Pathogenesis of ethanol-induced fatty liver: III. In vivo and in vitro effects of ethanol on hepatic fatty acid metabolism in rat

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SUMMARY Addition of ethanol to rat liver slices enhanced triglyceride, phospholipid, and fatty acid synthesis from acetate-1-C^{14} and pyruvate-2-C^{14} by liver slices. The type of fatty acid synthesized (i.e., primarily saturated) was not altered by the presence of ethanol.

These effects of ethanol on liver slices are probably not germane to the induction of fatty liver, since they were not observed after ethanol administration to the intact animal. The fatty acids accumulating in the liver after oral administration of ethanol consisted primarily of unsaturated fatty acids similar to those found in adipose tissue; the incorporation of circulating free fatty acids into hepatic triglycerides was increased. Also, the amount and rate of triglyceride formation from saturated and unsaturated fatty acids were significantly increased in liver homogenates and microsomes after ethanol administration but not upon in vitro addition of ethanol to liver slices.

In view of the differences between the in vitro and in vivo effects of ethanol, experiments based solely on the action of ethanol in vitro must be interpreted with caution in elucidating the pathogenesis of the ethanol-induced fatty liver.

KEY WORDS ethanol · fatty liver · adipose tissue · fatty acid · synthesis · mobilization · esterification · rat · liver · liver slices · microsomes

ETHANOL ADMINISTRATION is known to produce a fatty liver both in experimental animals and in man (1–3), probably as the result of several alterations in lipid metabolism. Fatty acids in the triglycerides that accumulate could be derived from peripheral fat depots or from the liver, either by increased hepatic fatty acid synthesis or decreased hepatic fatty acid oxidation. Previous studies from this laboratory (3) have indicated by elimination that in the rat the fatty acids found in the liver after ethanol ingestion arose for the most part from lipid mobilized from peripheral depots.

In addition to its effects on fatty acid metabolism, ethanol might also enhance hepatic *triglyceride* synthesis, or lead to an impaired release of triglycerides from the liver, or both. Recent studies from our laboratory (4) showed that when serum ethanol levels are very high, triglyceride release from the isolated perfused rat liver was indeed impaired; other studies (5) also indicate impaired hepatic triglyceride release and utilization. In the present report, evidence is presented that the administration of ethanol to rats also leads to enhanced esterification of fatty acids to triglycerides. These studies demonstrate, furthermore, marked differences between the effects of adding ethanol in vitro and those following its in vivo administration.

MATERIALS

Female Sprague-Dawley rats (Charles River Laboratories, Brookline, Mass.) weighing 150–280 g were maintained on Purina Lab Chow. In any one paired experiment, the weights varied no more than 10%. Palmitic acid-1-C^{14} (25 mc/mmole), sodium acetate-1-C^{14} (36.8 mc/mmole), sodium pyruvate-2-C^{14} (3.67 mc/mmole), and palmitic acid-9,10-H^3 (100 mc/mmole) were purchased from New England Nuclear Corp., Boston, Mass. Linoleic acid-1-C^{14} (32.5 mc/mmole) was obtained from Applied Science Laboratories, State College, Pa. Palmitic acid-1-C^{14} was purified by thin-layer chromatography (TLC) on silicic acid before

For first two papers in this series, see references 3 and 4.

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use. The remaining radiochemicals were used without further purification. Organic solvents were redistilled before use and contained 0.01% (w/v) of hydroquinone as antioxidant. Ethanol was a product of the U. S. Industrial Chemicals Company, New York, N.Y. DL-α-Glycerophosphate was obtained from Calbiochem, New York, N.Y.; L-α-glycerophosphate was a generous gift of Dr. E. Baer. Adenosine triphosphate disodium salt was obtained from Sigma Chemical Co., St. Louis, Mo., Coenzyme A from Pabst Laboratories, Milwaukee, Wis., and twice crystallized bovine albumin from Nutritional Biochemicals Corp., Cleveland, Ohio.

METHODS

Lipids were extracted from plasma and tissue according to the method of Folch, Lees, and Sloane Stanley (6). The phase containing lipid was evaporated to dryness under a stream of nitrogen at 40°. The lipid was redissolved in chloroform and aliquots were used for further analysis.

Triglycerides were determined by the method of Van Handel and Zilversmit (7), or for liver, as modified by Butler et al. (8).

In studies of the incorporation of radioactive precursors, lipid classes were separated by TLC on Silica Gel G, using 20 × 20 cm glass plates. Aliquots of the lipid extract were applied with a 50 μl syringe. For complete resolution of all lipid classes each sample was separated in two TLC solvent systems, namely petroleum ether (bp 30–60°)–diethyl ether–glacial acetic acid 90:15:1.5 and chloroform–cyclohexane–ethyl acetate–glacial acetic acid 150:50:25:4. Compounds were detected with iodine vapor and identified by comparison with simultaneously run standards (The Hormel Foundation, Austin, Minn.). After the iodine had sublimed, the areas containing lipid were transferred to vials; 12 ml of a toluene solution containing 0.01% (w/v) *p*-bis-[2-(5-phenyl-oxazolyl)] benzene and 0.3% 2,5-diphenyloxazole was added. Radioactivity was determined in a Packard Tri-Carb liquid scintillation counter at an efficiency for C^{14} of 65%. Results from the two plates for any one sample agreed within 3%. Samples containing phospholipid were always quenched on liquid scintillation counting, but the degree of quenching as determined by the channels ratio method (9) was constant. Use of internal standards showed that the quenching resulted in only a 3% loss of counting efficiency and thus no correction was made. When it was desired to isolate only triglycerides the solvent employed was chloroform–cyclohexane–glacial acetic acid 75:25:1.

The fatty acid composition of lipid extracts was determined by gas–liquid chromatography (GLC). Methyl esters were prepared by refluxing the lipid extract for 4 hr with concd HCl–anhydrous methanol (5:95) (10). The total amount of fatty acids present was determined by the internal standard method (11) using margaric acid (17:0). The methyl esters were separated on a Barber-Colman Model 25-C or Model 15 gas chromatograph equipped with an argon detector (tritium foil, 100 mc). Coiled or U-shaped glass columns, 6 ft × 3 mm i.d., were packed with Anakrom ABS, 80–90 mesh coated with 15% (w/w) ethylene glycol succinate polyester, and operated at 173°. Quantitative results with National Heart Institute Fatty Acid Standards agreed with the stated compositions with a relative error of less than 5% for major components and less than 12% for minor components. Specific activities of fatty acids were determined by a method similar to that described by Karmen, McCaffrey, and Bowman (12). A calibrated stream-splitter was introduced between the column and detector. While a peak was being recorded by the mass detector, the methyl ester was collected by condensation in a 9 inch length of Teflon tubing ($^1/_{16}$ inch i.d.) attached to the other arm of the streamsplitter. The ester was flushed out of the tubing into a counting vial with 15 ml of scintillation solution. Peak area was determined by triangulation. All operating parameters were maintained constant throughout any one experiment. Thus, specific activities of each fatty acid present in the original sample could be calculated. By this technique 90% of the injected radioactivity was accounted for and the reproducibility was within 20%.

Protein was determined as described by Lowry et al. (13).

EXPERIMENTAL DESIGN

1. *Effects of Ethanol on Rat Liver and Adipose Tissue: Fatty Acid Composition and Synthesis.* Unanesthetized rats (200–280 g) were given either 7.5 g of ethanol per kg or an isocaloric amount of glucose by stomach tube and then fasted for 16 hr. They were killed by decapitation; the liver and perirenal and perimetrial adipose tissue were quickly removed and the lipids immediately extracted. Lipid content and fatty acid composition were compared for the two groups of rats. Alternatively, liver slices from these animals, after incubation with radioactive substrates as described in section 3, served as the source of the lipid extracts. Specific activities of individual fatty acids were determined in order to compare their several rates of synthesis.

2. *Effect of Ethanol Administration on the Incorporation of Palmitic Acid-1-C^{14} into Liver Triglycerides by the Intact Animal.* Rats (170–200 g) were fasted overnight. Ethanol (7.5 g/kg) or an isocaloric amount of glucose was given by stomach tube to the unanesthetized animal. Four hours later, under light ether anesthesia, 14 μc of palmitic acid-1-C^{14} which had been homogenized in 0.5 ml of 5% albumin in 0.154 M NaCl was injected into the

femoral vein of each animal. Twenty minutes later an abdominal incision was made and the liver was quickly removed, blotted, and placed on ice. After thorough mincing and mixing with scissors, the lipid from a representative sample (1 g) was extracted. The triglycerides were isolated by TLC and their radioactivity and chemical amount determined. Preliminary experiments showed that there was 95% recovery of applied tripalmitin-C^{14} under these conditions.

3. *In Vivo and in Vitro Effects of Ethanol on Triglyceride and Phospholipid Formation from Acetate-1-C^{14}, Pyruvate-2-C^{14}, and Palmitate-1-C^{14} by Rat Liver Slices.* Liver slices were prepared in the cold with a Stadie-Riggs slicer (14) from rats given either ethanol (7.5 g/kg) or an isocaloric amount of glucose 16 hr previously. Randomized liver slices, 0.5 g, from the appropriately treated animal were incubated in Krebs-Ringer bicarbonate buffer (1/2 calcium), pH 7.4, which had been gassed for 10 min with 5% CO_2 in O_2. The medium was either 5 mM with respect to glucose or 10 mM with respect to ethanol. The appropriate radioactive substrate in buffer or, in the case of palmitate, bound to albumin was added 5 min after the flasks had been placed in a Dubnoff metabolic shaker at 37°. Final albumin concentration of the medium for all experiments was 5% and total volume of the medium was 5 ml. Incubations were for 3 hr in an atmosphere of 5% CO_2 and 95% O_2. The reaction was stopped by placing the flask on ice, decanting the medium, washing the slices three times with ice-cold distilled water, and homogenizing in 20 volumes of chloroform–methanol (2:1). The lipid was transferred quantitatively with chloroform to a 5 ml volumetric flask. Aliquots were taken for GLC and TLC. Results were expressed as counts per minute incorporated into total lipids or into individual lipid classes per milligram of liver slice. Results obtained from duplicate flasks agreed within 6%.

The four experimental conditions employed are shown in Fig. 1. A number of comparisons were possible. The effects exerted by ethanol in the medium on slices derived from glucose-treated animals were observed by comparing flasks *1* and *2;* one could superimpose the effects of ethanol in vitro upon those already produced by ethanol administration on comparing flasks *3* and *4;* and the effects of ethanol administration alone were observed by comparing flasks *1* and *3*.

4. *Effects of Ethanol on Triglyceride Formation from Fatty Acids in Homogenates and Subcellular Fractions of Rat Liver.* Unanesthetized rats were given by stomach tube either ethanol (7.5 g/kg) or an isocaloric amount of glucose. The rats were then fasted for 16 hr and killed by decapitation; the livers were immediately removed and weighed. In most experiments the livers from three treated animals were pooled. In all experiments the activities of homogenates prepared from these two sources as regards triglyceride and phospholipid formation were compared. The livers were kept at 0–4° and minced with scissors. A 20 or 33% (w/v) homogenate was prepared in M KCl–0.05 M nicotinamide, using a Potter-Elvehjem tissue grinder with a Teflon pestle. The homogenate was strained through double thickness gauze and centrifuged for 20 min at 500 × *g* in a Servall refrigerated angle centrifuge at 4°. The supernatant solution was used for the incubations. In experiments utilizing microsomes, the supernatant solution was further centrifuged for 20 min at 9000 × *g*, the sediment was discarded, and the supernatant solution was recentrifuged for 1 hr at 105,000 × *g* in a Spinco Model L ultracentrifuge. The microsomal pellet was reconstituted with the KCl–nicotinamide solution so that 1 ml represented the microsomes from 0.5 g liver. Each milliliter of incubation mixture, detailed in the section on experimental results, contained 20–60 mμmole of fatty acid-C^{14} complexed to 30 mg of albumin. Incubations, in duplicate, were carried out at 37° in air in a Dubnoff metabolic shaker and were terminated by adding 1 ml of the incubation mixture to 20 ml of chloroform–methanol 2:1 contained in a 125 ml Squibb separation funnel. The mixture was intermittently shaken for 1 hr, 4 ml of distilled water was added, and the mixture after shaking was centrifuged for 10 min at 3000 rpm in an International Model R centrifuge with a 239 head. The lipid from the lower phase was dissolved in a known volume of chloroform and aliquots were applied to thin-layer plates for separation of lipid classes.

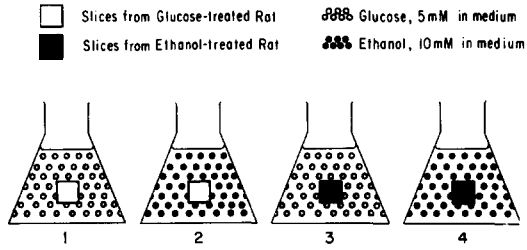

FIG. 1. Studies of hepatic lipid synthesis: experimental design. Incubation medium present in all flasks consisted of 5% twice crystallized bovine serum albumin in Krebs-Ringer bicarbonate buffer and either 25 μc of acetate-1-C^{14}, 1 μc of palmitate-1-C^{14}, or 2.5 μc of pyruvate-2-C^{14}. Total volume 5 ml. In flasks *3* and *4*, 0.5 g liver slices were derived from a rat given 7.5 g of ethanol per kg 16 hr before. In flasks *1* and *2* the slices were derived from an animal given an isocaloric amount of glucose. In addition, the medium in flasks *1* and *3* was 5 mM with respect to glucose, and in flasks *2* and *4*, 10 mM with respect to ethanol. Flasks were incubated with shaking for 3 hr at 37° in an atmosphere of 5% CO_2 and 95% O_2.

RESULTS

1. *Effects of Ethanol on Rat Liver and Adipose Tissue; Fatty Acid Composition and Synthesis.* Figure 2 shows that in these short-term experiments, ethanol administration did not

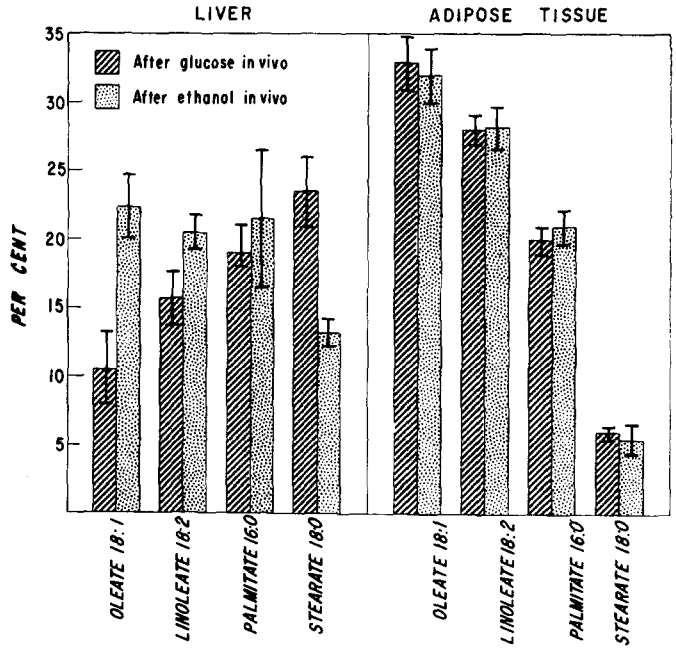

FIG. 2. Effect of ethanol in vivo on fatty acid composition of rat liver and adipose tissue. The percentages of the four quantitatively significant acids are shown for rat liver and adipose tissue 16 hr after administration of ethanol or isocaloric amounts of glucose. Results shown in the bar graphs are the mean ± SD of duplicate analyses on 6a nimals of each treatment group for liver and 3 animals for adipose tissue.

significantly alter the fatty acid composition of adipose tissue. However, marked changes in fatty acid composition of liver occurred, the most striking being an increase in oleic (18:1) and linoleic (18:2) acids, and a decrease in stearic acid (18:0). Adipose tissue was especially rich in unsaturated (oleic and linoleic) acids, whereas palmitic and stearic acids predominated in liver from glucose-treated rats. Dietary ethanol treatment resulted in a striking increase in the proportion of oleic and linoleic acids in the liver. Since the total fatty acid content of the liver, as determined by the internal standard technique, increased 2- to 6-fold after ethanol administration, this change represented a marked increase in the actual amount of oleic and linoleic acids. Further, the fatty acid pattern in the liver after ethanol administration bears a striking resemblance to that of adipose tissue. This similarity strongly suggested that the fatty acids in rat liver after ethanol ingestion were derived predominantly from adipose tissue.

TABLE 1 EFFECT OF ETHANOL ON FATTY ACID SYNTHESIS BY LIVER SLICES

Labeled Product	Ethanol-Treated Animals		Glucose-Treated Animals	
	Glucose Medium	Ethanol Medium	Glucose Medium	Ethanol Medium
	specific activity, cpm/mg			
Palmitic acid	27	155	70	330
Stearic acid	15	56	28	64
Oleic acid	3	11	13	32

Ethanol (7.5 g/kg) or an isocaloric amount of glucose was given to female rats 16 hr before sacrifice. Liver slices prepared from these animals were incubated as in Fig. 1 with 25 μc of acetate-1-C^{14} as substrate and either glucose, final concn 5mM, or ethanol, final concn 10 mM. Lipids were extracted and the specific activity of each fatty acid was determined after separation by GLC. Typical results are shown.

Since it is well recognized that addition of ethanol in vitro enhances fatty acid synthesis by the liver, it was important to determine which fatty acids were synthesized under these circumstances. For if the addition of ethanol in vitro simulates the effect of its administration in vivo, and if enhanced hepatic fatty acid synthesis were the predominant mechanism whereby ethanol produces a fatty liver, ethanol addition should preferentially stimulate the synthesis of those fatty acids which were found to accumulate. This, however, was not the case. Table 1 shows that palmitic and stearic acids were the predominant fatty acids synthesized from acetate by liver slices when the medium contained glucose. Although the addition of ethanol to the medium enhanced fatty acid synthesis, this pattern of fatty acid synthesis was not altered. (No synthesis of linoleic acid from acetate could be demonstrated.) The effects of ethanol in vitro on slices from glucose-treated animals

were similar to the results on slices from ethanol-treated animals except for the higher specific activities in the former due to the lesser amount of endogenous fatty acid.

Under these conditions any conversion of saturated to unsaturated fatty acids might not be observed. Therefore, slices were incubated with palmitate-1-C^{14} instead of acetate-1-C^{14}. Here, 95% of the label was recovered in the palmitate and stearate fraction and neither previous ethanol administration nor addition of ethanol to the medium resulted in enhanced hepatic conversion of palmitate to palmitoleate or oleate.

2. *Effect of Ethanol on Palmitate Incorporation into Liver Triglycerides in Intact Rats.* In the animals given ethanol, hepatic triglyceride concentration had increased 2-fold after 4 hr (Table 2); three times as much label derived from palmitate-1-C^{14} was incorporated into the liver triglycerides. The increase was sufficiently great to result in an apparent though not statistically significant increase in specific activity of the hepatic triglycerides. An increased labeling of hepatic triglycerides in the ethanol-treated animal was also found either 10 or 30 min after the injection of palmitate.

3. *Effects of Ethanol on Triglyceride and Phospholipid Formation from Acetate, Pyruvate, and Palmitate by Rat Liver Slices.* In order to study and compare the in vitro and in vivo effects of ethanol on lipid synthesis, the four experimental conditions shown in Fig. 1 were employed. All incubations were performed in duplicate. Three sets of experiments were performed in which acetate-1-C^{14} served as lipid precursor and one in which pyruvate-2-C^{14} was used. The results of these four experiments were similar. Since no significant formation of monoglycerides, diglycerides, or cholesterol esters was observed and no consistent change in cholesterol formation was seen, only triglyceride and phospholipid synthesis is reported. The results of a typical experiment are shown in Fig. 3. No increase in total glycerolipid synthesis from acetate (or pyruvate) by rat liver slices was observed as a result of prior ethanol administration, although a small (34%) but consistent increase in triglyceride formation, with a concomitant decrease in phospholipid formation, was seen.

These findings are in marked contrast to the effects of ethanol in vitro, which increased both triglyceride and phospholipid formation 3-fold (Fig. 3). In vitro addition of ethanol to liver slices from glucose-treated rats stimulated glycerolipid formation up to 2-fold (not shown in Fig. 3).

The in vitro and in vivo effects of ethanol on glycerolipid formation by liver slices from preformed fatty acid (palmitate) are shown in Fig. 4. Ethanol did not result either in vitro or in vivo in an increase in the incorporation of palmitate into total glycerolipids. This was also

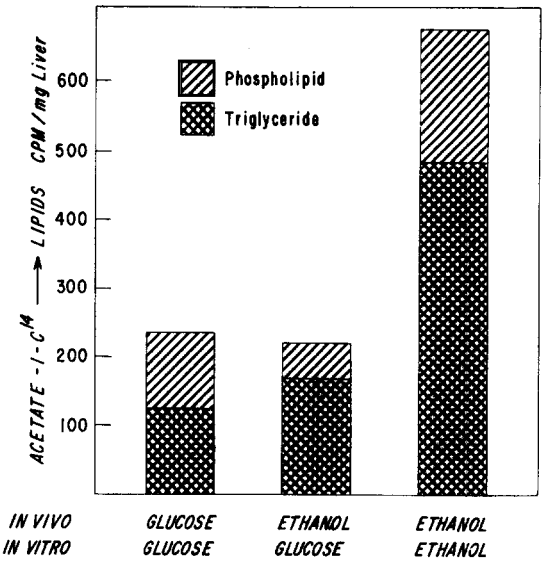

FIG. 3. Effect of ethanol vs. glucose on the incorporation of acetate-1-C^{14} into lipid by liver slices. Flasks *1*, *3*, and *4* of Fig. 1 are compared. Substrate, 25 μc of sodium acetate-1-C^{14}. Results are expressed as counts per minute of precursor incorporated into triglycerides and phospholipids per milligram wet weight of liver slice.

true of the in vitro effect of ethanol after glucose in vivo (not shown). However, ethanol in vivo resulted in a preferential conversion of palmitate to triglyceride, which was then further enhanced upon addition of ethanol to the medium.

4. *Effects of Ethanol on Triglyceride Formation from Fatty Acids in Homogenates and Subcellular Fractions of Rat Liver.* Triglyceride synthesis was studied in liver homogenates, where the complicating effects of ethanol on lipid transport across cell membranes were absent. Results of one experiment in this series are shown in Fig. 5. Both Coenzyme A and α-glycerophosphate were

TABLE 2 HEPATIC TRIGLYCERIDE FORMATION FROM PALMITATE-1-C^{14} INJECTED INTRAVENOUSLY

	In vivo Treatment		
	Glucose	Ethanol	*P*
No. of animals	5	6	
Total liver triglyceride (mg/g liver)	6.4 ± 2.5	13.4 ± 3.5	<0.01
Palmitate-C^{14} incorporated into liver triglyceride (cpm/g liver)	269,000 ± 177,000	880,000 ± 190,000	<0.001
Specific activity of triglyceride (cpm/mg)	45,320 ± 28,200	66,600 ± 10,800	N.S.

Rats were fasted for 16 hr, given ethanol (7.5 g/kg) or isocaloric amounts of glucose by intubation, and 4 hr later 14 μc of palmitate-1-C^{14} was injected into a femoral vein. Animals were killed 20 min later. Results are expressed as mean ± SD.

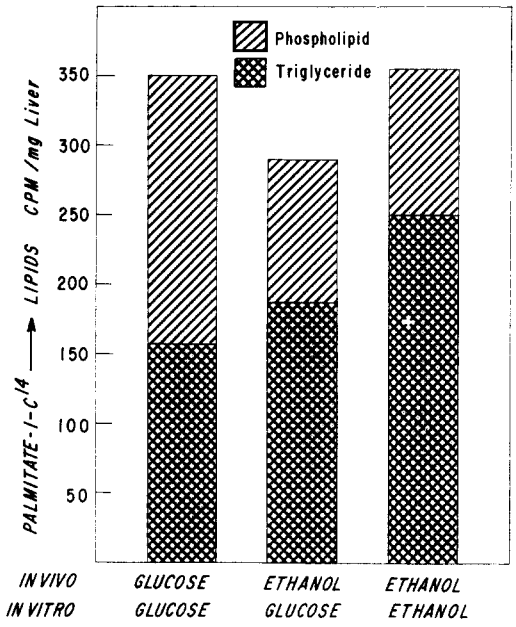

FIG. 4. Effect of ethanol vs. glucose on the incorporation of palmitate-1-C^{14} into lipid by liver slices. Flasks *1*, *3* and *4* of Fig. 1 are compared. Substrate, 1.0 μc of palmitate-1-C^{14} (25 mμmoles). Results are expressed as counts per minute of precursor incorporated into triglycerides and phospholipids per milligram wet weight of liver slices.

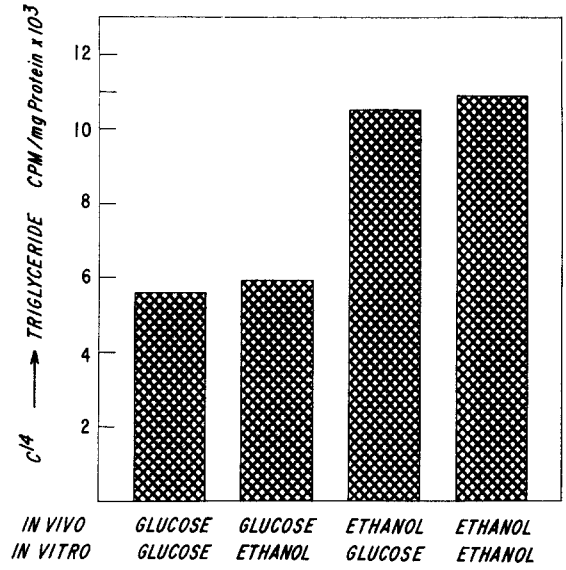

FIG. 5. Effect of ethanol vs. glucose on incorporation of palmitate-1-C^{14} into triglycerides by liver homogenates. 33% homogenates in KCl–nicotinamide were prepared from the livers of rats given either ethanol (7.5 mg/kg) or an isocaloric amount of glucose 16 hr previously. The incubation mixture consisted of 10 μmoles of ATP, 20 μmoles of KPO_4 buffer, pH 7.5, 10 μmole of $MgCl_2$, 10 μmoles of cysteine (neutral), 0.15 μmole of CoA, 10 μmoles of L-α-glycerophosphate, 26 mμmoles of palmitate-1-C^{14} bound to 90 mg of albumin in 1 ml of KCl–Tris buffer, pH 7.5 (19 parts of 0.154 M KCl and 1 part of 0.5 M Tris, pH 7.5). One milliliter of homogenate (protein concentration 35.4 mg/ml for both) was added to start the reaction. In this experiment, the final mixture of 3 ml was either 5 mM with respect to glucose or 10 mM with respect to ethanol. Incubation was for 30 min in a Dubnoff metabolic shaker in air at 37°. Results are expressed as radioactivity incorporated into triglyceride per milligram of homogenate protein.

required for esterification to proceed, but no requirement was shown for a sulfhydryl donor (cysteine) or pyridine nucleotides. Addition of ethanol in vitro had no effect on the esterification of palmitate-1-C^{14} to triglycerides by liver homogenates prepared from either glucose- or ethanol-treated animals. However, the homogenate from the ethanol-treated animals formed 87% more triglycerides than did the homogenate from the control animals. In ten paired experiments, ethanol administration resulted in an average of 74% (range, 18–200%) more triglycerides being formed from palmitate-1-C^{14}. This finding was not correlated with decreased phospholipid formation: only 5% (range, −25 to +41%) less label was found in the phospholipid fraction. In the three experiments in which ethanol feeding resulted in increased phospholipid formation, a still greater increase in triglyceride labeling was found. The addition of 100 μmoles of cytidine diphosphate choline to the incubation mixture led to increased phospholipid and decreased triglyceride formation. The magnitude of this change was the same in homogenates from glucose- as from ethanol-treated animals.

The rate of triglyceride formation from palmitate-1-C^{14} by liver homogenates prepared from rats given ethanol and from rats given glucose was studied and compared. Three experiments were performed with similar results. One such experiment is illustrated in Fig. 6, where it is seen that the rate was linear for 30 min in both homogenates. However, the rate as well as the total amount formed had been increased considerably by the in vivo treatment with ethanol. After 30 min, triglyceride labeling remained more or less constant for 90 min in both homogenates, indicating that the rate of triglyceride oxidation or of transfer of fatty acids from triglycerides to other lipid classes was not altered by ethanol administration.

Because of the finding that unsaturated rather than saturated fatty acids accumulate in the liver after ethanol administration, the relative effectiveness of linoleic and palmitic acids as substrates for triglyceride formation was studied. A preliminary experiment had shown that triglyceride formation from linoleic acid-1-C^{14} was enhanced by ethanol feeding. We next incubated equimolar amounts (15.4 mμmoles each) of linoleic acid-1-C^{14} and palmitic acid-9,10-H^3 with the homogenate. These results are shown in Fig. 7. Palmitate and linoleate served equally well as precursors for triglyceride formation. Furthermore, twice as much label from each isotope was incorporated into triglyceride as a result of ethanol administration.

In order to avoid any simultaneous effects of lipid oxidation and to further localize the subcellular site of

this in vivo effect of ethanol, the microsomal fraction was examined. Fig. 8 shows that liver microsomes from an animal given ethanol 16 hr previously formed five times as much triglyceride from palmitate-1-C^{14} as did liver microsomes prepared from the glucose-treated control animal. Ethanol in vitro was again without effect on triglyceride formation. This in vivo effect of ethanol on liver microsomes was observed as early as 4 hr after ethanol administration.

DISCUSSION

It has been repeatedly observed that single large doses of ethanol as well as more chronic ethanol administration produce alterations in lipid metabolism which result in triglyceride accumulation in the liver (1–3, 5, 15, 16). These alterations no doubt depend on the experimental conditions employed. It was hoped that these studies might resolve some of the discrepancies reported in the literature (17).

Ethanol added to liver slices not only enhances the hepatic synthesis of saturated fatty acids (Table 1) from acetate-1-C^{14}, but also enhances phospholipid and triglyceride formation (Fig. 3). Prior treatment of the animal with ethanol was not required for the demonstration of this in vitro effect of ethanol. Previous studies from this laboratory (3) indicated that ethanol in vitro may, in addition, inhibit fatty acid oxidation.

Several objections may be raised, however, to the conclusion that the major mechanism in the production of the fatty liver by ethanol is enhanced hepatic synthesis of fatty acids and glycerolipids or diminished hepatic utilization. It has been shown (3) that glucose administration or the incubation of liver homogenates with agents such as sorbitol, xylitol, or nicotinamide produces an equivalent or even greater depression of fatty acid oxidation than does ethanol, although these agents do not produce a fatty liver. Sorbitol administration produces an increase in fatty acid synthesis, but here again, a fatty liver is not produced.

Lieber and Schmid (18) were the first to demonstrate clearly that when liver slices from rats previously treated with ethanol were incubated in a medium to which ethanol had been added at a concentration of 10 mM the synthesis of fatty acids from tracer amounts of acetate-1-C^{14} was enhanced. In this experiment the control was a flask containing equimolar amounts of unlabeled acetate instead of ethanol. This study has recently been

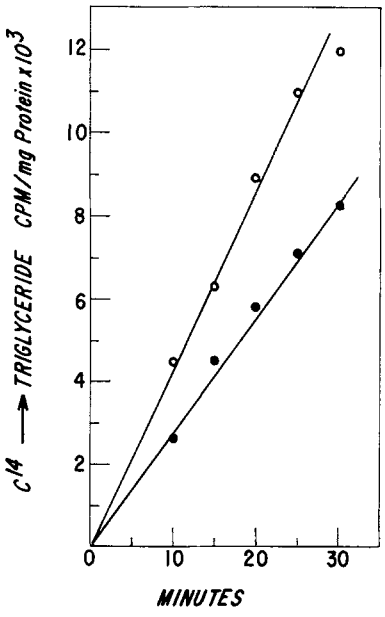

FIG. 6. In vivo effect of ethanol vs. glucose on the rate of triglyceride formation from palmitate-1-C^{14}. Two rats were given ethanol and two glucose, and 16 hr later 33% homogenates were prepared. Incubation mixture consisted of 1 ml of homogenate (48.4 mg protein per milliliter from glucose-treated animals and 43.5 mg/ml from ethanol-treated animals), 25 μmoles of DL-α-glycerophosphate, 10 μmoles of ATP, 20 μmoles of KPO_4 buffer, pH 7.5, 10 μmoles of $MgCl_2$, 0.15 μmole of CoA, 5 μmoles of glucose, and 26 mμmoles of palmitate-1-C^{14} bound to 90 mg of albumin in KCl–Tris, pH 7.5. Total volume 3 ml. Each point on the graph represents an individual flask incubated at 37° in air for the indicated period of time. Results are expressed as counts per minute incorporated into triglyceride per milligram of homogenate protein. ○-○, ethanol-treated; ●-●, glucose-treated.

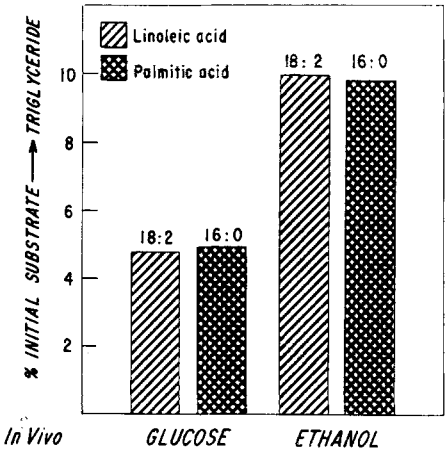

FIG. 7. In vivo effect of ethanol vs. glucose on triglyceride formation from palmitate-9,10-H^3 and linoleate-1-C^{14} by liver homogenates. The livers from 3 rats treated with ethanol were pooled, as were the livers from 3 rats given glucose, and 33% homogenates were prepared. The incubation mixture was the same as in Fig. 6 except that the substrate was 15 mμmoles each of linoleic acid-1-C^{14} and palmitic acid-9,10-H^3. One milliliter of the appropriate homogenate was added to start the reaction. Total volume 3 ml. The protein concentration of the homogenate prepared from the glucose-treated animals was 45.2 mg/ml and from the ethanol-treated animals, 47.6 mg/ml. Flasks were incubated in duplicate for 30 min at 37° in air. Dual isotope assay was performed at an efficiency of 24.2% for tritium and 41.3% for C^{14}. The percentage of initial radioactivity of each isotope incorporated into triglyceride is plotted.

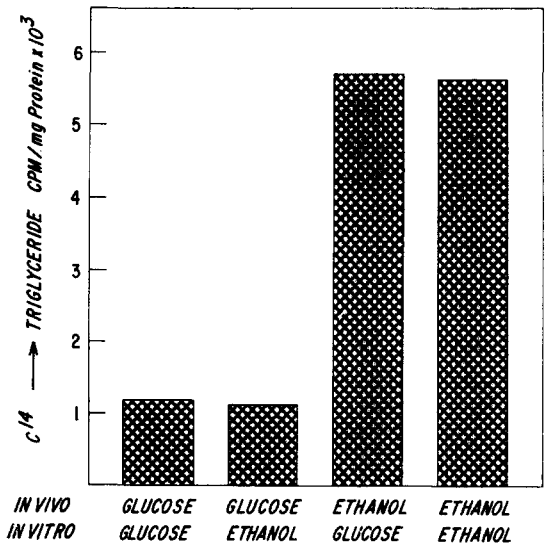

FIG. 8. Effect of ethanol vs. glucose on incorporation of palmitate-1-C^{14} into triglycerides by liver microsomes. The incubation mixture of 3.2 ml consisted of 1.5 ml of microsomal solution, 25 μmoles of DL-α-glycerophosphate, 10 μmoles of ATP, 10 μmoles of cysteine (neutral), 20 μmoles of KPO_4, 10 μmoles of $MgCl_2$, 0.15 μmole of CoA, and palmitate-1-C^{14} complexed to albumin in KCl–Tris, pH 7.4. Incubations were for 30 min in air at 37°.

criticized by Majchrowicz (19), who pointed out that the proper control flask is one containing only trace amounts of acetate-1-C^{14}. Under these conditions he showed that ethanol suppresses fatty acid synthesis, an effect that can be attributed to isotope dilution of the labeled acetyl CoA derived from acetate-1-C^{14} by unlabeled acetyl CoA derived from ethanol. Since it has not yet been established that the only pathway of ethanol metabolism is via acetyl CoA (20), experiments of this design do not yield a definitive interpretation.

The major source of the increased fatty acid content of the liver after ethanol administration was revealed by observing the effect of ethanol ingestion on hepatic fatty acid composition. Ethanol administration resulted in an accumulation of unsaturated fatty acids, especially oleic and linoleic, in the liver. The extent to which this occurred was such that there developed a remarkable similarity between the fatty acid composition of liver after ethanol administration and that of adipose tissue. These findings are consistent with those of Horning, Williams, Maling, and Brodie (16), who demonstrated that the linoleic acid content of the liver triglycerides increased strikingly after ethanol administration as compared with a saline-treated control animal. Since the rat is unable to synthesize this essential fatty acid, the presence in increased amounts of linoleic acid in the liver in these fasted rats after ethanol administration indicated that mobilization from extrahepatic stores had occurred.

While increased mobilization of fatty acids per se can produce a fatty liver (21), the work of Horning, Wakabayashi and Maling (22) suggested that ethanol may specifically enhance hepatic triglyceride formation, although in their experiments this occurred at the expense of phospholipid formation. We have been able to show in both liver slices and homogenates that ethanol administration does indeed enhance triglyceride formation (Figs. 3, 5, 6), both when the precursor fatty acids were synthesized de novo and when they were derived from other sources. Though diminished phospholipid formation occurred in liver slices from rats given ethanol, we have been able to show by using liver homogenates that enhanced triglyceride formation was not dependent on diminished phospholipid formation. Differences in the amount of substrate fatty acid may explain this discrepancy between the studies of Horning and co-workers and ourselves. We believe that this effect of ethanol on triglyceride formation is not related to diminished phospholipid synthesis for the following reasons. The addition of CDP choline to the homogenates stimulated phospholipid formation, suggesting that the phospholipid-synthesizing system was reasonably intact. Furthermore, this effect was also seen in the homogenates prepared from glucose-treated animals. In addition, while the rate of triglyceride formation from palmitate-1-C^{14} was increased by ethanol administration, the rate of phospholipid formation was not reciprocally depressed.

Confirmation of these in vivo effects of ethanol on triglyceride formation was derived from a study utilizing hepatic microsomes as enzyme source. Phospholipid formation was not altered by ethanol, but triglyceride formation was enhanced 5-fold.

The effects of ethanol in vitro on lipid synthesis by the liver appear to be at the level of the mitochondria and cell sap: fatty acid oxidation is depressed and synthesis of saturated fatty acids is enhanced. The effect of ethanol in vivo, however, is at the level of the hepatic microsome, where triglyceride formation from fatty acids is increased. The dichotomy between the in vivo and in vitro effects of ethanol is consistent with the concept advanced by Horning et al. (22) that in vivo effects of ethanol may be indirect and mediated through the pituitary–adrenal axis. Experiments designed to elucidate the biochemical mechanisms of this effect of ethanol, whether direct or indirect, on the hepatic microsome are in progress.

We wish to acknowledge the expert technical assistance of Miss Rita Quinn and Mrs. Gladys Drummey.

This investigation was supported by grants from the John A. Hartford Foundation, Inc. and the National Institutes of Health (AM-01392).

Manuscript received August 3, 1964; accepted October 26, 1964.

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