

## Fatty Liver in the Rat after Prolonged Intake of Ethanol with a Nutritionally Adequate New Liquid Diet<sup>1,2</sup>

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**ABSTRACT** To determine whether prolonged ethanol intake can produce a fatty liver, even when associated with a diet containing adequate amounts of protein, minerals and vitamins, rats were given liquid diets containing 18% of calories as casein, supplemented with methionine (0.3 mg/kcal) and cystine (0.5 mg/kcal), choline (0.25 mg/kcal), fat (35% of total calories), adequate minerals and vitamins and, in the control diet, 47% of the calories as carbohydrates. A littermate of each control rat was pair-fed with the same diet in which carbohydrates had been isocalorically replaced with alcohol to the extent of 36% of the total calories. These diets assured continued growth in all animals and normal liver in the controls, whereas in the rats fed with alcohol, fatty liver developed, which was evident both morphologically and on chemical analysis; after 24 days of alcohol, hepatic triglycerides had increased on the average sixfold and cholesterol esters fivefold, compared with those of the controls. These studies demonstrate that prolonged alcohol intake can produce a fatty liver even when given with a diet with nutritionally adequate content of protein, vitamins and minerals, and an amount of fat less than that of the average American diet.

There is a widespread belief that when alcohol is ingested with an adequate diet, it produces no liver damage. This concept was challenged when we showed previously that both in man and in rats, fatty liver could be produced by prolonged alcohol intake despite diets with adequate content in nutrients (1, 2). To overcome the natural aversion of rats for alcohol, totally liquid diets, containing in one formula the necessary nutrients, as well as alcohol, were used. Our former purified diet contained a complete amino acid mixture as a substitute for protein, sucrose as carbohydrate and an amount of fat comparable to that of an average American diet (43% of total calories). With such a diet, isocaloric substitution of carbohydrates with ethanol to the extent of 36% of total calories resulted, after 24 days, in an average 7- to 8-fold increase of hepatic triglycerides (1, 2). The present study was undertaken to determine whether a similar ethanol effect could be demonstrated with a diet containing, instead of amino acids, a protein (casein), and instead of sucrose, a dextrin-maltose mixture which more closely resembles carbohydrates commonly found in food. The fat content of the diet was also reduced to 35% of the calories.

### MATERIALS AND METHODS

Male rats of a Sprague-Dawley strain (CD) were used in 11 groups of 2 littermates each. They were maintained with a commercial laboratory ration<sup>4</sup> and tap water ad libitum until they had reached a weight of 100 to 150 g, at which time they were housed in individual wire-bottom cages and given liquid diets in drinking tubes as the only source of food and water.

The overall composition of the new diet is shown in figure 1, together with our previous formula, to facilitate comparison of the changes made. The composition of the ethanol and control diets was as follows: casein<sup>5</sup> (supplemented with methionine 0.3 mg/kcal, and cystine 0.5 mg/kcal), 18% of the total calories; fat, 35% of total calories; adequate vitamins and minerals;

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<sup>4</sup> Purina Laboratory Chow, Ralston Purina Company, St. Louis.

<sup>5</sup> Vitamin-free Micro pulverized Casein, Nutritional Biochemicals Corporation, Cleveland.

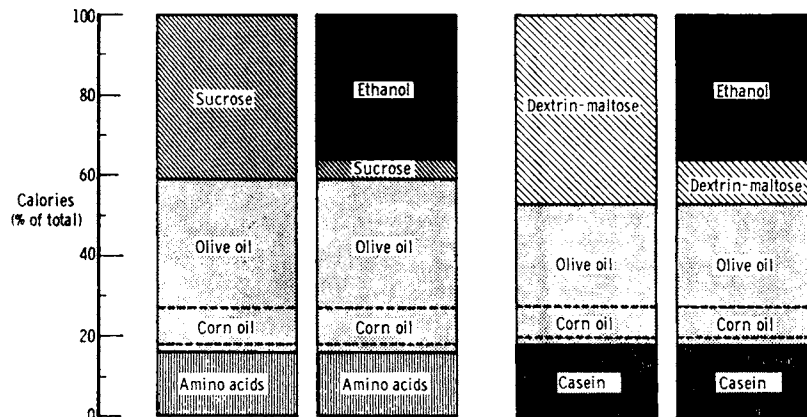


Fig. 1 Composition of liquid diets fed to the rats; amino acid, vitamin and mineral content was as described previously (22). Gray section below corn oil: ethyl-linoleate, 2 mg/kcal. Casein supplemented with methionine, 0.3 mg/kcal and cystine, 0.5 mg/kcal.

and in the control diet, carbohydrate,<sup>6</sup> 47% of the total calories. In the ethanol formula, carbohydrate was isocalorically replaced to the extent of 36% of the total calories.

The choline content and the calculated methionine composition were 0.25 and 1.5 mg/kcal, respectively, which is equivalent to concentrations of 0.1 and 0.6% in solid diets, amounts found to be optimal for the rat (3). The diets contained 1 kcal/ml, an adequate water-to-calorie ratio for the rat (4). Sodium carrageenan<sup>7</sup> (2 g/liter) was used to stabilize the liquid diet.

Animals were fed in groups of 2 littermates each: one rat was given the control diet, while the other received the same regimen in which dextrin-maltose, to the extent of 36% of calories, was isocalorically replaced with ethanol. Loss of ethanol by evaporation was negligible in the diets kept in the graduated drinking tubes (5) for periods of up to 48 hours. The diet was changed every 24 hours and, in some experiments, every 12 hours. Ethanol was introduced into the diet gradually. The final concentration of 50 g/liter of ethanol was achieved on the fifth day of feeding. During the first 2 days, the animals were given the liquid diet with 30 g/liter ethanol which was increased to 40 g/liter for the third and fourth days. Observation during the initial days indicated in each pair of littermates which of the animals had

the lowest spontaneous food intake. This rate-limiting rat (which was usually the one given ethanol) received the liquid diet ad libitum and the corresponding littermate was fed isocaloric amounts of the other diet on the following day. During the 24 hours preceding killing of the rats, the diets were given in three divided doses at approximately 8-hour intervals.

The body weight of the rats was determined at least twice a week. After 24 days, the animals were decapitated and blood was collected from the neck vessels in heparinized tubes and plasma was obtained by immediate centrifugation in the cold. The liver was quickly excised and approximately one gram was weighed into tubes containing chloroform:methanol (2:1). The plasma and remaining liver were stored at  $-18^{\circ}$ .

Total hepatic lipids were extracted (6) and quantitated by the method of Amenta (7). An aliquot of the total lipid extract, containing approximately 20 mg of fat, was evaporated under nitrogen to a volume of about 0.5 ml, and applied to a 0.5-mm thick silica gel chromatoplate (8) and developed in hexane:diethyl ether:acetic acid (83:16:1). The triglycerides, cholesterol esters and free cholesterol were eluted by the method of Goldrick and Hirsch (9); triglycerides were quantitated by determi-

<sup>6</sup> Dexin, generously supplied by Dr. Singleton, Burroughs Wellcome and Company, Tuckahoe, New York.  
<sup>7</sup> Viscarin, Marine Colloids, Inc., P.O. Box 70, Springfield, New Jersey.

nation of ester linkages by the procedure of Snyder and Stephens (10), and cholesterol and cholesterol esters were determined by the procedure of Searcy and Bergquist (11). Plasma alcohol was determined by the method of Bonnichsen (12). Samples of hepatic tissue were fixed in 10% neutral formalin until they were processed for histological examination.

The results obtained from each animal were compared with the corresponding values in the pair-fed control littermate. The means ( $\pm$  SE) and individual differences were calculated and the degree of significance was determined by Student's *t* test (13).

#### RESULTS

Twenty-four days of isocaloric replacement of carbohydrate by ethanol resulted in a significant increase of total hepatic lipids to  $96.8 \pm 6.6$  mg/g, compared with only  $46.1 \pm 1.3$  mg/g ( $P < 0.001$ ) in the controls. This increase in hepatic total lipids resulted primarily from a change in hepatic triglycerides and cholesterol esters as indicated in figures 2 and 3, respectively. The average triglyceride increase was almost sixfold, from  $10.8 \pm 0.64$  to  $56.8 \pm 4.6$  mg/g, whereas the average increase in cholesterol esters was fivefold,

from  $0.59 \pm 0.05$  to  $2.92 \pm 0.14$  mg/g. Free cholesterol changed only slightly as indicated in figure 3.

Alcohol levels, determined on the plasma taken at the time of killing, varied

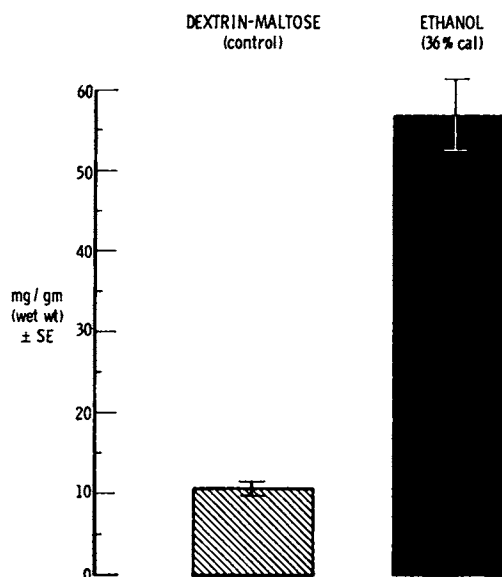


Fig. 2 Hepatic triglyceride concentration in rats pair-fed for 24 days with liquid diets containing either dextrin-maltose (control) or isocaloric amounts of ethanol (36% of calories).

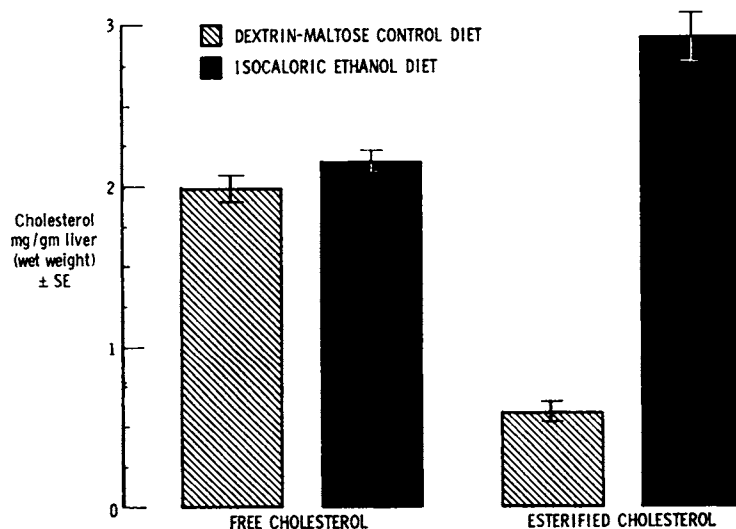


Fig. 3 Hepatic cholesterol concentration in rats pair-fed for 24 days with liquid diets containing either dextrin-maltose (control) or isocaloric amounts of ethanol (36% of calories).

widely with a mean of  $145.1 \pm 23.7$  mg/100 ml.

With both diets, rats continued to grow, with an average daily weight gain of 3.05 g.

With hematoxylin and eosin stains, hepatic morphology was found to be normal in controls, whereas fat accumulation was evident in the rats given alcohol, substantiating the chemical determinations.

#### DISCUSSION

The purpose of the present paper was to determine whether prolonged ethanol intake can result in fatty liver, despite a diet adequate in protein, vitamins and minerals. With conventional feeding techniques, that is, incorporation of alcohol in drinking water and administration of solid food, rats limited their alcohol intake, in most studies, to 20% of total calories or even less (14, 15); only in one study was the intake as high as 30% of total calories (16). With these techniques, however, as long as the dietary intake was adequate, no pronounced accumulation of fat in the liver was detected (14-16). This lack of steatosis is not unexpected, since 20% of total caloric intake as alcohol is a very low dose which, in our laboratory, not only failed to produce an appreciable fatty liver, but also did not result in any significant blood level of alcohol (2). No blood alcohol levels were reported in the one study in which 30% of total calories as alcohol failed to produce a fatty liver (16). To determine whether hepatic steatosis can be produced in rats with ethanol in the absence of dietary deficiency, an experimental method was needed to increase the amount of alcohol consumed by the rats. Alcohol, when given acutely without food in a large single dose by gastric tube, was found to produce fat accumulation in the liver (17-22) but the mechanisms involved in these acute experiments do not necessarily apply to more prolonged alcohol intake such as observed in chronic alcoholic patients (2, 22, 23). In our previous studies (1, 2, 24), prolonged intake of substantial amounts of alcohol was achieved by overcoming the natural aversion of rats for alcohol by incorporation of the ethanol in a completely liquid diet. This previous diet, however, contained, as

a substitute for protein, an expensive amino acid mixture. The present study demonstrates that a similar effect can be obtained with alcohol when the amino acids are replaced by casein, enriched with methionine and cystine. To eliminate possible direct effects of sucrose on hepatic lipid metabolism which have been described by some (25, 26) but not observed by others (27), sucrose was replaced by a mixture of dextrin-maltose. The diet was further changed by decreasing its fat content from 43 to 35% of total calories, an amount less than that of the average American diet (28). This decrease in fat content allowed an increase of the carbohydrate from 41 to 47% in the control diet; in the ethanol diet this resulted in a doubling of the remaining carbohydrate after incorporation of the ethanol. In addition to a composition in all known required nutrients in amounts considered to be adequate or optimal for the rat (29), the quality of the diet was evidenced by the continued growth of the rats as well as normal hepatic fat content and morphology in the controls.

Lipotrope content of our diet (0.25 mg of choline and 1.5 mg of methionine/kcal, including the methionine present in casein) was equivalent to the amount of lipotropic substances reported by Klatskin et al. (30) and Best et al. (31) to fully protect against fatty liver development in rats given a choline-deficient diet, with or without ethanol in drinking water. The amount of choline was also reported by others to be optimal for the rat (3). Since the possibility has been raised that ethanol may increase choline requirements (30), and since the ethanol intake in the present experiments was greater than that in the studies of Klatskin et al. (30), the possibility has to be considered that choline requirements may have been increased even further. It is unlikely, however, that simple enhancement of choline requirements could fully explain our results, since in one of our previous studies, massive choline supplementation with 20 times the amount present in our diet failed to fully protect against steatosis; hepatic triglyceride accumulation, although reduced, still represented a threefold increase compared with the controls (24). It is therefore likely

that ethanol produces steatosis through effects other than or in addition to those related to lipotrope metabolism. The present study demonstrates that this hepatic steatosis can be produced by ethanol even when our original formula (1, 2, 24) is modified to more closely resemble conventional diets by replacing sucrose by dextrin-maltose, amino acids by protein, and by decreasing the fat content to an amount less than that of the average American diet. This improved procedure for the experimental production of a fatty liver on prolonged alcohol ingestion is proposed as a convenient and inexpensive tool for further studies of the pathogenesis and possibly the treatment and prevention of alcoholic liver disease.

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