albumin. The anatomical and functional characteristics of these lymph nodes indicate that they are comparable to lymph nodes of mammals.

The authors express their grateful acknowledgement to Dr. Thomas E. Hunt and Dr. Clarence E. Klapper for helpful suggestions during preparation of the manuscript.

1. Jordon, H. E., Speidel, C. C., *Am. J. Anat.*, 1929, v43, 77.

2. Jordon, H. E., *Quart. Rev. Biol.*, 1933, v8, 58.

3. Ramer, A. S., W. B. Saunders Co., Phil. & London, 1949.

4. Andrew, W., New York, Oxford Univ. Press, 1959.

5. Evans, E. E., Horton, S. L., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 71.

6. Evans, E. E., *Fed. Proc.*, 1963, v22, 1132.

7. ———, PROC. SOC. EXP. BIOL. AND MED., 1963, v112, 531.

8. Sainte-Marie, G., *J. Histochem. & Cytochem.*, 1962, v10, 250.

9. Coons, A. H., Leduc, E. H., Connally, J. M., *J. Exp. Med.*, 1955, v102, 61.

10. McManus, J. F. A., Mowry, R. W., Paul B. Hoeber, Inc., New York, 1960.

11. Gridley, M. F., *Am. J. Clin. Path.*, 1951, v21, 879.

12. Unpublished data.

Received March 10, 1964. P.S.E.B.M., 1964, v116.

Phospholipid Patterns in Livers Accumulating Fat. (29279)

FRED SNYDER AND WALTER LEWIS*†
Oak Ridge Institute of Nuclear Studies, Medical Division, Oak Ridge, Tenn.
(Under contract with U. S. Atomic Energy Commission)

Although the rare earths so far have not been found in mammalian cells, these elements have been studied as anticoagulants and as possible internal emitters for therapeutic radiation; these earlier investigations have led to many reports of their biological effects(1-4). Our laboratory has been interested in the mechanism of triglyceride accumulation in fatty livers caused by intravenous administration of certain metals of the rare-earth series(5-9), since the fatty livers induced by these elements return to normal after one week and since the cycle of fatty infiltration can be repeated. The dual role of phosphatidic acid in triglyceride and lecithin synthesis in isolated enzyme systems (10) suggests that whereas we did not find a change in the total phospholipid phosphorus of the rare-earth fatty liver in an earlier study (5), alterations in the liver phospholipids probably occur under these conditions of rapid triglyceride accumulation in the liver. This paper reports our observations regarding the phospholipid components of fatty livers produced by cerium injections.

* Summer participant from Univ. of West Virginia; deceased 1963.

Methods. Exerimental treatment of rats. The rats used in these experiments were CFN female rats weighing between 150 and 250 g. Fatty livers were produced by injecting cerous chloride (2 mg/kg) intravenously(5). The animals were decapitated 24 and 48 hours after the injection; all rats were fasted 24 hours before death with the exception of one group of control animals that were fed.

Extraction of liver lipids. The whole liver was removed from each rat promptly after killing and placed into an Osterizer blender bowl containing 100 ml of methanol. The blender was operated for approximately 5 sec to reduce the livers to small particles; longer periods produced emulsions or very fine suspensions that were difficult to separate in later stages. The contents of the bowl were transferred to a beaker and placed in a boiling water bath until the methanol had boiled for approximately 3 minutes. Then 100 ml of chloroform was added and the mixture was stirred. After the solids had settled, the clear supernatant was removed quantitatively by decantation into a course sintered glass funnel. The extraction was repeated 2 more times. The combined filtrates were trans-

TABLE I. Distribution of Phosphorus Among Phospholipid Fractions.

Treatment	% of total phosphorus						
	A + A_1	B	E	F	G	H	I
Normal—unfasted	3.4	20	8.4	63	2.7	1.2	1.5
fasted 24 hr	4.9	21	8.5	54	4.5	2.4	1.5
Cerium injected 24 hr	6.4	20	7.3	55	3.3	2.2	2.8
48 hr	3.9	21	8.8	57	3.6	1.4	2.5

A, Polyglycerol phosphatides, including phosphatidic acid; B, Phosphatidyl ethanolamine + phosphatidyl serine; C and D, Unidentified; E, Phosphatidyl inositol; F, Phosphatidyl choline; G, Sphingosine derivatives; H or I, probably lysolecithin. Fraction A + A_1 from livers of animals sacrificed 16 and 96 hr after cerium had 8.6 and 5.8%, respectively, of the total phosphorus in an experiment in which only Fraction A + A_1 and total phosphorus were measured.

ferred to a 500-ml round-bottom flask and the solvent was removed at diminished pressures with a Labline rotary evaporator attached to a water aspirator. The dry residue was extracted 4 times with chloroform, which was decanted through a fine sintered glass filter into a tared 100-ml round-bottom flask. The chloroform was removed and weight of the total lipids was determined.

Usually 4 animals comprised a group but the liver of each was handled and extracted separately as described. Finally, the weighed lipids from the same group were combined to give a final concentration of 100 mg lipid/ml chloroform for chromatographic analysis.

Silicic acid chromatography. A 35 g portion of Bio-Rad silicic acid was suspended in approximately 55 ml of 1:1 chloroform-methanol solution and poured into a water-jacketed chromatographic column described by Hirsch and Ahrens(11). The silicic acid formed a column 20 mm × 240 mm. The column was maintained at 25°C ±0.5° by the water jacket mounted on a fraction collector, which was programmed to deliver 10.0 ml fractions. Bound water in the silicic acid was displaced by first passing 200 ml of chloroform-methanol (1:1) through the column. This was followed by 200 ml of pure chloroform. Sufficient extract was then applied to the top of the column to provide 2 to 12 mg of phospholipid phosphorus (usually contained in 5 to 8 ml of the total lipid extract). Another 200 ml of chloroform was used to wash the neutral lipids through the column leaving the phospholipids absorbed near the top. Next, 495 ml of chloroform-methanol solution (8:1) was put through the column, followed by 700 ml (2.5:1), 200 ml (1:1), and finally 200 ml of pure methanol. The solvent ratios are expressed on a volume for volume basis; the eluting sequence of solvents was based primarily on the work of Hanahan *et al*(12) and Marinetti *et al*(13). Flow rates as high as 5 ml/min using head pressures not exceeding 10 lb/sq in. were obtainable with the Bio-Rad silicic acid preparation; the rate was normally adjusted to 4 or 5 ml/min. Under these conditions, runs were completed in less than 8 hours. Phosphorus was determined on an aliquot from each tube by essentially the method of Harris and Popat(14). The color developed was read at a wavelength of 820 mμ in the Beckman Model B Spectrophotometer. Fatty acid esters(15) were also measured in the polyglycerol phosphatide and lecithin fractions.

Results and discussion. Fig. 1 and 2 show the silicic acid elution curves for phospholipids separated from control livers and fatty livers induced by cerium. The various peaks are designated in the legend for each figure. The main peaks in both samples were (A) polyglycerol phosphatide fraction, (B) phosphatidyl ethanolamine and phosphatidyl serine, (E) phosphatidyl inositol and (F) phosphatidyl choline. The percentage of phosphorus for the particular fractions is given in Table I. A new peak (A_1) occurred in all animals with fatty livers, but not in the controls. Glenn *et al*(16) reported an increase in the ester/phosphorus ratio of their cardiolipin fraction in a cerium fatty liver (36 hr after injection); perhaps the reason they did not observe a double first peak in their column elution was that their first stepwise elution mixture was more polar than ours (chloroform:methonal, 4:1 in their experiment). The

most striking patterns seen in livers accumulating fat were (a) this double peak in the first step of the elution, (b) the small but consistent increase in fraction A in cerium (24 hr) and fasting livers, and (c) the slight but consistent decrease in phosphatidyl choline in all of the fatty livers. The increase in the polyglycerol phosphatides of the cerium fatty livers is similar to the finding of Cornatzer and Walser(17), who have reported on phospholipid changes in rats maintained on 5% casein diets for extended periods. They found a progressive increase in the liver phosphatidic acid, whereas most of the other phosphatides decreased after 4 to 9 weeks on the low protein diet.

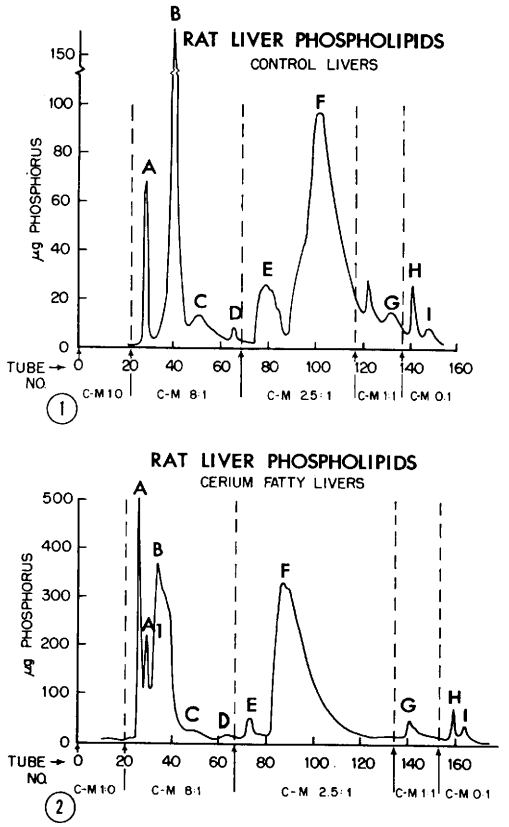

FIG. 1. A typical stepwise elution curve of liver phospholipids from control rats. Ratios of chloroform : methanol for each elution step are shown between the vertical broken lines set off by arrows. Letters above the various peaks designate: A, polyglycerol phosphatides; B, phosphatidyl ethanolamine + phosphatidyl serine; C and D, unidentified; E, phosphatidyl inositol; F, phosphatidyl choline; G, sphingosine derivatives; H or I, probably lysolecithin. Unmarked peak at right of lecithin (F) is thought to be an artifact caused by a change of solvents at tube 117 (not seen in other samples).

FIG. 2. A typical stepwise elution curve of liver phospholipids from cerium-injected rats. Ratios of chloroform : methanol for each elution step are shown between the vertical broken line set off by arrows. Letters above the various peaks designate: A, polyglycerol phosphatides, ester/phosphorus ratio 4.0 and 4.3; A_1 a secondary peak appearing with the polglycerol phosphatide fraction isolated from fatty livers, ester/phosphorus ratio 4.4 and 4.6; B, phosphatidyl ethanolamine + phosphatidyl serine; C and D, unidentified; E, phosphatidyl inositol; F, phosphatidyl choline, ester/phosphorus ratio 1.9 and 2.1; G, sphingosine derivatives; H or I, probably lysolecithin. The ester/phosphorus ratios given are for two separate samples.

Summary. Fatty livers produced by cerium injection have shown 3 main alterations in the liver phospholipid pattern separated on silicic acid columns: (1) the appearance of a secondary peak in the polyglycerol phosphatide fraction (eluted with chloroform: methanol, 8:1); (2) a small but consistent increase in the phosphatidic acid-polyglycerol phosphatide fraction; and (3) a decrease in the lecithin fraction for all fatty livers measured in this study.

The authors express appreciation to Miss Pauline Finney for valuable technical assistance.

1. Steidle, H., Seltene Erdmetalle, in *Handbuch der Experimentallen Pharmakologie,* Springer, Berlin, 1935, v3, pt. 4, p2189.

2. Trapmann, H., *Arzneim. Forsch.,* 1959, v9, 341, 403.

3. Kyker, G. C., Andersen, E. B., Eds., *Rare Earths in Biochemical and Medical Research,* US AEC Report, ORINS-12 (1956).

4. Kyker, G. C., *Rare Earths,* Chap. 36 in Mineral Metabolism, II B, *The Elements,* eds. C. L. Comar & F. Bronner, Academic Press, N. Y., 1962, p499.

5. Snyder, F., Cress, E. A., Kyker, G. C., *J. Lipid Research,* 1959, v1, 125.

6. ———, *Nature,* 1960, v185, 480.

7. Snyder, F., Baker, F., Rafter, J., Kyker, G. C., *Biochim. Biophys. Acta,* 1960, v43, 554.

8. Snyder, F., Stephens, N., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 202.

9. Snyder, F., Cress, E. A., *Experientia,* 1961, v17, 303.

10. Kennedy, E. P., *Fed. Proc.,* 1957, v16, 847.

11. Hirsch, J., Ahrens, E. H., Jr., *J. Biol. Chem.,* 1958, v233, 311.

12. Hanahan, D. J., Dittmer, J. C., Warashina, E., *ibid.,* 1957, v228, 685.

13. Marinetti, G. V., Erbland, J., Kochen, J., *Fed. Proc.,* 1957, v16, 837.

14. Harris, W. D., Popat, P., *J. Am. Oil Chemists' Soc.*, 1954, v31, 124.
15. Snyder, F., Stephens, N., *Biochim. Biophys. Acta*, 1959, v34, 244.
16. Glenn, J. L., Opalka, E., Tischer, K., *J. Biol. Chem.*, 1963, v238, 1249.
17. Cornatzer, W. E., Walser, A. H., U. S. AEC Rep., TID-16223, 1961.

Received February 27, 1964. P.S.E.B.M., 1964, v116.

Preservation of Cell Cultures by Freezing in Liquid Nitrogen Vapor.* (29280)

ARTHUR E. GREENE, RUTH K. SILVER, MARIE KRUG AND LEWIS L. CORIELL
(Introduced by Werner Henle)
South Jersey Medical Research Foundation, Camden, N. J. and University of Pennsylvania, Philadelphia, Pa.

The use of low temperature for preservation of live, viable mammalian cells has become an accepted procedure in the past decade for preservation of bovine sperm, red blood cells, bone marrow and cell cultures (1,2). The procedures described by Scherer and Hoosgasian(3), Swim *et al*(4), Hauschka *et al*(5), and Stulberg *et al*(6) have been followed by most workers for storage of cell cultures at low temperatures to prevent accidental loss of the culture, microbial contamination or chromosomal mutation which frequently occur when continuous cell lines are transferred every few days.

A temperature fall of 1°C/min was found to be the optimal rate for freezing bull sperm (7), and this information has been adopted as standard procedure by most cell biologists, even though few detailed freezing rate studies on other mammalian cells have been presented. Kite and Doebbler(8) showed that automatically controlled freezing rates between 1° and 45°C/min gave comparable cell recoveries with HeLa and strain L cells. Other cell lines were also frozen successfully in liquid nitrogen vapor at approximately 20°C/min. Optimal freezing rates were not determined, and the method of cell recovery was based on an inoculum of 7,500 cells which does not give a sensitive index of cell viability such as is provided by growth of individual cells in a cell plating technique(9). Greaves and Nagington(10) recently developed a small polystyrene plug for freezing in liquid nitrogen vapor and successfully used it with several serially passed and primary cell lines. However, optimal cooling rates for many cells remain to be determined(11).

In view of the expanding use and the many advantages of liquid nitrogen for preservation and storage of cell cultures and biological materials, this report concerns experiments to obtain more precise quantitative data on optimal freezing rates of different cell lines, and to ascertain the practicability of freezing and storing cells directly in liquid nitrogen vapor without elaborate programming equipment.

Materials and methods. The 15 cell types to be reported consist of the 9 continuous cell lines, 4 primary cell strains and 2 diploid strains listed in Table I. Cell cultures were grown in Eagle's medium(12) in Earle's balanced salt solution in an atmosphere of 5% CO_2 - 95% air, with the exception of Hayflick WI-38 and Human Lung S.J. which were grown in Eagle's medium in Hanks' balanced salt solution. Ten percent calf serum was used as the serum source with the exception of HeLa 229 and Detroit-6, which received 10% human serum. Culture media contained 100 units/ml of penicillin and 100 μg/ml of streptomycin.

Cells to be frozen were grown in Blake bottles for 4-6 days and the medium changed 24 hours before harvest with 0.25% trypsin. Three to five million viable cells/ml were suspended in fresh growth medium containing 5% glycerol, distributed into ampuls and sealed with an oxygen-propane torch.

Experimental groups of ampuls were suspended in a Linde liquid nitrogen refrigerator 300 by attaching the test ampuls to metal

* Work was supported by grants from Nat. Cancer Inst., U.S.P.H.S.