

Effects of Chronic Ethanol Feeding on Serum Lipoprotein Metabolism in the Rat

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ABSTRACT In rats, chronic ethanol feeding was found to enhance the postprandial hyperlipemia and to increase the incorporation of dietary palmitic acid-³H and intravenously injected L-lysine-¹⁴C into serum lipoproteins. The main increases of total amount, labeling, and specific activity of lipid and protein occurred in the $d < 1.019$ lipoprotein fraction. Fat absorption and the clearance of injected chylomicrons were not affected by ethanol feeding. Blocking of lipoprotein and chylomicron removal with Triton did not prevent the action of ethanol on serum lipids, indicating that the ethanol effect is not likely due to defective removal of lipids from the circulation. Ethanol enhanced the incorporation of chylomicron fatty acids into newly synthesized very low density lipoproteins, as shown by an increased reappearance of the fatty acid label into the lipids of this fraction after injection of palmitate-¹⁴C/glycerol-³H doubly labeled chylomicrons. These results indicate that alcoholic hyperlipemia is due, at least in part, to an increase in newly synthesized lipoproteins. The hyperlipemia produced by ethanol was accompanied by hepatic steatosis. The simultaneous production of fatty liver and hyperlipemia makes it unlikely that defective lipoprotein synthesis or secretion is a primary mechanism for the pathogenesis of the alcoholic fatty liver.

INTRODUCTION

Hyperlipemia has been observed in association with alcoholism. An exaggeration of the postprandial hyperlipemia after acute administration of ethanol¹ has been

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¹Ethanol and alcohol (ethyl alcohol) are used synonymously in this paper.

described both in man (2, 3) and in rats (4). An increase of circulating triglycerides can also be produced in fasting individuals after ingestion of ethanol for several hours (5-7) as well as during administration of alcohol containing diets for several days (8-10). This hyperlipemia could result primarily either from increased hepatic production of lipoproteins or from decreased removal of serum lipids. The present study was undertaken to determine which of these mechanisms can be incriminated in the pathogenesis of the hyperlipemia induced by prolonged ethanol intake.

METHODS

Materials. L-Lysine-¹⁴C (uniformly labeled) (237 mCi/mmole) of a radiochemical purity greater than 99% was obtained from New England Nuclear Corp., Boston, Mass., and diluted with 0.9% NaCl to 1 μ Ci/ml for intravenous injection. Palmitic acid-9,10-³H (432 mCi/mmole) with tripalmitin carboxyl-¹⁴C (126 mCi/mmole), the purity of which was verified by thin-layer chromatography, were obtained from New England Nuclear Corp. and dissolved in chloroform. Aliquots of this solution were dried under nitrogen and redissolved in the diets described below at a concentration of 1 μ Ci/ml of diet. Triton WR-1339 (*p*-isooctylpolyoxyethylenephenol polymer) was obtained from Winthrop Laboratories, New York, and diluted in 0.9% NaCl to 100 mg/ml for intravenous injection.

Chylomicrons were labeled by intragastric administration of the appropriate precursors to rats with thoracic duct fistula. Doubly labeled chylomicrons were prepared according to the method of Borgström and Jordan (11). Thoracic duct fistulae were established in rats (300 g body wt) fed Purina Chow and in rats given the ethanol-containing diet described below for 24 days, using the technique of Bollman, Cain, and Grindlay (12) with minor modifications. At operation, gastrostomy cannulae were also inserted. 0.5 mCi of palmitic acid-1-¹⁴C (10.3 mCi/mmole), 1 mCi of palmitic acid-9,10-³H or a combination of 0.5 mCi of palmitic acid-1-¹⁴C and 1.0 mCi of tripalmitin glyceryl-2-³H (255 mCi/mmole), all of them obtained from New England Nuclear Corp. (assessed with regard to purity as described above), were dissolved in 2 ml of olive oil and administered to dif-

ferent groups of rats through the gastrostomy tube at least 24 hr after operation. The lymph was collected at room temperature in sterile tubes during the period of maximal labeling (within 4-8 hr after intragastric administration of the precursors) and stored at room temperature for a maximum of 72 hr. The day of the experiment, chylomicrons were separated by ultracentrifugation as previously described (13). Lipid content and radioactivity were measured in aliquots of these preparations. More than 90% of the chylomicron lipid was shown to be triglyceride by thin-layer chromatography. In the doubly labeled chylomicron preparations, the isotopes ^{14}C and ^3H were incorporated into the chylomicron lipids of the two preparations used with a $^{14}\text{C}/^3\text{H}$ ratio of 2.386 and 3.401 respectively. These preparations were injected at a dose of 3.5 mg of chylomicron lipid per 100 g body weight. For each preparation, the changes that occurred in the $^{14}\text{C}/^3\text{H}$ ratio during the clearing of the injected chylomicrons were related to the initial ratio which, arbitrarily, was considered to have a value of 1 for purposes of comparison. The two singly labeled preparations used (one labeled with ^3H and the other with ^{14}C) had the same clearance rate when injected simultaneously to normal rats. They were given in a dose of 7 mg of chylomicron lipid per 100 g body weight.

Animal procedures. Male rat littermates of a Sprague-Dawley strain (CD), weighing 100-150 g, and purchased from Charles River Breeding Laboratories (Wilmington, Mass.), were pair fed once or twice daily a liquid diet previously described (14). These diets supply 18% of the total calories as protein, 35% as fat, 11% as carbohydrate, (Dextri-Maltose, generously supplied by Mead Johnson & Co., Evansville, Ind.) and 36% either as additional carbohydrate (controls) or as isocaloric amounts of ethanol. Alcohol was introduced in the diet gradually, as described before (14). Rats were treated for a total period of 10 or 24 days, 18 and 6 hr before the experiments, the liquid diets were given by gastric intubation (6 ml/100 g body weight) to assure equal timing and rate of food intake.

In the first set of experiments, rats fed the liquid diets for 10 days (eight pairs) and 24 days (24 pairs) were injected with 0.5 μCi of L-lysine- ^{14}C per 100 g body weight into the femoral vein under light ether anesthesia. This procedure was immediately followed by intragastric administration of the diet (6 ml/100 g body weight) containing 1 μCi of palmitic acid- ^3H per ml. 0, 30, 60, 90, and 120 min thereafter in the 24 days fed animals and 90 min thereafter in the 10 days fed animals, the blood was drained from the aorta without anticoagulants and allowed to clot at room temperature. The liver was removed. Cardias and rectum were ligated and the entire gastrointestinal tract was also excised by separating it from the mesenterium as close to the intestinal insertion as possible. Intestinal fat absorption was assessed after 24 days of alcohol feeding by subtracting the fatty acid radioactivity remaining in the gastrointestinal tract from that given in the diet 0, 30, 60, 90, 120 min or 6 hr before. Lipid absorption was also determined in this manner in six additional pairs of rats using tripalmitin carboxyl- ^{14}C as a marker of dietary fat 90 min before sacrifice. Blood samples were obtained from the tip of the tail at 60-min intervals for alcohol determinations.

In a second set of experiments, 24 rats were fed for 24 days in groups of four littermates each: two rats in each group were given the ethanol-containing diet, whereas the other two received the same amount of the diet in which ethanol was isocalorically replaced by carbohydrate. The feeding schedule described before was followed except that

50 mg of Triton per 100 g body weight were injected into the femoral vein in one of the controls and in one of the ethanol fed animals of each group 90 min before sacrifice. The other two were injected intravenously with a similar volume of saline. Intestinal fat absorption was also assessed as described above.

In a third set of experiments, no radioactive precursor was given in the diet to four pairs of rats fed for 10 days and 12 for 24 days. Labeled chylomicron preparations were injected into the femoral vein under light ether anesthesia at the time of the last gastric intubation or 30 min thereafter. From the tip of the tail, 15- to 50- μl samples of blood were collected in previously calibrated and heparinized capillary tubes at 1, 2, 3, 4, 6, 8, 15, 30, and 60 min after the injection of chylomicron. At 10 and at 90 min, samples of blood were also taken for microhematocrit determinations to allow calculation of the volume of plasma sampled. The hematocrit fell similarly in the controls and alcohol-fed rats from $47.4 \pm 0.1\%$ at 10 min to $37.1 \pm 1.0\%$ at the time of sacrifice. The blood samples were washed with 1 ml of serum (obtained from normal nonfasted rats) from the capillary into cellulose acetate tubes for ultracentrifugation. The total volume of blood drawn during this 90 min period varied between 0.3 and 1.0 ml. 90 min after doubly labeled chylomicron injection, the remainder of the blood was collected from the aorta. In the other eight pairs of rats in which singly labeled chylomicrons were used, the experiment was repeated 6 hr later by using differently labeled chylomicrons 30 min after Triton had been administered intravenously (50 mg/100 g body weight). Chylomicrons were diluted in 1% Triton solution in 0.9% NaCl immediately before injection.

Analysis. Three serum lipoprotein fractions were separated successively by ultracentrifugation according to the method of Havel, Eder, and Bragdon (15). The density of the serum samples was adjusted to 1.019 and that of the successive infranates to 1.063 and 1.210 respectively. Serum samples of 2-4 ml were carefully layered under saline of the appropriate density so that the lipoproteins migrated to the top of the tube through a protein-free medium (16). Under these conditions, protein or lipid labeling of lipoproteins, as well as their protein- ^{14}C /lipid- ^3H ratio, did not change after a second ultracentrifugation. No attempt was made to separate dietary chylomicrons from endogenous particles or very low density lipoproteins.

In the series of animals in which Triton was injected, total serum lipids and their labeling were measured. No lipoprotein fractionation was attempted because of the reported interference by Triton with the standard lipoprotein separation procedures (17).

The isolated lipoprotein fractions and the liver samples (homogenized in saline) were precipitated with trichloroacetic acid and the precipitates were washed and delipidized with ethanol-acetone and ether as described by Radding and Steinberg (16). The dried protein precipitates were dissolved in 1 N NaOH, and the protein content was measured by the method of Lowry, Rosebrough, Farr, and Randall (18). 0.1 ml of the alkaline solution was directly counted in Bray's liquid scintillator (19).

Hepatic lipids were extracted according to the procedure of Folch, Lees, and Sloane-Stanley (20). The ethanol-acetone and ether extracts of the precipitated serum lipoprotein fractions were combined and dried under nitrogen, redissolved in chloroform-methanol, and washed according to Folch et al. (20). Aliquots of the lipid extracts were counted and their lipid content measured as previously reported (14). Tissue lipid labeling was corrected for the

radioactivity contributed by the plasma, using values reported by Dewey (21) for plasma content of tissues. Liver lipids were further fractionated by thin-layer chromatography; the triglycerides were quantitated (14) and their labeling counted.

The gastrointestinal tract and samples of the diets were digested and saponified by autoclaving in 90% KOH for 1 hr at 120°C (22). The fatty acids were extracted in petroleum ether after acidification and an aliquot of these extracts was dried under nitrogen and counted.

Blood alcohol was measured by the method of Bonnichsen (23).

Statistics. In all experiments, the values obtained in the alcohol-fed animals were compared to those obtained in their pair-fed controls and the mean of the individual differences was tested by the Student *t* test (24). All values were expressed by their mean \pm SEM.

RESULTS

The feeding of the ethanol-containing diet (6 ml/100 g body weight) resulted in rapidly increasing levels of blood alcohol. The highest values were found 2 hr after gastric intubation (153.4 ± 8.8 mg/100 ml of blood). Afterwards there was a progressive decline, as shown in Fig. 1.

Effects of ethanol feeding on lipid absorption. As determined by the radioactivity of the tritiated palmitate remaining in the gastrointestinal tract, the net intestinal absorption was not affected significantly by ethanol feeding for 24 days (Fig. 2). The recovery of radioactivity from the gastrointestinal tract immediately removed after administration by gastric tube of the control and alcohol-containing diet was $99.0 \pm 7.3\%$ and $104.8 \pm 7.8\%$ respectively. In six pairs of animals, tripalmitin carboxyl- 14 C was used as a marker of dietary fat. The percentage of the initially administered radioactivity remaining in the gastrointestinal tract 90 min after administration by gastric tube, was also unchanged by

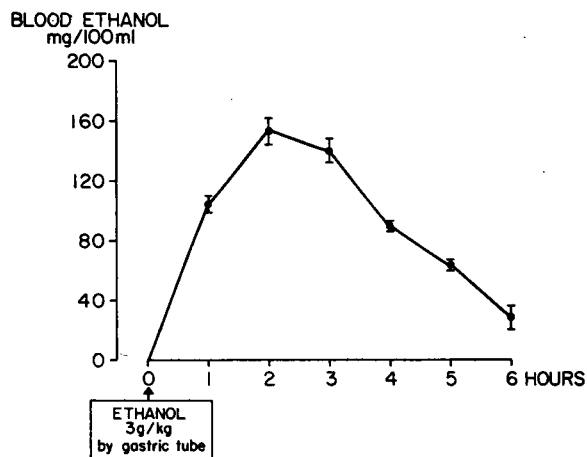


FIGURE 1 Blood ethanol concentrations after administration of the ethanol-containing diet by gastric tube to rats fed the same diet for 24 days.

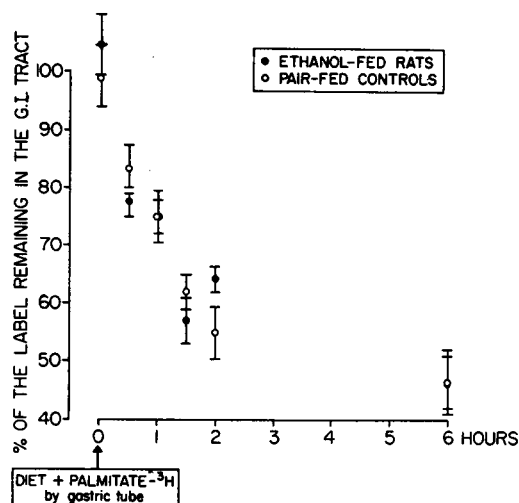


FIGURE 2 Percentage of radioactivity remaining in the gastrointestinal tract at different times after administration (by gastric tube) of ethanol or isocaloric control diets labeled with palmitic acid- 3 H. Both groups of rats were fed the corresponding diets for 24 days before the experiments.

ethanol feeding for 24 days ($45.0 \pm 3.9\%$ in the ethanol-treated rats and $43.0 \pm 1.6\%$ in their pair-fed controls).

The intestinal fat absorption was also unaffected by Triton (with or without ethanol); 90 min after the labeled feeding, radioactivity remaining in the gastrointestinal tract was $56.5 \pm 8.1\%$ after Triton in the alcohol-fed rats (compared to $53.8 \pm 6.9\%$ after Triton in their pair-fed controls) and $56.3 \pm 8.6\%$ after saline plus ethanol (compared to $57.1 \pm 3.0\%$ in their pair-fed controls).

Effects of ethanol feeding on liver lipids. As expected, alcohol feeding resulted in a progressive accumulation of fat in the liver. After 10 days of ethanol administration (only 6 days on the full dose of alcohol) moderate but significant ($P < 0.01$) increases in liver total lipid and triglyceride concentrations were observed (Table I). In the alcohol-fed animals the total liver lipids increased 48.1% and the hepatic triglycerides 75%, compared to their pair-fed controls.

After 24 days, the lipid concentration of the liver in the ethanol-fed animals doubled and liver triglycerides increased more than 3-fold (compared to their controls). Both changes were significant ($P < 0.01$) (Table I).

When tritiated palmitate was included in the diets given by gastric tube 60 and 90 min before sacrifice, the ethanol-fed rats incorporated significantly more radioactivity in the liver total lipids and triglycerides than their pair-fed control ($P < 0.01$), while at other times this difference was not significant. The magnitude of the increase in liver lipid labeling after ethanol was such that, despite the preexisting steatosis, the specific ac-

TABLE I
Effects of Ethanol Feeding on Liver Lipid Concentration and Labeling 90 min after the Administration of Diets Containing Palmitic Acid-³H

	Control rats (mean ±SEM)	Alcohol-fed rats (mean ±SEM)	P
A. 10 day pair feeding			
Total lipid concentration, mg/g of liver	37.52 ± 1.14	55.58 ± 4.13	<0.01
Total lipid labeling, dpm × 10 ⁻³ /g of liver	20.55 ± 4.14	54.84 ± 12.34	<0.02
Triglyceride concentration, mg/g of liver	11.76 ± 1.87	20.58 ± 1.87	<0.01
Triglyceride labeling, dpm × 10 ⁻³ /g of liver	6.01 ± 0.73	19.49 ± 1.74	<0.01
B. 24 day pair feeding			
Total lipid concentration, mg/g of liver	41.28 ± 3.01	84.30 ± 7.65	<0.01
Total lipid labeling, dpm × 10 ⁻³ /g of liver	21.06 ± 3.22	41.04 ± 7.15	<0.02
Triglyceride concentration, mg/g of liver	14.51 ± 1.90	47.29 ± 10.45	<0.01
Triglyceride labeling, dpm × 10 ⁻³ /g of liver	5.32 ± 0.96	12.22 ± 2.08	<0.05

tivity of the liver total lipids and triglycerides was not significantly lower in the ethanol-fed rats than in the controls at the time of maximal labeling. After 10 days of alcohol feeding and 90 min after administration of the label, the specific activity of the hepatic triglycerides actually increased ($P < 0.01$). After 24 days, no significant difference in the specific activity of the hepatic triglycerides was found between the controls and alcohol-treated rats. The data observed in the animals sacrificed 90 min after administration of the label are shown in Table I.

A similar increase in hepatic triglyceride labeling was observed 90 min after injection of labeled chylomicrons: 38.4 ± 1.2 dpm × 10⁻³/g of liver in the rats fed alcohol for 10 days compared to 11.1 ± 2.7 in their controls ($P < 0.01$) and 34.8 ± 1.4 in the rats fed alcohol for 24 days compared to 14.1 ± 1.6 in their controls ($P < 0.01$). Again, the specific activity of the hepatic triglycerides increased after 10 days of alcohol feeding (from 761

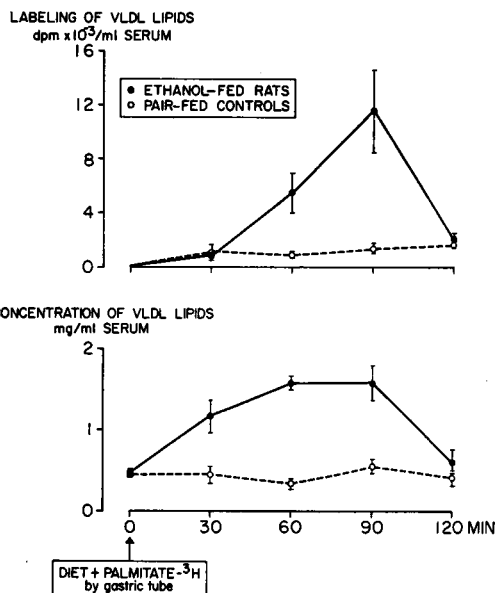


FIGURE 3 Concentration and labeling of the lipid moiety of serum very low density lipoproteins (VLDL; $d < 1.019$) at different times after administration (by gastric tube) of ethanol or isocaloric control diets labeled with palmitic acid-³H. Both groups of rats were fed the corresponding diets for 24 days before the experiments.

± 183 dpm/mg in the controls to 2135 ± 313 in the alcohol-treated animals) ($P < 0.01$), but this difference was not present after 24 days of administration of ethanol, probably because of the greater degree of pre-existing hepatic steatosis.

Triton administration did not alter the increase in hepatic lipid concentration or labeling produced by ethanol (Table II).

Effects of ethanol feeding on liver proteins. Total liver proteins and protein labeling after intravenous injection of L-lysine-¹⁴C were not significantly changed by ethanol feeding. Total protein concentrations were 195.4 ± 12.2 mg/g of liver in the controls, and 204.1 ± 9.4 mg/g of liver after ethanol. Incorporation of lysine-¹⁴C into total liver proteins was maximal 60 min after intravenous injection both in the alcohol-fed rats

TABLE II
Effects of Triton on Hepatic Triglyceride Concentration and Labeling of Control and Alcohol-Fed Rats (24 days) 90 min after the Administration of Diets Containing Palmitic Acid-³H

	Triton	Control rats (mean ±SEM)	Ethanol-fed rats (mean ±SEM)	P
Triglyceride concentration, mg/g of liver	+	11.56 ± 3.61	38.48 ± 12.54	<0.05
	-	12.10 ± 2.44	33.72 ± 7.68	<0.01
Triglyceride labeling, dpm × 10 ⁻³ /g of liver	+	2.32 ± 0.31	7.21 ± 1.73	<0.05
	-	3.83 ± 0.47	9.52 ± 1.61	<0.01

TABLE III
Effects of Ethanol Feeding on Lipid and Protein Content and Labeling of Serum Lipoproteins 90 min after the Administration of Diets Containing Palmitic Acid-³H and the Intravenous Injection of L-Lysine-¹⁴C

	Serum lipoprotein, fractions	Control rats (mean ±SEM)	Ethanol-fed rats (mean ±SEM)	P
A. 10 day pair feeding				
Lipid content, mg/ml of serum	<1.019	0.800 ±0.099	1.728 ±0.364	<0.02
	1.019-1.063	0.485 ±0.041	0.493 ±0.031	NS
	1.063-1.210	1.452 ±0.114	1.772 ±0.187	NS
Lipid labeling, dpm × 10 ⁻³ /ml of serum	<1.019	1.461 ±0.261	4.934 ±1.146	<0.01
	1.019-1.063	0.230 ±0.045	0.241 ±0.069	NS
	1.063-1.210	0.257 ±0.036	0.328 ±0.047	NS
Protein content, mg/ml of serum	<1.019	0.057 ±0.004	0.130 ±0.013	<0.01
	1.019-1.063	0.361 ±0.066	0.311 ±0.032	NS
	1.063-1.210	1.151 ±0.149	1.339 ±0.126	NS
Protein labeling, dpm × 10 ⁻³ /ml of serum	<1.019	0.177 ±0.049	0.922 ±0.196	<0.01
	1.019-1.063	0.148 ±0.028	0.202 ±0.054	NS
	1.063-1.210	1.037 ±0.243	1.357 ±0.054	NS
B. 24 day pair feeding				
Lipid content, mg/ml of serum	<1.019	0.599 ±0.070	1.573 ±0.153	<0.01
	1.019-1.063	0.414 ±0.035	0.698 ±0.082	<0.01
	1.063-1.210	1.981 ±0.302	2.981 ±0.559	<0.01
Lipid labeling, dpm × 10 ⁻³ /ml of serum	<1.019	1.341 ±0.249	11.773 ±3.208	<0.01
	1.019-1.063	0.374 ±0.119	0.933 ±0.290	NS
	1.063-1.210	0.316 ±0.029	0.550 ±0.054	<0.01
Protein content, mg/ml of serum	<1.019	0.031 ±0.005	0.078 ±0.013	<0.02
	1.019-1.063	0.124 ±0.012	0.187 ±0.023	NS
	1.063-1.210	1.246 ±0.105	1.685 ±0.254	NS
Protein labeling, dpm × 10 ⁻³ /ml of serum	<1.019	0.128 ±0.031	0.658 ±0.142	<0.01
	1.019-1.063	0.355 ±0.089	0.511 ±0.083	NS
	1.063-1.210	1.904 ±0.413	2.679 ±0.506	NS

(159 ±10 dpm × 10⁻³/g liver) and in the controls (133 ±22 dpm × 10⁻³/g liver). At 90 min, 126 ±5 dpm × 10⁻³/g liver were incorporated into the liver protein of alcohol-fed rats compared with 116 ±2 in the controls.

Effects of ethanol feeding on serum lipoproteins. After intragastric feeding, the serum became turbid or frankly lactescent in the rats fed alcohol for 10 or 24 days, whereas in the controls it was either clear or only slightly turbid.

After alcohol feeding, significant increases in the serum fraction of density less than 1.019 were observed 60 and 90 min after administration of the diets by gastric tubes. Compared with the values obtained in the control group, the lipid and protein content of this fraction doubled in the alcohol-fed rats. The incorporation of palmitic acid-9,10-³H and L-lysine-¹⁴C into the lipid and protein moieties, respectively, of this very low density fraction, as well as their specific activities, also significantly increased (Figs. 3 and 4). In a limited

number of samples, lipid composition of this fraction was determined and was found to consist of at least 90% glycerides, primarily triglycerides.

After 10 days of alcohol feeding, no significant changes were observed in the lipid and protein content and labeling of the other serum lipoprotein fractions collected 90 min after gastric intubation (Table III A).

After 24 days of ethanol feeding (Table III B), significant increases in the lipid content of all serum lipoprotein fractions were observed. The incorporation of palmitic acid-³H into the lipoprotein lipids was found to be significantly increased not only in the very low density fraction but also in lipoproteins of density higher than 1.063. The main increases occurred in the very low density fraction with resulting enhanced specific activities. Alcohol feeding for 24 days also resulted in increased protein content, labeling, and specific activity of the very low density lipoprotein fraction. By contrast, the protein concentration and the incorporation of

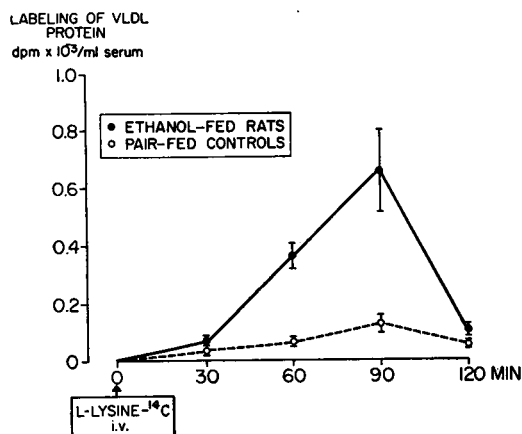


FIGURE 4 Incorporation of L-lysine-¹⁴C into the protein moiety of serum very low density lipoproteins (VLDL; $d < 1.019$) at different times after intravenous injection.

L-lysine-¹⁴C into serum nonlipoprotein proteins ($d > 1.210$) were unaffected by ethanol feeding: the concentration was 52.9 ± 2.8 mg/ml of serum in the alcohol-treated animals and 53.4 ± 3.6 in the controls. The incorporation 90 min after intravenous L-lysine-¹⁴C injection was 38.88 ± 8.19 dpm $\times 10^{-3}$ /ml of serum in the alcohol-fed animals and 39.89 ± 8.22 in the controls, resulting in specific activities four times and 11 times lower than that of the $d < 1.019$ lipoproteins in the controls and in the rats fed alcohol, respectively.

The Triton effect on chylomicron removal was confirmed by the observation of a striking reduction of the clearance rate of injected singly labeled (¹⁴C or ³H) chylomicrons (Fig. 5). Under our experimental conditions, the Triton effect appeared rapidly, was maintained for the duration of the experiment, and was comparable in the alcohol-fed and control rats. The experiments of Fig. 5 also indicate that in the absence of Triton, the clearance of chylomicron obtained from ethanol-fed rats was not significantly altered by ethanol

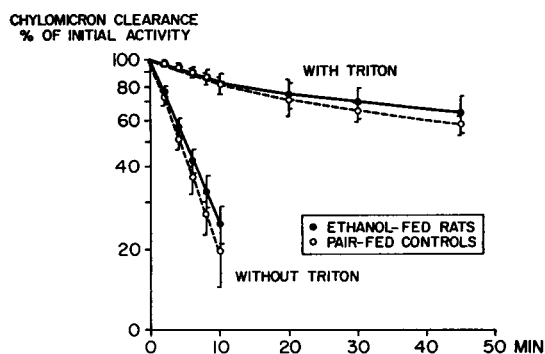


FIGURE 5 Blood clearance of labeled chylomicrons in ethanol-treated and control rats; effect of Triton.

feeding: its half-life was 4.05 ± 0.58 min in the controls vs. 4.88 ± 0.50 min after ethanol.

Although alcohol feeding did not modify the Triton block, its effect on serum lipids was not prevented (Fig. 6). In the controls, the addition of Triton increased blood lipids and their labeling 90 min after intragastric administration of labeled palmitate, as expected. Alcohol feeding (in the presence of Triton) increased blood lipids even further, including lipid labeling ($P < 0.05$) despite similar fat absorption and similar block of chylomicron clearance.

When doubly labeled chylomicrons were injected into eight pairs of rats, the alcohol feeding again did not significantly change the rate of disappearance of the fatty acid label of the chylomicrons (Fig. 7). The half-life of the chylomicrons was 2.47 ± 0.37 min in the controls and 2.79 ± 0.38 in the alcohol-fed rats. Contrasting with the lack of significant change in the disappearance of the chylomicron fatty acid label, there was a highly significant difference ($P < 0.01$) in the reappearance of the palmitic acid-¹⁴C in the very low density fraction of the serum. In the ethanol-fed animals, there was five times more ¹⁴C labeling in the lipids of the $d < 1.019$ fraction than in the controls. In the latter, $1.77 \pm 0.52\%$ of the initial serum ¹⁴C radioactivity was

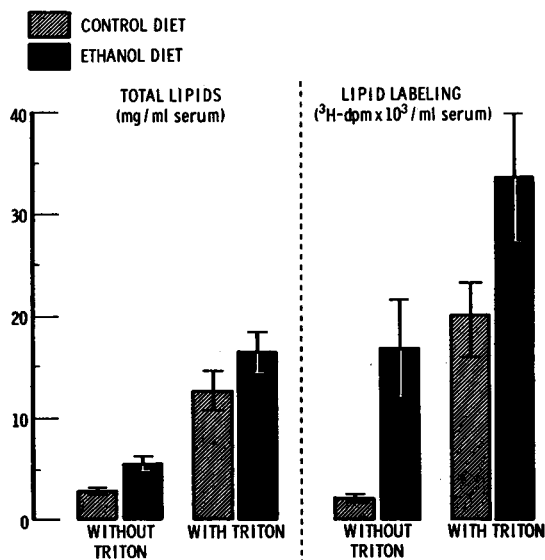


FIGURE 6 Effect of ethanol on Triton-enhanced postprandial hyperlipemia. Rats were treated during 24 days in groups of four animals: in each group, two rats received the ethanol-containing diet (black bars) whereas the other two were fed equal amounts of a diet in which ethanol was isocalorically replaced by carbohydrate (shaded bars). Serum lipids and their labeling (mean \pm SEM) were measured 90 min after intragastric feeding of the corresponding diets containing a trace amount of palmitic acid-³H, preceded by intravenous injection of either Triton or saline.

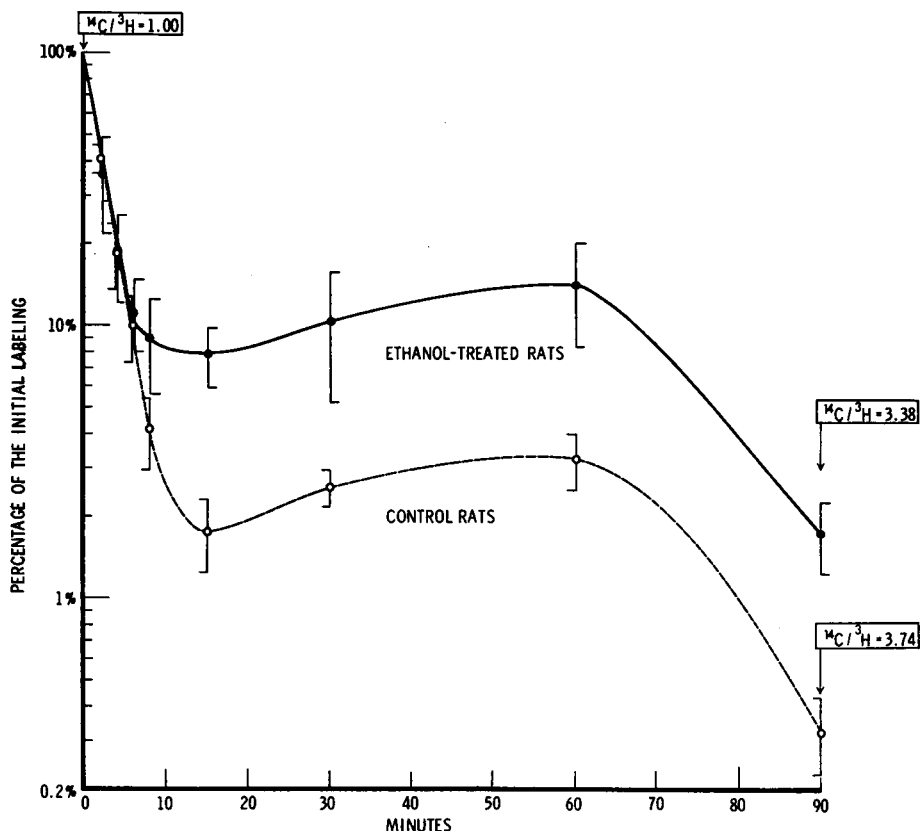


FIGURE 7 Labeling of $d < 1.019$ serum lipoproteins after intravenous injection of doubly labeled chylomicrons. ^{14}C radioactivity and $^{14}\text{C}/^3\text{H}$ ratio were measured in the lipids of the $d < 1.019$ lipoprotein fraction during the 90 min after the injection of chylomicrons (palmityl- ^{14}C , glycerol- ^3H). Controls: dotted line; alcohol-fed rats: solid line.

present in this fraction 15 min after injection of the chylomicrons. At 60 min, $3.29 \pm 0.66\%$ of the initial radioactivity reappeared in the very low density fraction. In the alcohol-fed rats, $7.80 \pm 1.92\%$ was present at 15 min and increased up to 14.80 ± 5.71 by 60 min after chylomicron injection (Fig. 7).

During the 2–4 min after chylomicron administration, the $^{14}\text{C}/^3\text{H}$ ratio was maintained close to the original value in both groups of animals. After this short initial period, a rapid increase of the ratio could be detected in most of the animals and, after 8 or 10 min, the tritium labeling fell so low that often it could not be counted accurately in the small volume of the plasma sampled. When enough serum was available at the time of sacrifice, an accurate measurement of the tritium became possible; it showed that the $^{14}\text{C}/^3\text{H}$ ratio had significantly ($P < 0.01$) and similarly increased in both groups of rats. The $^{14}\text{C}/^3\text{H}$ ratio in the lipids of the serum fraction of density less than 1.019 was 3.38 ± 0.93 in the alcohol-treated animals and 3.74 ± 1.24 in their pair-fed controls compared to the initial value of 1.

DISCUSSION

The results of the present study show that ethanol feeding enhances postprandial hyperlipemia in the rat and increases the incorporation of dietary palmitic acid- ^3H and intravenously injected L-lysine- ^{14}C into serum lipoproteins. Although the main changes occurred in the $d < 1.019$ serum fraction (which includes dietary chylomicrons), significant increases in the lipid content of lipoproteins of density higher than 1.019 were also observed, indicating that the lipids accumulated in the serum did not only represent dietary chylomicrons. Moreover, the observed differences could not be accounted for by altered intestinal fat absorption which was not significantly affected by ethanol under these conditions. The hyperlipemia, therefore, had to result either from increased hepatic production of lipoproteins or from decreased removal of serum lipoproteins (including that of the dietary chylomicrons) or from both. Actually, the chylomicron clearance was unaffected by ethanol feeding, confirming previous observations carried out with slightly different procedures (13). The

latter study also showed that hepatic uptake of chylomicrons was unchanged by ethanol. Moreover, the considerable hyperlipemia present in the ethanol-fed animals during the clearing of the injected labeled chylomicrons would have slowed down the disappearance rate of chylomicrons due to the larger pool, if chylomicrons had indeed greatly contributed to the original hyperlipemia; removal of chylomicrons from the blood was indeed found to be slowed down by increasing chylomicron loads (25).

Since chylomicrons obtained from ethanol-fed as well as from normal rats were used in this study, the possibility that the clearance of chylomicrons obtained from alcohol-fed rats might be different than those from control rats can be ruled out. A selective block of chylomicron removal by ethanol is also unlikely as a primary cause for the hyperlipemia, because the hyperlipemic effect of alcohol was still present although removal of chylomicrons was almost completely suppressed by Triton. The degree of the Triton block was not affected by ethanol and the fat absorption was unaltered. Since Triton also blocks the removal of other serum lipoproteins (26), it is unlikely that the alcohol effect was due primarily to a selective block in the removal of the latter. Furthermore, the increase in the specific activity of the serum lipoprotein protein in the alcohol-treated rats could not be explained on the basis of a defective removal and suggests instead an increased hepatic production of serum lipoproteins.

The increased reappearance of the labeled chylomicron fatty acids into the serum lipoproteins of $d < 1.019$, with a concomitant increase of the palmitate- ^{14}C /glycerol- ^3H ratio, shows that the palmitate- ^{14}C was reesterified with unlabeled glycerol (27) and represents additional evidence for an increased synthesis of very low density lipoproteins.

In addition to the hyperlipemia, fatty liver also developed after alcohol feeding. The increased incorporation of labeled dietary fatty acids, as well as chylomicron-fatty acids, into the liver triglycerides indicates that the processes that lead to the hepatic accumulation of dietary fat after alcohol were taking place during the 90 min of study. It is significant that increases in the specific activity of hepatic triglycerides and serum lipids were observed in the ethanol-fed rats despite isotopic dilution by the fat accumulated in the liver. This observation is compatible with the existence of at least two triglyceride pools in the liver (28): a storage pool with slow turnover in which only a fraction of the incorporated fatty acids was diluted, and a more rapidly exchangeable pool which became much more labeled with the dietary palmitic acid- ^3H in the alcohol-fed animals than in their

pair-fed controls. The highly labeled lipoproteins probably reflect the activity of this rapidly exchangeable pool.

The simultaneous production of steatosis and hyperlipemia has a direct bearing on the problem of the pathogenesis of the alcoholic fatty liver. A variety of toxic agents (29, 30), such as carbon tetrachloride, and deficiency states (31) produce fat accumulation in the liver by interfering with lipoprotein production or release. A similar mechanism has been postulated for ethanol. In contrast with the *in vitro* perfusion of normal livers with large amounts of ethanol (32), acute administration of one large dose of ethanol *in vivo* did not result in impaired protein or lipoprotein synthesis (33, 34) or in altered Triton hyperlipemia (35). Similarly, our data of simultaneous fatty liver development and increased lipoprotein production after chronic feeding of ethanol tend to rule this mechanism out, at least, for the initial steps of the hepatic lipid accumulation. Actually, it appears that the fatty liver rather than resulting from a block in lipoprotein secretion, could be a cause for the hyperlipemia, or, alternatively, increased lipoprotein production and fat accumulation could have a common pathogenesis. Decreased fatty acid oxidation in the liver or increased fatty acid synthesis (36) or both, would increase the availability of substrate for lipoprotein synthesis. Such increased availability of substrate in the liver itself has to be postulated, since we found accumulation of lipids in both liver and blood, without evidence that ethanol enhances the clearance of chylomicrons by the liver or affects lipid absorption or peripheral utilization. Thus, assuming that methods used in the present and preceding studies were sensitive enough to detect small but meaningful differences in these parameters, the most likely explanation we can offer for the simultaneous accumulation of fat in the liver and increased lipoprotein secretion after ethanol is increased availability of fatty acids in the liver itself. This can be achieved through decreased lipid oxidation; it has indeed been shown that ethanol reduces lipid oxidation in liver slices incubated with fatty acids (36), in isolated livers perfused with fatty acids or chylomicron (37) or *in vivo* in rats (38), and in man (39). Furthermore, whereas normally oxygen is utilized in the liver primarily for lipid oxidation (40), when ethanol is given, it becomes the main fuel for the liver (41); its oxidation thus supplants that of lipids. Moreover, the endoplasmic reticulum is the site of lipid esterification and lipoprotein production (42, 43). It is possible that the proliferation of the smooth endoplasmic reticulum induced by ethanol (44-46) plays a role in the increased capacity of the alcoholic fatty liver to produce serum lipoproteins.

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REFERENCES

1. Baraona, E., and C. S. Lieber. 1968. Effect of ethanol on hepatic lipoprotein production. *Clin. Res.* 16: 279. (Abstr.)
2. Talbott, G. D., and B. M. Keating. 1962. Effects of preprandial whiskey on postalimentary lipemia. *Geriatrics*. 17: 802.
3. Brewster, A. C., H. G. Lankford, M. G. Schwartz, and J. F. Sullivan. 1966. Ethanol and alimentary lipemia. *Amer. J. Clin. Nutr.* 19: 255.
4. DiLuzio, N. R., and M. Poggi. 1963. Abnormal lipid tolerance and hyperlipemia in acute ethanol-treated rats. *Life Sci.* 2: 751.
5. Jones, D. P., M. S. Losowski, C. S. Davidson, and C. S. Lieber. 1963. Effects of ethanol on plasma lipids in man. *J. Lab. Clin. Med.* 62: 675.
6. Nestel, P. J., and E. Z. Hirsch. 1965. Mechanism of alcohol-induced hypertriglyceridemia. *J. Lab. Clin. Med.* 66: 357.
7. Verdy, M., and A. Gattereau. 1967. Ethanol, lipase activity, and serum lipid level. *Amer. J. Clin. Nutr.* 20: 997.
8. Losowski, M. S., D. P. Jones, C. S. Davidson, and C. S. Lieber. 1963. Studies of alcoholic hyperlipemia and its mechanism. *Amer. J. Med.* 35: 794.
9. Lieber, C. S., D. P. Jones, J. Mendelson, and L. M. DeCarli. 1963. Fatty liver, hyperlipemia and hyperuricemia produced by prolonged alcohol consumption, despite adequate dietary intake. *Trans. Ass. Amer. Physicians Philadelphia*. 76: 289.
10. Schapiro, R. H., R. L. Scheig, G. D. Drummey, J. H. Mendelson, and K. J. Isselbacher. 1965. Effect of prolonged ethanol ingestion on the transport and metabolism of lipids in man. *N. Engl. J. Med.* 272: 610.
11. Borgström, B., and P. Jordan. 1959. Metabolism of chylomicron glyceride as studied by C¹⁴-glycerol-C¹⁴-palmitic acid labeled chylomicrons. *Acta Soc. Med. Upsal.* 64: 185.
12. Bollman, J. L., J. C. Cain, and J. H. Grindlay. 1948. Techniques for the collection of lymph from the liver, small intestine, or thoracic duct of the rat. *J. Lab. Clin. Med.* 33: 1349.
13. Lieber, C. S., N. Spritz, and L. M. DeCarli. 1966. Role of dietary, adipose, and endogenously synthesized fatty acids in the pathogenesis of the alcoholic fatty liver. *J. Clin. Invest.* 45: 51.
14. DeCarli, L. M., and C. S. Lieber. 1967. Fatty liver in the rat after prolonged intake of ethanol with a nutritionally adequate new liquid diet. *J. Nutr.* 91: 331.
15. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* 34: 1345.
16. Radding, C. M., and D. Steinberg. 1960. Studies on the synthesis and secretion of serum lipoproteins by rat liver slices. *J. Clin. Invest.* 39: 1560.
17. Scanu, A., and P. Oriente. 1961. Triton hyperlipemia in dogs. I. In vitro effects of the detergent on serum lipoproteins and chylomicrons. *J. Exp. Med.* 113: 735.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265.
19. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* 1: 279.
20. Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* 226: 497.
21. Dewey, W. C. 1959. Vascular-extravascular exchange of I¹²⁵ plasma proteins in the rat. *Amer. J. Physiol.* 197: 423.
22. Siperstein, M. D., and V. M. Fagan. 1958. Studies on the relationship between glucose oxidation and intermediary metabolism. I. The influence of glycolysis on the synthesis of cholesterol and fatty acid in normal liver. *J. Clin. Invest.* 37: 1185.
23. Bonnichsen, R. 1963. Ethanol determination with alcohol dehydrogenase and DPN. In *Methods of Enzymatic Analysis*. H.-U. Bergmeyer, editor. Academic Press Inc., New York. 285.
24. Snedecor, G. W. 1956. *Statistical Methods Applied to Experiments in Agriculture and Biology*. The Iowa State College Press, Ames. 5th edition.
25. Edgren, B., and H. C. Meng. 1962. The removal of dietary chylomicrons and artificial fat emulsions from the circulation of rats. *Acta Physiol. Scand.* 56: 237.
26. Otway, S., and D. S. Robinson. 1967. The effect of a non-ionic detergent (Triton WR 1339) on the removal of triglyceride fatty acids from the blood of the rat. *J. Physiol.* 190: 309.
27. Olivecrona, T. 1962. Metabolism of chylomicrons labeled with C¹⁴-glycerol-H³-palmitic acid in the rat. *J. Lipid Res.* 3: 439.
28. Nikkilä, E. A., M. Kekki, and K. Ojala. 1966. Kinetic analysis of triglyceride metabolism in single rats. *Ann. Med. Exp. Biol. Fenn.* 44: 348.
29. Recknagel, R. O. 1967. Carbon tetrachloride hepatotoxicity. *Pharmacol. Rev.* 19: 145.
30. Roheim, P. S., S. Switzer, A. Girard, and H. A. Eder. 1966. Alterations of lipoprotein metabolism in orotic acid-induced fatty liver. *Lab. Invest.* 15: 21.
31. Lombardi, B., P. Pani, and F. F. Schlunk. 1968. Choline-deficiency fatty liver: impaired release of hepatic triglycerides. *J. Lipid Res.* 9: 437.
32. Schapiro, R. H., Y. Shimizu, G. D. Drummey, Y. Shimizu, and K. J. Isselbacher. 1964. Studies on the pathogenesis of the ethanol-induced fatty liver. II. Effect of ethanol on palmitate-1-C¹⁴ metabolism by the isolated perfused rat liver. *J. Clin. Invest.* 43: 1338.
33. Ashworth, C. T., C. F. Johnson, and F. J. Wrightsman. 1965. Biochemical and morphologic correlations of hepatic protein synthesis in acute ethanol intoxication in rat. *Amer. J. Pathol.* 46: 757.
34. Seakins, A., and D. S. Robinson. 1964. Changes associated with the production of fatty livers by white phosphorus and by ethanol in the rat. *Biochem. J.* 92: 308.
35. DiLuzio, N. R. 1965. An evaluation of plasma triglyceride formation as a factor in the development of the ethanol-induced fatty liver. *Life Sci.* 4: 1373.

36. Lieber, C. S., and R. Schmid. 1961. The effect of ethanol on fatty acid metabolism; stimulation of hepatic fatty acid synthesis *in vitro*. *J. Clin. Invest.* 40: 394.
37. Lieber, C. S., A. Lefevre, N. Spritz, L. Feinman, and L. M. DeCarli. 1967. Difference in hepatic metabolism of long and medium-chain fatty acids: the role of fatty acid chain length in the production of the alcoholic fatty liver. *J. Clin. Invest.* 46: 1451.
38. Rebouças, G., and K. J. Isselbacher. 1961. Studies on the pathogenesis of the ethanol-induced fatty liver. I. Synthesis and oxidation of fatty acids by the liver. *J. Clin. Invest.* 40: 1355.
39. Barnes, E. W., N. J. Cooke, A. J. King, and R. Passmore. 1965. Observations on the metabolism of alcohol in man. *Brit. J. Nutr.* 19: 485.
40. Fritz, I. B. 1961. Factors influencing the rates of long-chain fatty acid oxidation and synthesis in mammalian systems. *Physiol. Rev.* 41: 52.
41. Lundquist, F., N. Tygstrup, K. Winkler, K. Mellemgaard, and S. Munck-Petersen. 1962. Ethanol metabolism and production of free acetate in the human liver. *J. Clin. Invest.* 41: 955.
42. Stein, Y., and B. Shapiro. 1958. Glyceride synthesis by microsome fractions of rat liver. *Biochim. Biophys. Acta.* 30: 271.
43. Jones, A. L., N. B. Ruderman, and M. G. Herrera. 1967. Electron microscopic and biochemical study of lipoprotein synthesis in the isolated perfused rat liver. *J. Lipid Res.* 8: 429.
44. Iseri, O. A., C. S. Lieber, and L. S. Gottlieb. 1966. The ultrastructure of fatty liver induced by prolonged ethanol ingestion. *Amer. J. Pathol.* 48: 535.
45. Lane, B. P., and C. S. Lieber. 1966. Ultrastructural alterations in human hepatocytes following ingestion of ethanol with adequate diets. *Amer. J. Pathol.* 49: 593.
46. Rubin, E., and C. S. Lieber. 1967. Experimental alcoholic hepatic injury in man: ultrastructural changes. *Fed. Proc.* 26: 1458.