Effect of Ethanol Ingestion on Liver Microsomal Phosphatidyl Choline Biosynthesis[1] (36496)

DAVID N. SKURDAL AND W. E. CORNATZER

Guy and Bertha Ireland Research Laboratory, Department of Biochemistry, University of North Dakota School of Medicine, Grand Forks, North Dakota 58201

The manner in which ethanol ingestion leads to fatty infiltration of the liver is not clear. Triglycerides which accumulate in the fatty liver can originate from the gut as chylomicrons; adipose tissue lipids, which are transported to the liver as free fatty acids; and lipid synthesized and/or oxidized in the liver itself (1). It has been shown that ethanol enhances the fat synthesis, depresses the oxidation of fatty acids and triglycerides in the liver, stimulates mobilization of fatty acids from adipose tissue and interferes with hepatic release of triglycerides (2, 3). Robinson (4) has shown that ethanol administration increases the liver triglycerides and does not affect the plasma lipoprotein or liver protein synthesis.

The removal of triglyerides of the liver occurs by fatty acid oxidation in mitochondria and by lipoproteins release to plasma containing triglycerides (5). Phosphatidyl choline is lipotropic and is involved in this removal of triglycerides. The mechanisms of the triglyceride removal and role of phosphatidyl choline are yet unknown. Phospholipids represent 25% of the dry weight of mitochondria, 30% of the dry weight of microsomes (6, 7). Cellular lipoprotein fractions have been isolated and shown to contain 73–82% phospholipids of total lipids (8). Phosphatidyl choline occurs as 45% of total lipid P of liver mitochondria and 48.5% in microsomes (9). The plasma phospholipids which are part of the lipoproteins containing triglycerides released from the liver are synthesized and metabolized only in the liver (1, 10).

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Liver phosphatidyl choline biosynthesis is known to occur by two different major pathways in microsomes. The Kennedy and Weiss (11) pathway involves cytidine diphosphocholine and α-β-diglycerides to form phosphatidyl cholines. The Bremer and Greenberg pathway (12) involves the methylation of phosphatidyl ethanolamine from adenosylmethionine to form phosphatidyl choline. The specificity of the incorporation of 1,2-^{14}C-choline and 1,2-^{14}C-ethanolamine into these phosphatidyl choline fractions as a means of measuring these two biosynthetic pathways of lecithin synthesis has been determined (13). Phosphatidyl cholines of fractions 1 and 2 are chiefly incorporated from 1,2-^{14}C-ethanolamine and provides a lecithin rich in polyunsaturated fatty acids (13). The phosphatidyl cholines of fractions 3 and 4 are mainly synthesized by the 1,2-^{14}C-choline pathway (13). The effect of ethanol ingestion on the biosynthesis of liver microsomal phosphatidyl choline fractions has not been determined.

Methods. Choline-1,2-^{14}C (sp act 3.7 mCi/mmole) and ethanolamine-1,2-^{14}C (sp act 3.51 mCi/mmole) was purchased from Mallinckrodt Nuclear, St. Louis, MO. Female albino rats of Sprague-Dawley strain weighing 150 $\pm$ 22 g were divided into two groups. Group I was fed the alcohol liquid diet for 2 weeks (14). The animals of Group II served as controls. They were pair-fed the isocaloric diet containing sucrose instead of ethanol. A liquid-alcohol diet, expressed as grams per 100 ml; was as follows (g): essential amino acids mixture, 2.307; nonessential amino acids, 2.422; salt mixture (Hegsted IV), 1; vitamin mixture, 1.250; Mazola corn oil,

1.250; cod liver oil, 0.375; Pastene olive oil, 3.000; sodium carrageenate (Viscarin), 0.400; ethanol (95% laboratory alcohol), 5.000; distilled water, up to 100 ml.

The composition of the essential amino acid mixture in grams per 100 g was as follows (g): L-lysine HCl, 13.434; L-arginine HCl, 8.125; DL-tryptophan, 2.166; DL-phenylalanine, 9.750; DL-leucine, 17.334; DL-isoleucine, 10.834; DL-valine, 15.167; L-histidine HCl, 5.850; DL-methionine, 6.500; DL-threonine, 10.834.

Nonessential amino acid mixture, grams per 100 g; was as follows (g): L-glutamic acid, 20.639; DL-serine, 5.159; glycine, 7.223; DL-tyrosine, 28.895; L-cystine, 2.063; L-proline, 9.287; L-asparagine monohydrate, 14.344; DL-alanine, 12.383.

Composition of the vitamin mixture in milligrams per 100 g was as follows (mg): thiamine HCl, 5; riboflavin, 10; pyridoxine HCl, 5; calcium pantothenate, 40; nicotinamide, 30; choline chloride, 2500; biotin, 0.2; folic acid, 2; inositol, 200; 2-methyl-1,4-naphthoquinone, 2; vitamin B_{12}, 0.2; *p*-aminobenzoic acid, 100; sucrose, 97,610. The controls were fed similar isocaloric diet containing 8.75 g/100 ml of sucrose instead of the 5 g of ethanol. This diet was similar to that reported by Porta, Hartroft and Dela Iglesia (14) and was purchased from General Biochemicals, Chagrin Falls, OH.

At the end of the dietary regimen, the control and alcohol-fed rats were injected intraperitoneally with 3.33 μCi/100 g of body weight of the isotopic compounds. The rats were killed by decapitation at 0.25, 0.5, 1, 2, 3 and 4 hr after injection of the isotopic compounds. Plasma was collected using heparin as an anticoagulant. The livers were removed, rinsed with cold water, blotted, weighed, and homogenized with ice-cold 0.25 *M* sucrose in a Potter-Elvehjem homogenizer with a Teflon pestle. The microsomal fraction was isolated by differential centrifugation (15). The nuclei and mitochondria was separated from the homogenate by centrifuging for 10 min at 14,500*g*. The supernatant solution was centrifuged at 78,450*g* for 45 min to sediment the microsomal pellet. The method of Folch, Lees and Stanley (16) was employed to extract and purify the lipids from microsomes and/or plasma. The lipids were stored in a dilute chloroform solution under nitrogen at —18°.

Phosphatidyl cholines were isolated from the lipid extract by thin-layer chromatography by the method of Parker and Peterson (17) using solvent chloroform:methanol:acetic acid:water, 65:25:4:1.4 (v/v/v/v). Phospholipids were identified by comparisons with purified standards. Plates were sprayed with 0.008% rhodamine 6G solution and viewed under ultraviolet light to identify and outline the band of gel containing the phospholipids. The silica gel containing the phosphatidyl cholines was scraped into a flask containing 20 ml of chloroform–methanol 2:1 (v/v). The phosphatidyl cholines were eluted from the gel by filtration of the chloroform–methanol solution with the aid of a sintered-glass funnel (medium porosity). The gel was washed twice with chloroform:methanol: water 200:97:3 (v/v/v) and once with chloroform and once with methanol. Quantitative recovery of the phospholipids was possible with this elution procedure. Recovery values represented 94% of the total phosphorus. The filtrate was washed with 0.2 vol of 0.04% calcium chloride solution. A dilute solution of the phospholipid extracts in chloroform was stored under dry nitrogen at —18°. On an aliquot of the chloroform solution, the total phosphatidyl choline phosphorus was determined (18, 19). Fractionation of the phosphatidyl choline fractions was carried out by thin-layer chromatography on silica gel H impregnated with silver nitrate (20). The phosphatidyl choline fractions were identified by spraying with 0.01% methanolic solution of 2,7-dichlorofluorescein and viewing under ultraviolet light (20). The phosphatidyl choline fractions were scraped into tubes containing 15 ml chloroform:methanol, 2:1 (v/v), and 9 ml 0.04% $CaCl_2$ added, shaken, and filtered with sintered-glass funnels (medium porosity) to remove the silver ion and collect the lipid–silica gel. The lipids were extracted from the gel with 15 ml chloroform:methanol:water, 200:97:3 (v/v/v), and

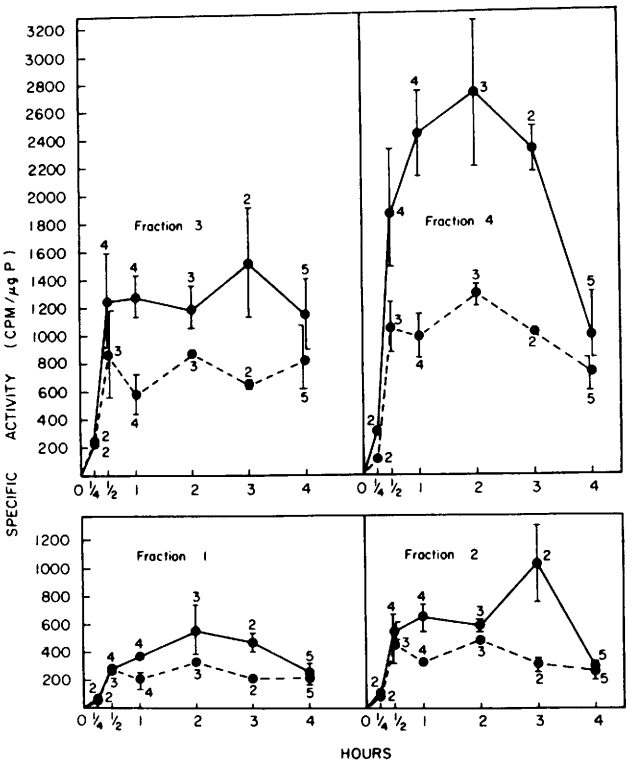

FIG. 1. The effect of alcohol ingestion on the incorporation of 1,2-^{14}C-choline into phosphatidyl choline fractions from liver microsomes of female rats. Each point on the graph represents the average. The vertical bars on graph represent the standard deviation whenever it was found to be larger than the symbol used to identify it. The number of animals are indicated above each value. (—) Control; (- - -) alcohol.

5 ml methanol; and chloroform was added to adjust the ratio to 2:1 (chloroform:methanol). The solution was shaken, centrifuged and aspirated to remove the methanol–water phase. Fifteen milliliters of methanol and 9 ml of 0.04% $CaCl_2$ were added and the washing procedure was repeated. Fifteen milliliters of chloroform were added to the lipid extract, cooled in a refrigerator, and the water layer was removed by aspiration. The volume of lipid extract was diluted to 50 ml with chloroform. Recovery values represented 98% of the phosphorus applied to silica gel impregnated with silver nitrate. Samples were taken for lipid phosphorus (18, 19) and radioactivity. All radioactivity measurements were made in a Packard Tri-Carb liquid scintillation counter. The scintillation solvent consisted of 0.4% of 2,5-diphenyloxazol (PPO) and 0.005% of 1,4-bis(5-phenyloxazolyl)benzene (POPOP) in toluene. Specific activity is expressed as counts per minute per microgram of lipid phosphorus.

Additional animals were pair-fed the liquid-alcohol diet for 2 weeks and controls received the isocaloric diet containing sucrose. Total liver lipids were extracted (21) and triglycerides were determined (22).

Results and Discussion. The total liver triglycerides in the female animals fed the alcohol-liquid diet for 2 weeks were 216 ± 6 mg. The liver from the control animals pair-fed the isocaloric diet containing sucrose gave 61.5 ± 1.5 mg of triglycerides. The fatty liver produced from the ingestion of ethanol-liquid diet is similar to that reported by Porta, Hartroft and Dela Iglesia (14). The liquid diet fed to the animals represents 36%

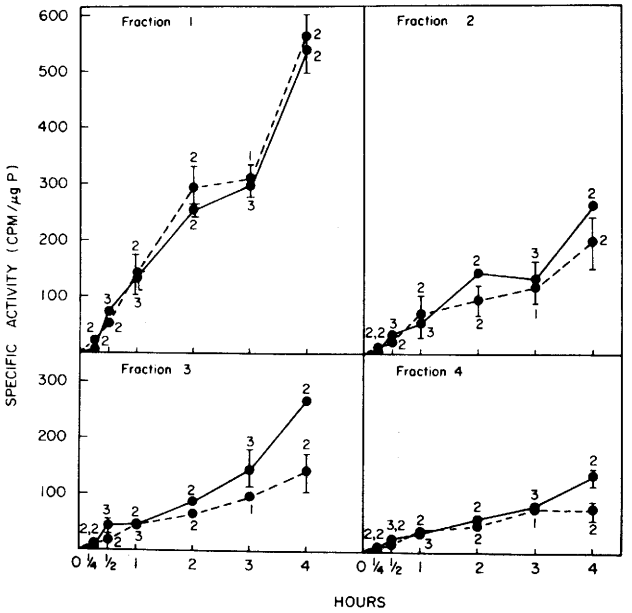

FIG. 2. The effect of alcohol ingestion on the incorporation of 1,2-^{14}C-ethanolamine into phosphatidyl choline fractions from liver microsomes of female rats. Each point on the graph represents the average. The vertical bars on graph represent the standard deviation whenever it was found to be larger than the symbol used to identify it. The number of animals are indicated above each value. (—) Control; (- - -) alcohol.

of total calories given as ethanol or sucrose. Sections of liver from the animals receiving the alcohol diet for 2 weeks, stained for fat, revealed numerous droplets of fat compared to the liver sections of the pair-fed control animals receiving the isocaloric sucrose diet. The histological picture was very similar to that published by Lieber *et al.* (23) for rats fed the same diet for 2 weeks.

Lieber, Jones and DeCarli (24) have observed a difference in phospholipid concentration in male rats fed ethanol and pair-fed isocaloric sucrose-supplemented controls after 10 and 24 days, respectively. French (25) reports a significant increase in the concentration of liver phospholipids in male and female rats by chronic ethanol feeding. The increase was observed in phosphatidyl ethanolamine and lysolecithin fractions. Fallon, Pesch and Klatskin (26) report that isolated liver slices from male rats fed ethanol in drinking water for 7 days incorporated more methyl-^{14}C-methionine into lecithin than control. However, the animals were not pair-fed and the lecithin fractions were not separated. It has been demonstrated that ethanol administration increases the dietary choline requirement in the rat (27, 28). Rats fed ethanol for a 24 day period were shown to have livers which on perfusion took up higher quantities of choline than control liver (29).

It has been demonstrated that 1,2-^{14}C-choline has a specificity for the incorporation into phosphatidyl choline fractions 3 and 4 in liver microsomes (13). It is apparent from the data in Fig. 1 that in the animals fed ethanol in the diet containing choline there is a decreased synthesis of fractions 3 and 4, when compared to those control animals pair-fed an isocaloric diet containing sucrose. The greatest inhibition occurred in these two phosphatidyl choline fractions. The biosynthesis of phosphatidyl choline by the methylation of phosphatidyl ethanolamine can be measured by the incorporation of 1,2-^{14}C-ethanolamine. The specificity of the incorporation of this molecule into phosphatidyl choline is in fractions 1 and 2 (13). The data in Fig. 2 and

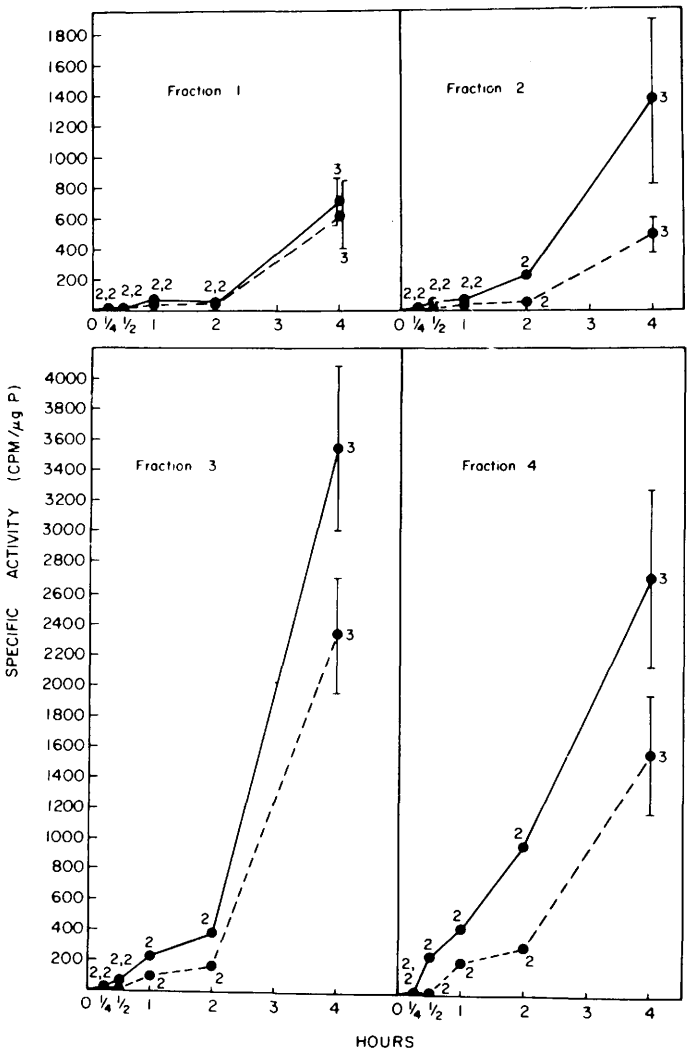

FIG. 3. The effect of alcohol ingestion on incorporation of 1,2-^{14}C-choline into phosphatidyl choline fractions from plasma of female rats. Each point on the graph represents the average of the data from pooled plasma. The vertical bars on the graph represent the standard deviation whenever it was found to be larger than the symbol used to identify it. The number of samples are indicated above each value. (—) Control; (- - -) alcohol.

Fig. 4 shows very little difference between the specific activities of the phosphatidyl choline fractions 1 and 2 from the 1,2-^{14}C-ethanolamine incorporation in the animals fed ethanol when compared to the isocaloric, pair-fed controls receiving sucrose. This observation would suggest that ethanol ingestion for 2 weeks does not affect the biosynthesis of phosphatidyl choline by the methylation of phosphatidyl ethanolamine.

Seventy to 80% of the total phospholipids of plasma contain choline (30). Nelson and Freeman (31) separated the serum lipoproteins by ultracentrifugation and have shown that phosphatidyl choline represents 68–80% of the total phospholipids in the various fractions. The liver is the main, if not the sole, source of phospholipids in plasma (10). The biochemical changes of phospholipids in the liver are reflected by corresponding changes

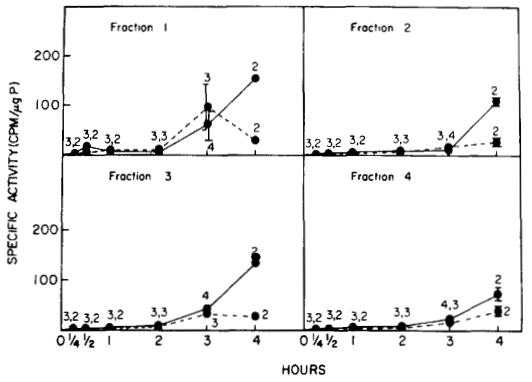

FIG. 4. The effect of alcohol ingestion on incorporation of 1,2-^{4}C-ethanolamine into phosphatidyl choline fractions from plasma of female rats. Each point on the graph represents the average of the data from pooled plasma. The vertical bars on the graph represent the standard deviation whenever it was found to be larger than the symbol used to identify it. The number of samples are indicated above each value. (—) Control; (- - -) alcohol.

in the plasma (32, 33). These observations are substantiated by comparing the time uptake of isotopic compounds into liver and plasma phospholipids (Figs. 1 and 3). The time uptake of the incorporation of 1,2-^{14}C-choline into liver microsomal phosphatidyl choline fraction 4 shows a peak of incorporation at 2 hr followed by decline at 4 hr (Fig. 1). The specific activity values of phosphatidyl cholines in plasma (Fig. 3) are progressively increasing throughout the 4 hr period of time uptake and the specific activity values are greater than those seen in liver. These data show the liver precursor–product relationship in the plasma (34–36). The decreased synthesis of phosphatidyl choline that was observed in the liver microsomes in the animal fed ethanol is reflected to a greater degree in the plasma (Fig. 3). The existence of two pathways of phosphatidyl choline biosynthesis in liver microsomes provides different lecithin molecules, and may be a sensitive means of controlling the availability of specific phosphatidyl cholines for the lipoproteins to transport triglycerides out of the liver. The decrease of the biosynthesis of phosphatidyl cholines in fractions 3 and 4 in animals fed ethanol may be the key in the development of a fatty liver.

Summary. The biosynthesis of phosphatidyl choline by two different pathways in liver microsomes from female rats fed alcohol has been investigated. The incorporation of 1,2-^{14}C-choline and 1,2-^{14}C-ethanolamine into the phosphatidyl choline fractions of liver microsomes and plasma at 0.25, 0.5, 1, 2, 3 and 4 hr after intraperitoneal injection of the isotopic compounds in female rats fed a liquid diet containing 5 g % ethanol for 2 weeks has been determined. Controls were pair-fed the isocaloric diet containing sucrose. This diet when fed for 2 weeks produces a fatty liver; the liver triglycerides increased in the ethanol fed 3.5 times the controls. There was a decreased biosynthesis of phosphatidyl choline in fractions 3 and 4 by the 1,2-^{14}C-choline pathway in liver microsomes and plasma in the ethanol fed animals. There was very little difference in the incorporation of 1,2-^{14}C-ethanolamine in the phosphatidyl choline fractions from liver microsomes and plasma in the animals fed ethanol when compared to controls.

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