determined by the Student's t-test. N.S. = Not Significant

TABLE 9

ACTIVITY OF HEPATIC MALIC ENZYME IN ETHANOL-FED AND CONTROL MICE

	Per mg protein	Per g liver	Per liver	Per g mouse
Control	.097 ± .009 (6)	8.30 ± .89 (6)	10.7 ± 1.2 (6)	.351 ± .040 (6)
Ethanol-fed	.042 ± .006 (6)	3.94 ± .56 (6)	4.78 ± .66 (6)	.180 ± .020 (6)
P	$< .001$	$< .01$	$< .01$	$< .01$

Values are reported as micromoles of NADPH formed per minute ± standard error of the mean for the number of observations in parentheses.
P is determined by the Student's t-test.

TABLE 10

ACTIVITY OF HEPATIC LACTATE DEHYDROGENASE IN ETHANOL-FED AND CONTROL MICE

	Per mg protein	Per g liver	Per liver	Per g mouse
Control	3.13 ± .35 (6)	269 ± 30 (6)	344 ± 36 (6)	11.3 ± 1.2 (6)
Ethanol-fed	1.64 ± .09 (6)	153 ± 4 (6)	185 ± 5 (6)	6.95 ± .18 (6)
p	$< .01$	$< .01$	$< .01$	$< .01$

Values are reported as micromoles of NADH degraded per minute ± standard error of the mean for the number of observations in parentheses.
P is determined by the Student's t-test.

Fig. 1 - Photomicrograph of liver of control mouse. Hematoxylin and eosin (H and E), 135X

Fig. 2 - Photomicrograph of liver of control mouse. H and E, 220X

Fig. 1

Fig. 2

Fig. 3 - Photomicrograph of liver of ethanol-fed mouse.
H and E, 135X

Fig. 4 - Photomicrograph of liver of ethanol-fed mouse.
H and E, 220X

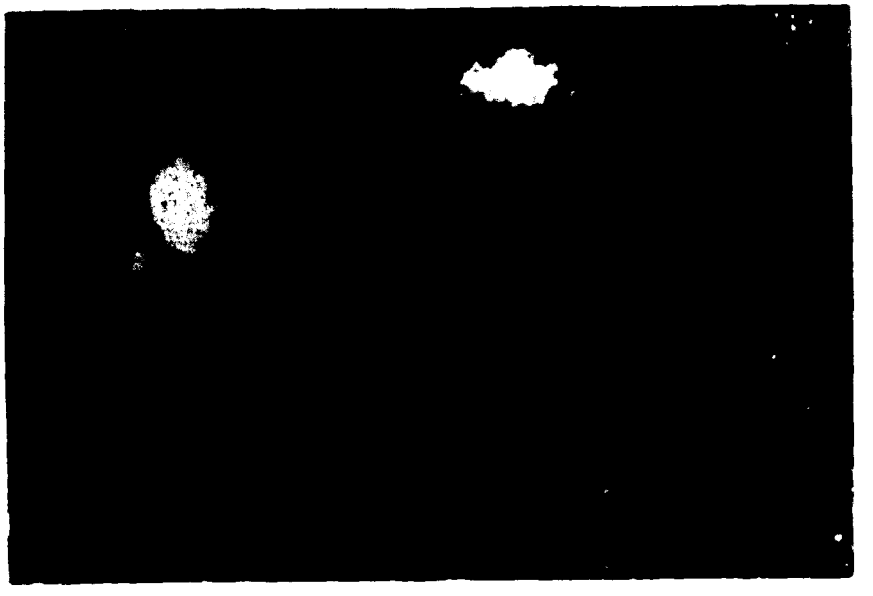


Fig. 3

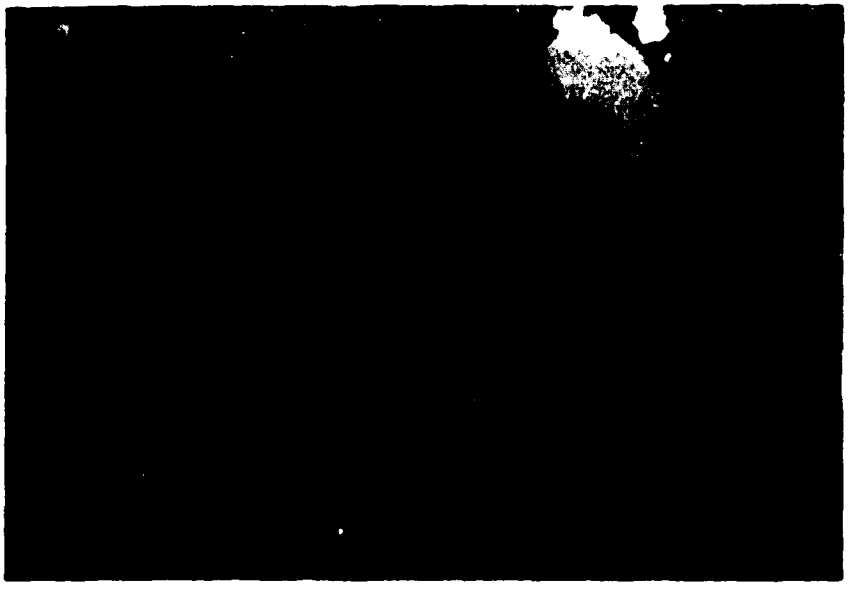


Fig. 4

Fig. 5 - Photomicrograph of liver of control mouse.
Periodic acid - Schiff (PAS), 135X

Fig. 6 - Photomicrograph of liver of control mouse.
PAS, 220X

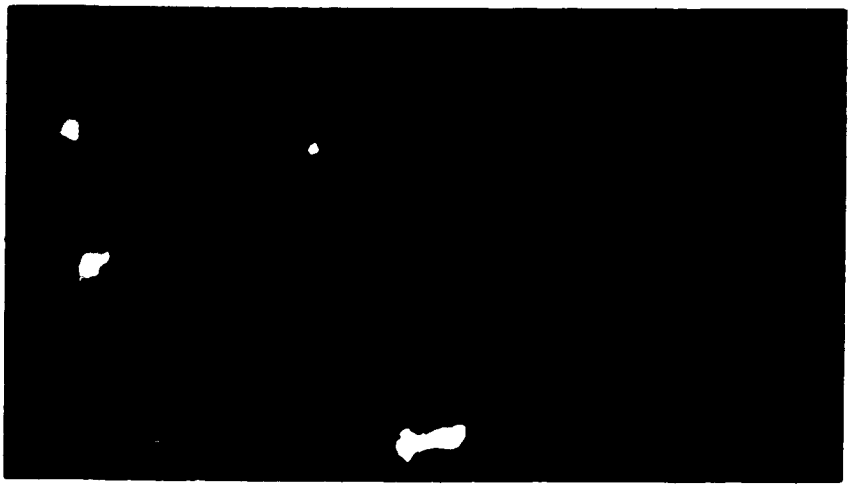


Fig. 5

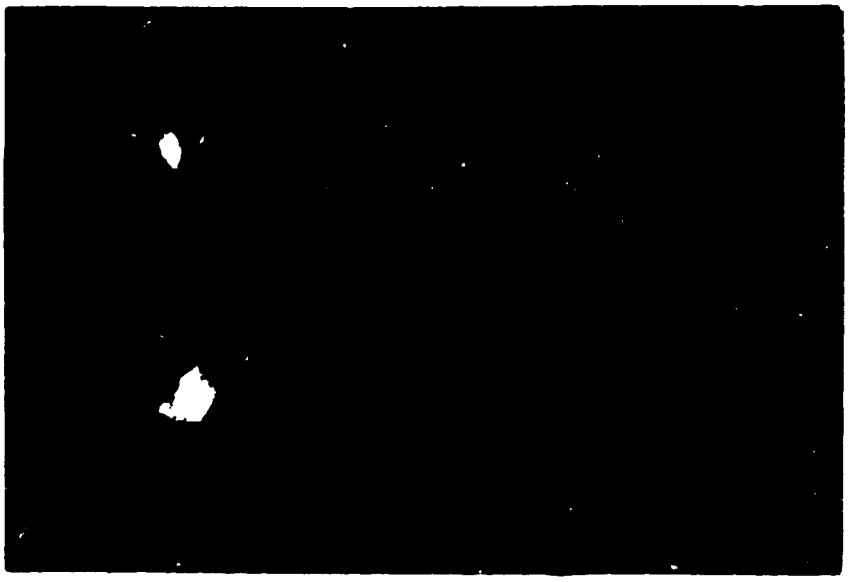


Fig. 6

Fig. 7 - Photomicrograph of liver of ethanol-fed mouse. PAS, 135X

Fig. 8 - Photomicrograph of liver of ethanol-fed mouse. PAS, 220X

Fig. 7

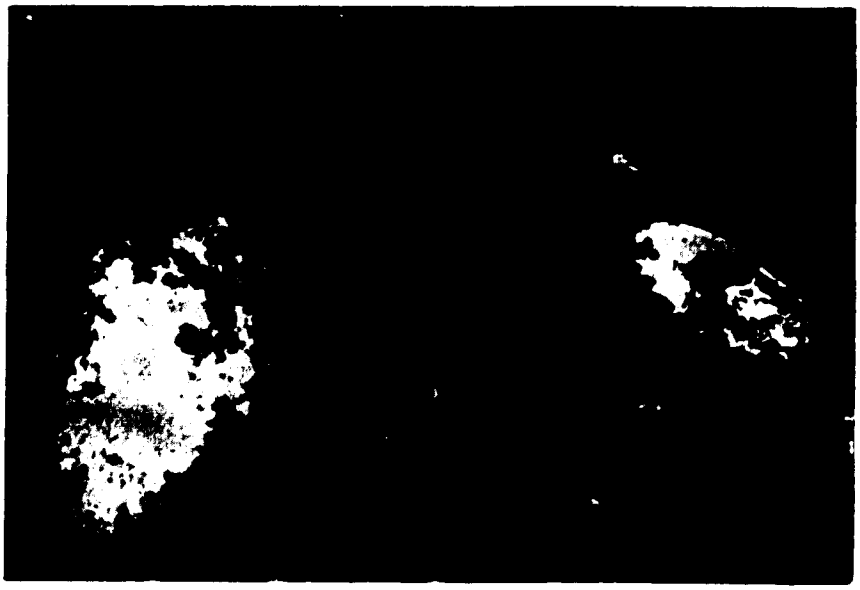


Fig. 8

Fig. 9 - Electron micrograph of hepatocyte of ethanol-fed mouse. Note numerous fat droplets and normal nucleus. 4500X

Fig. 10 - Electron micrograph of hepatocyte of ethanol-fed mouse. Note elongated and irreguarly shaped mitochondria, and small electron-lucent vesicles. 25,600X

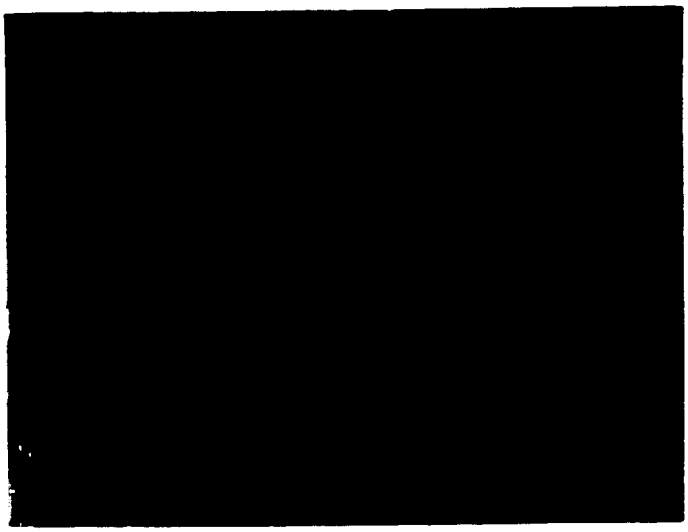


Fig. 9

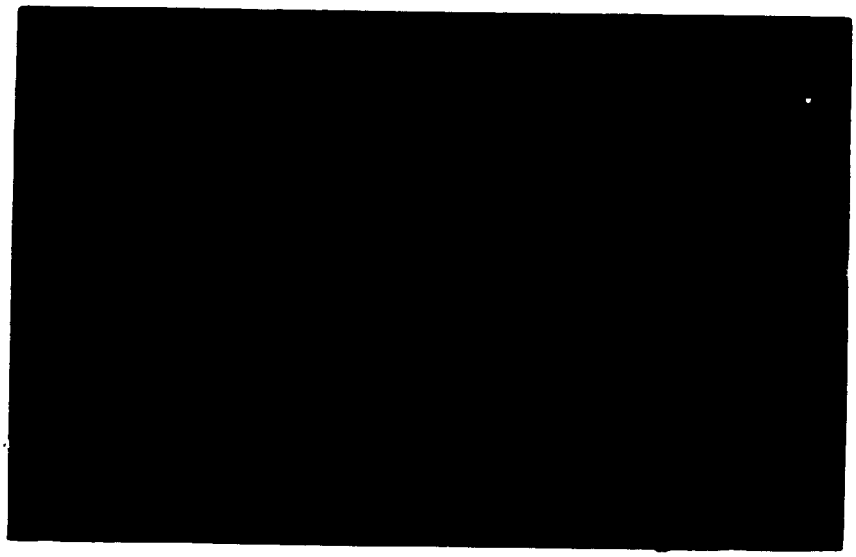


Fig. 10

Fig. 11 - Electron micrograph of hepatocyte of ethanol-fed mouse. Note irregularly shaped mitochondria and small electron-lucent vesicles. 25,600X

Fig. 12 - Electron micrograph of hepatocyte of control mouse. Note normal nucleus and mitochondria. 16,000X

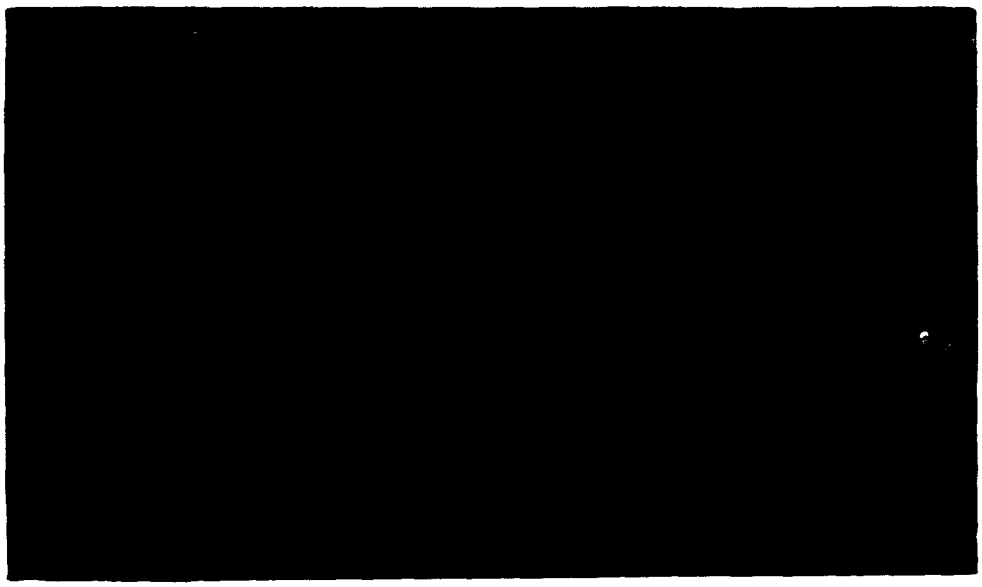


Fig. 11

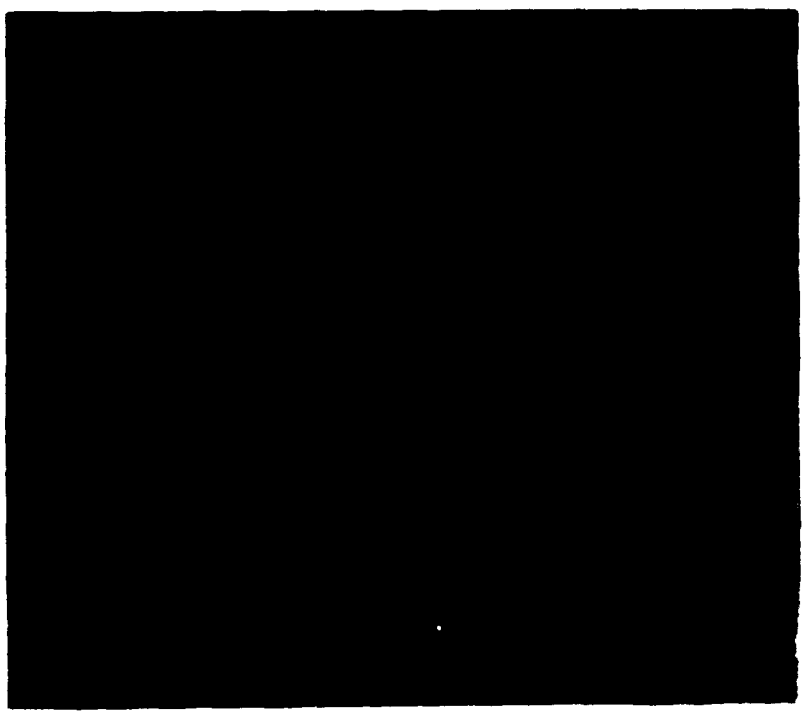


Fig. 12

Fig. 13 - Electron micrograph of hepatocyte of control mouse. Note normal mitochondria and rough endoplasmic reticulum. 23,750X

Fig. 13

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