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Plasmalogens and phosphatides of rat diaphragm after incubation with palmitate-1-C^{14} *in vitro*[*†]

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SUMMARY

Ethanolamine-containing plasmalogen was found to be the major type of plasmalogen in rat diaphragm. The molar ratio of total plasmalogen to total phosphatide phosphorus was 0.09. After incubation of rat diaphragm with palmitate-1-C^{14}, it was found that the specific activity of diglyceride always exceeded that of both cephalin and lecithin, and the specific activity of the phosphatides approached that of the diglyceride with increasing time of incubation. Lecithin specific activity always exceeded that of cephalin. Estimated turnover times were approximately 2 hours for lecithin, 3 hours for triglyceride and 10 hours for cephalin.

Stein *et al.* (1) found more radioactivity in neutral lipid than in phospholipid after incubating rat-liver mitochondria with C^{14}-palmitate for 30 minutes. In *in vitro* studies with rat diaphragm, Neptune *et al.* (2) found a more rapid incorporation of labeled palmitate into total neutral lipid than into phospholipid; however, after prolonged incubation, they found phospholipid radioactivity to exceed that of neutral lipid. The experiments reported here were performed to determine the time sequence of incorporation of radioactivity from palmitate-C^{14} and to measure simultaneously the plasmalogen content of specific phosphatides of rat diaphragm. The results are compared with those of Neptune *et al.* (3) who investigated the time sequence of incorporation of radioactivity into specific neutral lipids.

METHODS

Incubation Procedure and Lipid Extraction. Hemidiaphragms, taken from rats of the Long-Evans strain weighing between 125 and 135 g, were suspended in 3.0 ml of Ringer-phosphate buffer, pH 7.4, containing 0.5 μc of palmitate-1-C^{14} and 4.5 mg of crystalline bovine serum albumin. Diaphragms from 16 rats, approximately 3.2 g of muscle tissue, were used in each experiment (4 hemidiaphragms per flask). The tissues were incubated in 25-ml Erlenmeyer flasks in a Dubnoff metabolic shaker for a fixed period of time at 37°. Immediately following incubation, tissues were removed, blotted, and ground in a porcelain mortar with washed sea sand and chloroform—methanol 2:1. After centrifugation and rewashing of the precipitate with additional solvent, the pooled extracts were evaporated under nitrogen, and the residues were dissolved in 10 ml of chloroform.

Chromatographic Separations. Two-gram columns of silicic acid powder (Mallinckrodt Chemical Works, St. Louis, Missouri, 100 mesh, activated at 110° overnight) were prepared in 10-ml hypodermic syringes and used for separation of neutral lipid from phospholipid. A chloroform solution containing up to 10 mg of neutral lipid plus 5 mg of phospholipid was applied. Neutral lipid was eluted with 30 ml of chloroform, and phospholipid with 30 ml of methanol. Approximately 96% of the radioactivity applied was recovered. After removal of the methanol under nitrogen, the sample was dissolved in chloroform and then applied to a 4-g (1 × 10 cm) column of silicic

* The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the view of the Navy Department or the naval service at large.

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acid powder (Bio-Rad Laboratories, Richmond, California, activated overnight at 125°). Less than 25 mg of phospholipid was applied to each column. The phosphatides were eluted with increasing concentrations of methanol in chloroform by using a flow rate of approximately 2 ml per minute under positive nitrogen pressure.

Analytical Procedures. Phosphorus was measured by the method of Beveridge and Johnson (4). Plasmalogen aldehyde was determined by a modification of the technique of Wittenberg *et al.* (5). Phosphatide bases were qualitatively determined by high-voltage paper electrophoresis after hydrolysis in 6 N methanolic hydrochloric acid for 12 hours. Samples for radioassay were dried in 20-ml counting vials, then dissolved in a solution of 0.4% 2,5-diphenyloxazole and 0.01% 1,4-*bis*-2(5-phenyloxozolyl)-benzene in toluene. These were counted in a Tri-Carb liquid scintillation counter (Packard Instrument Company, LaGrange, Illinois) with 70% efficiency for carbon-14.

RESULTS

General. Figure 1 shows a typical elution pattern obtained from the silicic acid fractionation of phospholipids. Samples pooled from the 10% methanol fraction and the 20% methanol fraction both contained ethanolamine but no detectable serine. They are referred to as phosphatidyl ethanolamine I and phosphatidyl ethanolamine II, respectively. A synthetic dipalmitoylphosphatidylethanolamine was eluted exclusively in the 20% methanol fraction, and a synthetic dipalmitoylphosphatidylcholine exclusively in the 40% methanol fraction. The material in the pooled 40% methanol fraction is called phosphatidyl choline. Two small peaks in the 60% and 100% methanol fractions, which probably represent lysolecithin and sphingomyelin, contained very little radioactivity and are not included in the data reported here. The amount of phosphorus found in each of the three fractions at different incubation times is presented in Figure 2. The phosphatidyl ethanolamine II fraction contained a much smaller amount of material than either of the other fractions. The sum of phosphorus values for the three fractions, which represents recovery from the diaphragms of 16 rats, accounted for approximately 93% of the phosphorus eluted from the column.

Plasmalogen Content. The amount of aldehyde found in each fraction at the various incubation times is presented in Figure 3. The source of the aldehyde was assumed to be ethanolamine-containing and choline-containing plasmalogen, by virtue of the presence of material giving a positive reaction by the Wittenberg method in the fractions containing phosphatidyl ethanolamine and phosphatidyl choline. Most of the

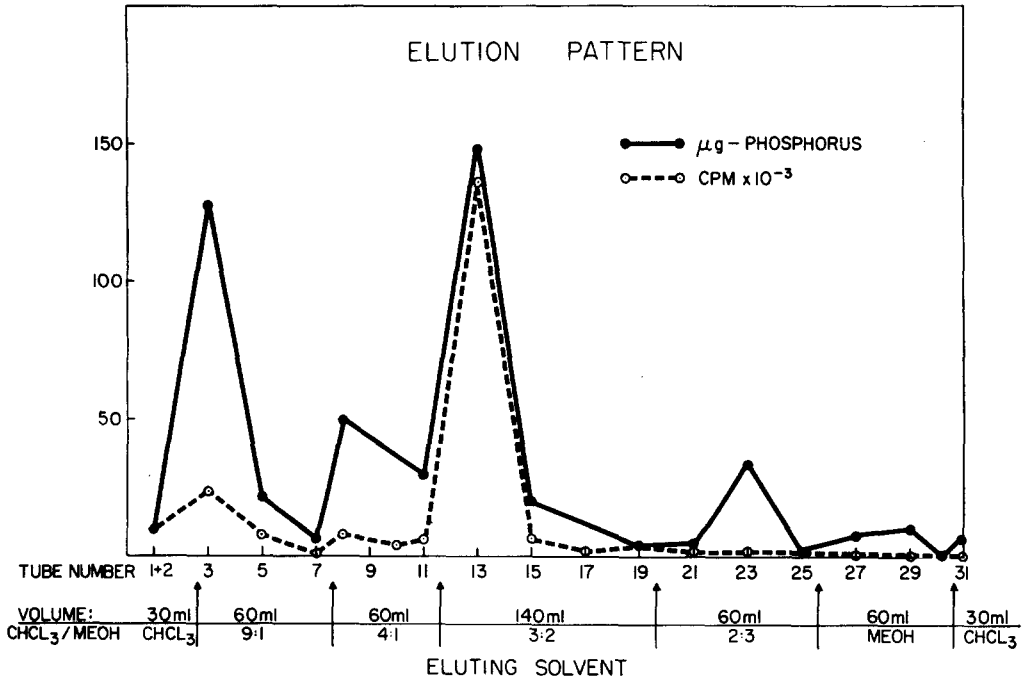

FIG. 1. Silicic acid chromatography of a phospholipid extract from rat diaphragm after a 4-hour incubation with albumin-bound palmitate-1-C^{14}. An aliquot of the tissue extract in chloroform containing 0.77 mg of phosphorus and 402,325 cpm was applied to a 4-g (1 x 10 cm) column. Overall recovery from the column was 101% for phosphorus, 93% for radioactivity.

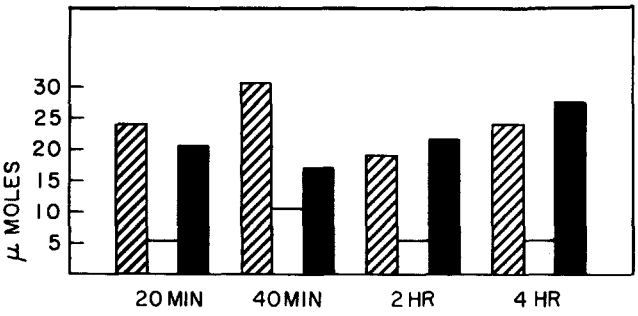

FIG. 2. Phosphorus content of phosphatide fractions from rat diaphragm isolated on silicic acid after incubation with albumin-bound palmitate-1-C^{14}. The diagonally striped bars represent phosphatidyl ethanolamine I; the open bars, phosphatidyl ethanolamine II; the solid bars, phosphatidyl choline. The ordinate indicates total μmoles of phosphorus recovered in the respective fractions. For each incubation time, this represents recovery from 32 hemidiaphragms or approximately 3.2 g of muscle tissue. Phosphorus applied to a 4-g column was quantitatively recovered, and approximately 93% of that recovered was in the three fractions represented in this figure.

plasmalogen was present in the phosphatidyl ethanolamine I fraction. Molar ratios of aldehyde to phosphorus ranged from about 0.11 to 0.20 for the phosphatidyl ethanolamine I fraction, while for the other two fractions this ratio usually was from 0.03 to 0.06.[1] The amount of plasmalogen found in each fraction did not change greatly at different incubation times. The molar ratio of aldehyde to phosphatide phosphorus for the sum of the three fractions was around 0.09 at all incubation times. The molar ratio of choline-containing plasmalogen to total plasmalogen was around 0.12 at each incubation time except 20 minutes, where it was 0.27.

Specific Activity. A semi-logarithmic plot of the relative specific activities found against time of incubation is presented in Figure 4. The values are normalized to the highest specific activity observed, which was that in the phosphatidyl choline fraction after a 4-hour incubation. This value was 24,900 cpm per μmole of phosphorus and represents an estimated incorporation of 6.5 mμmole of added fatty acid per μmole of phosphorus. Each fraction shows a progressive increase in specific activity with time. The specific activities of the lecithin fraction are higher than those of either of the cephalin fractions, and the specific activities of the plasmalogen-poor cephalin are somewhat higher than those of the relatively plasmalogen-rich cephalin. In Table 1, diglyceride and triglyceride specific activities found by Neptune *et al.* (3) (corrected for different amount of radioactivity and different counting instrument efficiency used in their experiments) are compared with the phosphatide specific activities.

[1] However, the 2-hour phosphatidyl ethanolamine II value is 0.14. This fraction contained a very small amount of phosphorus, and the value is of doubtful reliability.

DISCUSSION

The plasmalogen of rat diaphragm is found to be predominantly in the ethanolamine-containing fraction of phospholipids. Webster (6) recently reported the same finding for nervous tissue from several animal species where only traces of choline-containing plasmalogen were present. He also examined rat skeletal muscle where he found only 13% to 16% of the plasmalogen to be in the choline-containing fraction.

Rapport and Lerner (7) reported 11% to 13% of the lipid phosphorus of rat skeletal muscle to be accounted for by plasmalogen. Webster (6) obtained a similar value. The finding, in the experiments reported in this paper, that 9% of the phosphatide phosphorus isolated from rat diaphragm is represented by plasmalogen is in general agreement with the skeletal muscle values found by the above workers.

From Table 1, it may be seen that the diglyceride specific activity exceeds that of each of the phosphatides and of triglyceride at every incubation time for which comparable data are available. With increasing time of incubation, phosphatide and triglyceride specific activity approach that of diglyceride. Diglyceride specific activity remains relatively constant. This is the type of relationship one might expect if the Kennedy scheme (8) for phosphatide and triglyceride synthesis from a common diglyceride precursor were operating in this system. If we assume the Zilversmit (9) precursor-product criterion to hold and apply his

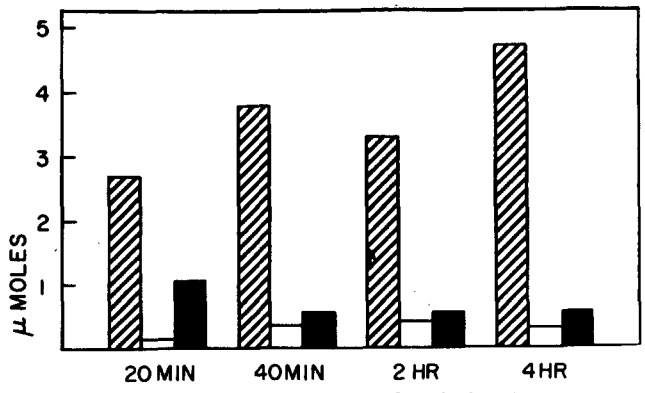

FIG. 3. Plasmalogen content of phosphatide fractions from rat diaphragm after incubation with albumin-bound palmitate-1-C^{14}. The striped bars represent phosphatidyl ethanolamine I; the open bars, phosphatidyl ethanolamine II; the solid bars, phosphatidyl choline. The ordinate indicates total μmoles of aldehyde for the entire fraction. See Figure 2 for amounts of tissue represented.

analysis for the case where precursor specific activity is a constant, turnover times can be estimated from the data in Table 1. Values thus obtained are 2 hours for lecithin, 3 hours for triglyceride, and 8 and 12.5 hours for the two cephalins.

Evidence has been presented recently (10, 11) indicating that S-adenosylmethionine may sequentially contribute three methyl groups to phosphatidyl ethanolamine to form lecithin. Bremer *et al.* (12) have proposed such a pathway as a general mechanism for the synthesis of lecithin. In the experiments reported in this paper, specific activity of lecithin is considerably higher than that of phosphatidyl ethanolamine at all time intervals after incubation with labeled palmitate. This finding makes it very unlikely that the methylation of phosphatidyl ethanolamine plays a major role in the formation of lecithin in rat diaphragm under the conditions employed for these studies. Since an appropriate methyl donor was not added to the system, however, these experiments do not rule out the possibility that the stepwise transmethylation of phosphatidyl ethanolamine to lecithin could be an important process in rat diaphragm under proper conditions.

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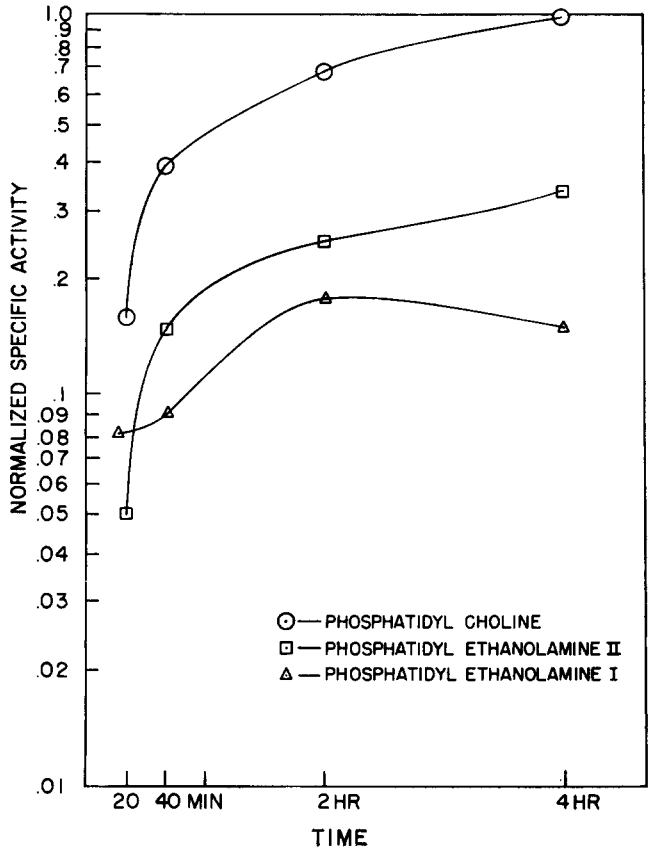

FIG. 4. Relative specific activity of phosphatides of rat diaphragm after incubation with albumin-bound palmitate-1-C^{14}. Values are corrected to the 4-hour phosphatidyl choline value of 24,900 cpm per μmole of phosphorus, which represents an estimated incorporation of 6.5 mμmoles of added fatty acid per μmole of phosphorus.

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TABLE 1. COMPARISON OF DIGLYCERIDE SPECIFIC ACTIVITY WITH THE SPECIFIC ACTIVITIES OF OTHER LIPID FRACTIONS IN RAT DIAPHRAGM INCUBATED WITH PALMITATE-1-C^{14}

Time of Incubation	Specific Activity of Diglyceride	Ratio of Specific Activities: Diglyceride / Triglyceride	Diglyceride / Phosphatidyl Ethanolamine I	Diglyceride / Phosphatidyl Ethanolamine II	Diglyceride / Phosphatidyl Choline
	cpm/μmole				
20 min	7,519	7.5	24.2	38.6	12.1
40 min	5,542	5.3	15.9	9.5	3.7
2 hrs	5,192	2.0	7.4	5.5	2.0