fatty acids in triglyceride synthesis (excluding position). Accordingly, these results do not define the specific source of the excess triglycerides that accumulate in the cerium liver, but they do show that gas-liquid chromatography of total liver lipids can reveal this hepatic accumulation of triglyceride within a few hours after intravenous cerium (Fig. 1) and long before its gross occurrence(1,2).

Summary. It has been demonstrated that the reduction in the release of esterified fatty acids from the liver is an insignificant factor in the mechanism of the rare-earth fatty liver. Intravenous cerium in rats caused the fatty acid composition of total liver lipids to become similar to that of adipose tissue even before the detection of the massive accumulation of fat in hepatic cells. This change is explained on the basis of the fatty acid composition of the triglycerides that accumulate in the liver.

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Biosynthesis of Liver Phospholipids During the Development of a Fatty Liver.* (29401)

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The lipotropic effect of lecithins appears to act on the metabolism of fatty acids in the liver, rather than enhancing their mobilization in the form of plasma phospholipids(1). Lecithins are probably an integral part of an enzyme system necessary for oxidation of fat and fatty acids since this is the only phospholipid which is lipotropic for neutral fat or fatty acids. In the liver of the dog (2) or rat(3) maintained on a choline-deficient diet there is a progressive decrease in concentration of the lecithins and a diminished ability to synthesize these phospholipids. Diets low in protein reduce the level of total liver phospholipids(4,5) and lecithin P and cephalin P in liver mitochondria(6). In this paper are reported studies on incorporation of P_i^{32} into mitochondria phospholipids and the concentration of these lipids during the development of a fatty liver. Evidence is presented that these phospholipids are involved in this process.

Materials and methods. Male albino rats of Sprague-Dawley strain weighing 100-150 g were maintained 1, 5, 7, 9 and 21 weeks on a 5% casein-5% fat diet(7). Controls were fed a 25% casein-5% fat diet(8). At the end of the dietary regime the rats were injected with 200 μc of $NaH_2P^{32}O_4$. Six hours later the animals were killed by decapitation, livers removed, weighed and divided into 2 fractions. The acid soluble phosphorus was removed from one fraction, 0.5-1.0 g of liver, by extracting with 10% trichloroacetic acid (TCA). Radioactivity(9) and phosphorus(10) were determined on the

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extract. Radioactivity measurements were made by counting an aliquot of the extract in steel planchet under an ultrathin window gas flow geiger tube (Tracerlab TGC-14). The 6-hour time interval corresponds to the ascending part of the specific activity time curve, where synthesis of phospholipids occurs to a degree greater than breakdown(11, 12). The other fraction was homogenized in a Potter-Elvehjem type tissue grinder with Teflon pestle in ice-cold methanol. Chloroform was added to the methanol-homogenate to bring a 1:1 (v/v) ratio. The lipids were extracted 4 times with methanol:chloroform 1:1 (v/v) by heating the liver homogenate on a water bath 40°C for 30 minutes, centrifuging and removing the solvent. The pooled lipid extracts were evaporated to dryness with Rinco Rotating Vacuum Evaporators (Rinco Instrument Co.), the water removed with Freeze-Dryer (Vir-Tis Co., Inc.) and residue dissolved with chloroform. Radioactivity(9) and phosphorus(10) were determined on the lipid extract. The phospholipids were chromatographed on silicic acid impregnated filter paper according to the method of Marinetti(15) using method of ascending chromatography. The solvent system was diisobutyl ketone:acetic acid:water 40:25:5 (v/v). Into a chromatography jar (Corning No. 6945) measuring 6 × 18 inches was placed 280 ml of the solvent and allowed to equilibrate for at least 6 hours. The silicic acid impregnated papers were introduced into the jars and allowed to remain at a constant temperature of 23°C for 21 hours. The chromatograms were dried, stained with Rhodamine 6G and the phospholipids identified with ultraviolet light. Synthetic standards (L-α-cephalin, β, γ-dipalmitoyl; L-α-lecithin, β, γ-dipalmitoyl, California Biochemicals, Los Angeles) were also used as reference materials. Additional purified standards of phosphatidyl inositol, phosphatidyl serine and sphingomyelin were prepared from rat spleen extracted 3 times with hot ethanol, ethanol:ether (2:1) chromatographed on silicic impregnated cellulose paper(15), identified by staining with Rhodamine 6G observing under ultraviolet light, chemical spot tests, ninhydrin, choline, glycerol and inositol and reextracted from the chromatogram as reference standards. Other lipids such as lysolecithins were not identified, although they would contain radioactive phosphorus. The encircled spots on the dried, stained, chromatograms were cut out and placed into 15 × 125 mm pyrex test tubes, and hydrolyzed in 4 ml 3 N methanolic HCl for 30 minutes in a water bath of 63°C. Glass marbles were placed over the mouths of the test tubes so as to prevent evaporation of the digestion mixture. The acid methanol extract was removed and evaporated to dryness under infra-red lamps. The extraction procedure was repeated 2 times and the phospholipid phosphorus and radioactivity were determined on the combined extracts.

The phospholipid extract was digested for 30 minutes with 0.2 ml of H_2SO_4:HNO_3 (1:1), allowed to cool, and 3 drops of HNO_3 added and the digestion continued for an additional 15 minutes. The samples were then neutralized to pH 6-7 with NH_4OH with phenol red as internal indicator. Inorganic phosphorus was determined by the method of Fiske and Subbarow(10). An aliquot of the colored solution of the phosphate analysis was pipetted into a steel planchet containing a drop of concentrated NaOH solution and dried under a heat lamp. The radioactivity was determined by placing the planchet under an ultrathin window, gas flow Geiger tube (Tracerlab TGC-14). From these results and the radioactivity measurements, relative specific activities of the individual phospholipids were calculated(13). Relative specific activity expresses the relationship between isotopic concentration in a compound and in its precursor(14). Under the conditions of our experiments an increase in formation of phospholipids may be reasonably assumed when higher values for relative specific activities are found. The relative specific activity is the ratio of the specific activity of the phospholipid P to that of the acid soluble P. The specific activity is defined as counts per minute per mg of phosphorus.

Additional groups of rats were maintained on the 5% casein-5% fat diet(7) 1 and 4 weeks. At the end of the dietary regime 250 μc radioactive $NaH_2P^{32}O_4$ was adminis-

TABLE I. Lipid Phosphorylation During Development of Fatty Liver in Animals Maintained on a Low Protein - Low Fat Diet.

Time on diet, days	No. of animals	Total lipids, mg/g wet wt	Liver phospholipids		
			Total phospholipid P, mg/g wet wt	Specific activity*	Relative specific activity†
17	5	103 ± 16.1	1.15 ± .10	67.3 ± 9.2	.169 ± .026
36	8	135 ± 51.8	1.16 ± .14	58.7 ± 13.7	.569 ± .046
64	10	335 ± 86.0	.60 ± .06	85.3 ± 24.1	.203 ± .041
96	4	371 ± 27.4	.64 ± .10	49.0 ± 14.6	.179 ± .038
114	8	301 ± 92.7	.74 ± .12	69.5 ± 11.3	.200 ± .047
164	12	282 ± 20.1	.60 ± .10	63.7 ± 22.2	.177 ± .021
185	4	290 ±102.7	.73 ± .09	67.8 ± 7.6	.183 ± .036
206	14	298 ± 73.6	.63 ± .19	66.9 ± 11.0	.181 ± .027
249	6	326 ± 16.8	.66 ± .03	80.3 ± 16.7	.168 ± .020
297	8	314 ± 15.3	.48 ± .05	71.1 ± 15.8	.150 ± .014

* c/m/mg of P.
† Specific activity of phospholipid P/specific activity of inorganic PO_4.
Figures preceded by ± sign indicate standard deviation.

tered and the animals sacrificed after 6 hours. The livers were removed, the mitochondria fraction was prepared according to the method of Hogeboom *et al*(16) and acid-soluble phosphorus and mitochondria lipid phosphorus fractionated. The phospholipids were extracted, chromatographed on silicic impregnated paper. Radioactivity and phosphorus were determined on the individual phospholipid fractions as described above. A third group of rats were maintained 17, 36, 64, 96, 114, 164, 185, 206, 249 and 297 days on a 5% casein-5% fat diet(7). At the end of the dietary regime the rats were injected with 200 μc of $NaH_2P^{32}O_4$ and 6 hours later were killed by decapitation, livers removed, weighed and divided into 3 fractions. The acid-soluble phosphorus was removed from one fraction 0.5-1.0 g of liver, by extracting with 10% trichloroacetic acid (TCA) and radioactivity(9) and phosphorus(10) were determined as previously described. A small sample was taken for histological examination. The other fraction was covered with alcohol, and lipids extracted with alcohol-ether and purified with chloroform(9). Radioactivity(9), phosphorus(10) and the weight of total lipids were determined on aliquots of the chloroform solution as previously described. From these results the specific activity and relative specific activity were calculated.

Results. Table I shows the liver lipids and the phospholipid synthesis during the development of a fatty liver in animals maintained on a 5% protein-5% fat diet at various intervals ranging from 17 to 297 days. It is apparent from the data that there is a decrease in total liver phospholipids concentration as total lipids increase. This is in agreement with previously reported data (4,5,6). A choline deficiency produced by feeding a low-protein diet in the rat results in a prompt fall in total serum phospholipid concentration with a corresponding rise in concentration of total liver lipids(18). There is an increase in the relative specific activity of the total liver phospholipids in the animals maintained on the diet for 36 days. An increase in the specific activity of the total liver phospholipids occurred after 64 days on the diet. During this time interval liver total lipids rose from 103 to 335 mg/g of wet liver. However, lipid phosphorylation decreases after this time interval and remains rather constant throughout the remainder of the study. Histological examination of the liver indicated fatty infiltration in the animals maintained on the diet for 17 days. Diffused, severe, fatty change was noted throughout the entire histological section in the animal fed the diet 96 days. The liver of the animals fed the diet 206 days demonstrated extensive fatty change and early fibrosis. Histological examination of the liver from the animals fed the diet for 297 days showed extensive fatty change, nodular hyperplasia, fibrosis and liver cell regeneration.

TABLE II. Effect of Dietary Choline Deficiency on Liver Phospholipids.

Time on diet, wk	No. of animals	μg of phospholipid P/g of wet tissue: Phosphatidyl ethanolamine	Phosphatidyl serine	Lecithin	Sphingomyelin	Phosphatidyl inositol
0	6	246 ± 70 (.412 ±.037)	77 ± 19 (.392 ±.06)	859 ± 110 (.286 ±.049)	128 ± 24 (.313 ±.062)	36 ± 2 (.398 ±.024)
1	6	256 ± 50 (.429 ±.049)	67 ± 15 (.424 ±.062)	761 ± 91 (.378 ±.009)*	103 ± 20 (.391 ±.080)	25 ± 4 (.546 ±.037)*
5	5	232 ± 27 (.278 ±.033)*	51 ± 8 (.273 ±.040)*	599 ± 12* (.248 ±.026)	85 ± 8* (.187 ±.029)*	33 ± 4 (.245 ±.044)*
7	6	207 ± 48 (.253 ±.026)*	63 ± 11 (.227 ±.025)*	542 ± 43* (.234 ±.030)	83 ± 17* (.180 ±.021)*	33 ± 7 (.245 ±.020)*
9	5	119 ± 6* (.265 ±.006)*	37 ± 7* (.172 ±.006)*	291 ± 18* (.223 ±.011)	46 ± 8* (.174 ±.014)*	16 ± 2* (.240 ±.015)*
21	6	161 ± 46 (.239 ±.041)*	29 ± 19* (.138 ±.027)*	331 ± 33* (.172 ±.024)*	72 ± 19* (.112 ±.021)*	18 ± 6* (.220 ±.022)*

Values in parentheses indicate relative specific activities. Figures preceded by ± sign indicate standard deviation. Test of significance was applied to difference between mean value of controls and experimental values(17).

* Probability for chance occurrence of this difference was <.01.

Table II shows the effect of dietary choline deficiency on concentration and radioactive turnover of the liver phospholipids; phosphatidyl ethanolamine, phosphatidyl serine, lecithin, sphingomyelin and phosphatidyl inositol. The data show that reduction of dietary protein for 5 weeks results in statistically significant decreased concentration of liver lecithin and sphingomyelin. The concentration of these 2 phospholipids continues to decrease as long as the animals are maintained on the low-protein diet. A statistically significant decrease in concentration of phosphatidyl ethanolamine and phosphatidyl inositol occurs at the end of 9 weeks on the dietary regime. These data indicate that lecithin and sphingomyelin concentration in liver is directly related to dietary protein intake.

The increase of lipid phosphorylation occurring in animals maintained on a choline-deficient diet for one week has been reported (3,19). Apparently there is a compensating mechanism as an increase in relative specific activity values occurs at this time interval. This would indicate an increase of phospholipid synthesis.

The data in Table III show that a reduction of dietary protein intake for 4 weeks results in statistically significant decreased concentration of phosphatidyl serine, lecithin, sphingomyelin and phosphatidyl inositol of liver mitochondria.

The oxidation of fatty acids(20) and the lipotropic action of choline(1) occur in liver

TABLE III. Effect of Choline Deficiency on Liver Mitochondria Phospholipids.

Time on diet, wk	No. of animals	μg of phospholipid P/g of liver: Phosphatidyl ethanolamine	Phosphatidyl serine	Lecithin	Sphingo-myelin	Phosphatidyl inositol	Phosphatidic acid
0	4	152 ± 17 (.22 ±.01)	41 ± 8 (.23 ±.04)	260 ± 21 (.23 ±.03)	56 ± 10 (.17 ±.02)	23 ± 2 (.21 ±.05)	1 ± 1
1	8	163 ± 15 (.21 ±.01)	38 ± 9 (.24 ±.02)	247 ± 40 (.23 ±.02)	48 ± 10 (.16 ±.04)	21 ± 6 (.31 ±.05)	4 ± 1 (.54 ±.14)
4	8	143 ± 5 (.25 ±.02)*	27 ± 3* (.19 ±.04)	192 ± 19* (.22 ±.02)	31 ± 6* (.17 ±.02)	13 ± 2* (.29 ±.04)	8 ± 2* (.17 ±.03)

Values in parentheses indicate relative specific activities. Figures preceded by ± sign indicate standard deviation. Test of significance was applied to difference between mean value of controls and experimental values(17).

* Probability for chance occurrence of this difference was <.01.

mitochondria, although the lipotropic mechanism is unknown. It is of interest that the decrease of liver phospholipids occurs in mitochondria during the development of a fatty liver. Phosphatidic acid is an intermediate common to lecithin synthesis and neutral fat(21). There is an increase in the concentration of liver mitochondria phosphatidic acid (Table III) as the choline deficiency progresses in the animals maintained on the low-protein diets. The accumulation of phosphatidic acid in the liver mitochondria must be associated with the decrease in the phospholipids in the liver. This accumulation of phosphatidic acid, a precursor of the lipotropic agent lecithin, in liver mitochondria of choline deficient animals despite a decreased turnover rate indicates that there may be a block of this phospholipid synthesis resulting in a decreased supply of lecithin. Since phosphatidic acid is a common pathway for lecithin and neutral fat synthesis(21) a block occurring at this level would make the excessive accumulation of neutral fat possible.

Summary. 1. Liver total lipids, phospholipid P and phospholipid synthesis has been investigated during the development of a fatty-cirrhotic liver in animals maintained on a low-fat, low-protein diet for 17 to 297 days. 2. There is an increase in the specific and relative specific activities of total liver phospholipids between the 36th and 64th days, *i.e.*, during the development of the fatty liver. 3. Chromatography of liver phospholipids and lipid phosphorylation during the development of a fatty liver was studied in rats maintained on low protein-low fat diets. 4. A statistically significant decrease of liver lecithin and sphingomyelin occurred at end of 5 weeks of dietary regime and continued to decrease as long as the animals were maintained on the low protein diet. 5. A statistically significant decrease of phosphatidyl ethanolamine and phosphatidyl inositol occurs at the end of 9 weeks on the dietary regime. 6. A reduction of dietary protein intake for 4 weeks results in a statistically significant decrease of phosphatidyl serine, lecithin, sphingomyelin and phosphatidyl inositol in liver mitochondria. There is a progressive increase of mitochondria phosphatidic acid as the choline deficiency progresses. The role of the reduction of phosphatidic acid in the production of a fatty liver is discussed.

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