Preferential Labeling of Phosphatidylcholine during Phospholipid Synthesis by Bovine Mammary Tissue

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ABSTRACT

Bovine mammary cells and tissue synthesize de novo the classes of phospholipids, found in mammary tissue and milk, from various precursor molecules. Several short term experiments were carried out in vitro, using labeled precursors, i.e., 1-^{14}C-fatty acids; 2-^{14}C-acetate; U-^{14}C-glycerol; 1,2-^{14}C-choline; 1,2-^{14}C-ethanolamine; 2-^{14}C-serine; and Me-^{14}C-methionine. All the phospholipid classes were labeled. The specific activity of tissue phosphatidylcholine was consistently three to six-fold greater than that of phosphatidylethanolamine. The results indicated that stepwise methylation of phosphatidylethanolamine with labeled methyl group of methionine was occurring to a minor extent, as was a negligible amount of choline exchange. Serine was incorporated into phosphatidylserine and sphingomyelin. Significant quantities of labeled phosphatidylserine were decarboxylated to phosphatidylethanolamine. Apparently phosphatidylcholine was synthesized de novo from choline via phosphorylcholine and CDP-choline. Based on the present observations and other data, it is suggested that there may be two pools of phosphatidylcholine in lactating mammary cells.

Phospholipids are secreted in bovine milk as phospholipoproteins complexed in the fat globule membrane and as discrete lipoprotein particles (1-19). A bovine mammary gland yielding 25 liters milk secretes ca. 10 ± 3 g phospholipids per day which corresponds to an average 5% of the mammary tissue phospholipids. The various phospholipid (PL) classes are secreted in different amounts. Approximately equal amounts of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are secreted in bovine milk (14,15,20). Based on their respective tissue concentrations, PE is expected to have a more rapid turnover rate than PC because its intracellular concentration is ca. 50% that of PC (14,15,17). However in vivo and in vitro studies using labeled fatty acids, glycerol and ^{32}P reveal that PC is the most rapidly labeled mammary and milk PL (17-19, 21-25). Because these results may be explained by the activity of a number of different biosynthetic routes or possibly exchange reactions and because in vivo studies may be confounded by possible extramammary PL precursors (though evidence is to the contrary [25]), studies with mammary tissue have been made to compare the relative rates of PC and PE labeling when radioactive

TABLE I

Incorporation of Long Chain Fatty Acids into Phospholipids by Dispersed Mammary Cells in Vitro[a]

	Incorporation (cpm x 10^3)							
	Cultured tissue				Culture medium			
	Incubation time, hr							
Substrate	1.5	3	6	12	1.5	3	6	12
Sodium-1-^{14}C-myristate	—	115.2	—	134.4	—	19.2	—	41.6
Sodium-1-^{14}C-palmitate	148.8	481.6	740.8	803.2	8.0	14.4	30.4	48.0
Sodium-1-^{14}C-stearate	144.0	224.0	505.6	624.0	4.8	11.2	22.4	24.0
Sodium-1-^{14}C-oleate	352.0	454.4	480.0	528.0	16.0	27.1	20.8	35.2

[a]Mammary cells, equivalent to 5.0 mg protein, were incubated with 40 μM of the sodium salts of each fatty acid (10μCi/mol). Lipids were isolated as described in methods.

TABLE II

Percentage Distribution of Radioactivity in Cellular Phospholipid Classes following Incubation of Mammary Cells with Labeled Fatty Acids (Experiment I)[a]

Phospholipid class	Percentage distribution											
	Myristic-1-^{14}C		Palmitic-1-^{14}C			Stearic-1-^{14}C			Oleic-1-^{14}C			
	Incubation period, hr											
	3	12	1.5	3	6	12	1.5	3	6	12	1.5	3	6	12
Lysophosphatidylcholine	4.2	2.4	2.1	2.8	1.3	0.9	0.3	0.4	0.2	0.2	tr	tr	0.8	0.9
Sphingomyelin	4.9	4.8	3.1	4.2	4.6	5.0	1.0	1.8	2.1	3.5	0.3	0.2	0.6	1.0
Phosphatidylcholine	78.1	80.0	62.8	61.8	62.1	68.5	65.6	64.0	69.7	70.0	71.8	73.9	74.0	76.1
Phosphatidylinositol	4.5	3.2	10.2	9.7	8.0	6.2	18.0	17.0	11.0	8.1	17.1	13.8	11.6	8.3
Phosphatidylserine	tr[b]	tr	2.1	2.3	2.4	2.6	2.0	2.8	2.5	3.0	1.3	1.8	2.1	2.3
Phosphatidylethanolamine	6.4	7.0	16.8	17.0	16.2	15.4	12.1	12.0	13.3	14.1	8.5	9.0	9.7	10.3
Others	1.9	2.6	2.9	2.2	1.4	1.4	1.0	1.2	1.2	1.1	1.0	1.3	1.2	1.1

[a]Phospholipids were separated by two dimensional thin layer chromatography (14). Lipid spots were transferred to scintillation vials and radioactivity determined as described (17).

[b]tr = trace.

precursors were used in vitro.

MATERIALS AND METHODS

Mammary cells from lactating cows were dispersed with collagenase and cultured under aseptic conditions as described previously (26,27). Each treatment contained ca. 5 x 10^7 cells, equivalent to 5.0 mg protein in 6 ml culture medium. The appropriate radioisotopes in sterile buffer solution were added to the culture flasks after dispensing the cells, and the stoppered flasks were incubated for specified periods at 37 C. After incubation the cells were separated from the culture media by gentle centrifugation (300 g for 10 min). The lipids were extracted by the procedure of Folch et al. (28). The lipids were fractionated by two dimensional thin layer chromatography (14). Lipid phosphorus was quantified by the method of Rouser et al. (29). Argentation chromatography was used to separate PC species according to degree of unsaturation (30).

The radioactivity in the lipid spots, identified from known standards (Applied Science Lab., State College, Pa.), was determined by liquid scintillation spectrometry as reported previously (21). Cellular protein was quantified by a microkjeldahl method.

Radiochemically pure (99%) substrates were purchased from New England Nuclear (Boston, Mass.). Chromatographic supplies were obtained from Brinkmann (Westbury, N.Y.) and redistilled solvents were used.

RESULTS

Experiment I. Fatty Acid Incorporation

The fatty acids commonly occurring in bovine milk PL were incubated with the cell preparations as their respective sodium salts and incorporation into phospholipids of tissue and culture media were monitored (Table I). Incorporation of radioactivity into cellular PL increased markedly with time, and labeled PL accumulated in the media also. Though oleic acid ($C_{18:1}$) was incorporated most rapidly initially, the saturated acids palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) were ultimately esterified to a greater extent. The ratio of radioactivity in the media PL showed that, compared to the intracellular PL there was a preferential secretion of PC, especially that labeled with $C_{14:0}$ and $C_{16:0}$. Analysis of the phospholipids revealed that the preponderance of the radioactivity was in the PC (Table II), though all of the PL classes were labeled in all experiments. The respective radioactivities and specific activities (SA) of cellular PC and PE (Table III) varied

TABLE III

Ratios of Radioactivity and Specific Activities of Phosphatidylcholine and Phosphatidylethanolamine Isolated from Mammary Tissue and Culture Media following Incubation with Labeled Fatty Acids (Experiment I)

Source	Ratio of radioactivity, PC/PE (cpm x 10^3)[a]													
	1-^{14}C-Myristic		1-^{14}C-Palmitic				1-^{14}C-Stearic				1-^{14}C-Oleic			
	Incubation time, hr													
	3	12	1.5	3	6	12	1.5	3	6	12	1.5	3	8	12
Cultured tissue	12.2	11.4	3.7	3.6	3.8	4.5	5.4	5.3	5.2	5.0	8.4	8.2	7.7	7.5
Culture media	21.9	15.0	9.4	8.8	11.4	14.0	5.4	5.6	5.6	5.1	—	4.6	—	5.2
Ratio of specific activity, PC/PE[b]	5.7	5.0	1.7	1.5	1.5	1.4	2.4	2.3	2.	2.1	3.6	3.6	3.0	3.3

[a]PC, phosphatidylcholine; PE, phosphatidylethanolamine.
[b]Specific activity expressed as cpm x $10^3/\mu$g phosphorus in specific phospholipid.

with each fatty acid substrate and the disparity in SA from $C_{14:0}$ and $C_{18:1}$ substrates was most marked. The SA ratio decreased with duration of each incubation and in general was lower than those obtained in subsequent experiments using other labeled substrates.

Experiment II. Acetate

The fatty acid labeling data were consistent with observations from in vivo experiments (19). Because of possible differences in intracellular location of PC and PE synthesizing enzymes and fatty acid activation, the relative extent of labeling of PC and PE by fatty acids of endogenous origin was examined (Table IV). Incorporation into both classes increased markedly with time but considerably more labeled fatty acids were acylated into the PC as shown by the SA ratios, which decreased with time. The SA ratios were similar to those obtained in experiment I for myristic acid.

Experiment III. Glycerol

Because the apparent preferential labeling of PC with exogenous and endogenous fatty acids could have been explained by active acyl transferase(s), the cells were incubated with labeled glycerol which was actively incorporated into the phosphoglycerides (Fig. 1). Initially PC contained four-fold more activity than the PE, but this difference decreased with duration of incubation. The SA of PE gradually increased with time, as observed in experiments I and II.

Experiment IV. Choline, Ethanolamine, Serine, Methionine

The above data unequivocally indicated that de novo labeling and probably net synthesis of PC exceeded that of PE. The possibility existed that PE was actually being synthesized within these cells but was rapidly being transformed to PC by transmethylation. To study this the cells were incubated with the series of different PL precursors listed in Table V. Significantly more choline was incorporated compared to the ethanolamine in all experiments. Methionine and serine were utilized in small quantities. Differences in utilization were observed between preparations; however the patterns of incorporation of the various precursors were consistent. Evidence of molecular interconversions, i.e., decarboxylation of phosphatidyl-

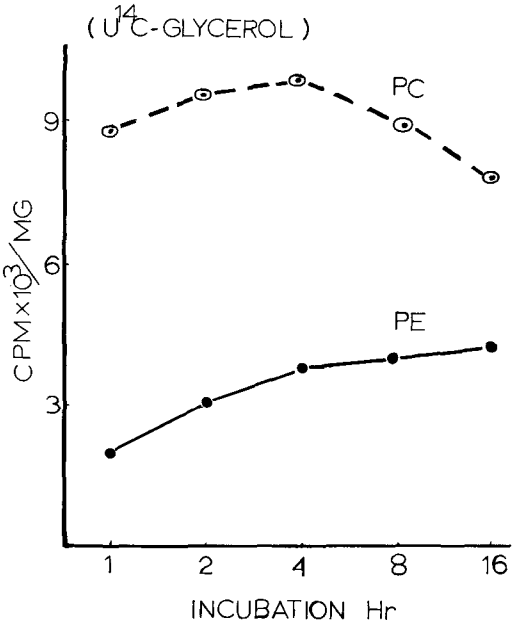

FIG. 1. Relative incorporation of U-^{14}C-glycerol into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of mammary cells. Approximately 5 x 10^7 cells were incubated with 50 μM of U-^{14}C-glycerol (8mc/mol) for specified periods and lipids then analyzed (Experiment II). Specific activity expressed as cpm x 10^3/mg phospholipid.

TABLE IV

Differential Incorporation of Radioactivity into Cellular Phospholipids following Incubation of Bovine Mammary Cells with Na-2-^{14}C-Acetate (Experiment II)[a]

	Incorporation (cpm x 10^3)			
	Incubation period, hr			
Lipid class	2	4	8	24
Total phospholipids	37.0	126.0	256.0	645.0
Phosphatidylcholine (PC)	21.0	80.6	174.1	451.5
Phosphatidylethanolamine (PE)	1.6	7.5	15.3	35.0
Ratio of specific activity, PC/PE	5.2	4.5	4.6	4.1

[a]Tissue dispersions (ca. 5 x 10^7 cells) were incubated with 50 μM Na-2-^{14}C-acetate (specific activity ca. 10mc/nmol) in each treatment. Lipids were analyzed as described in Methods.

serine (PS) to PE, and methylation of PE to PC was obtained (Table VI). The preponderance of the ^{14}C-methyl group of methionine utilized was incorporated into the choline moiety of PC. The serine was incorporated mostly into PS through measurable quantities of the radioactive carbon were transformed into sphingomyelin, PE and some into neutral lipids. These data revealed that while stepwise methylation of PE was occurring in mammary cells, it was small compared to the rate of incorporation of choline into PC and probably could not account for the differential rate of labeling of PC and PE in these mammary cells when common precursors were used as substrates.

Experiment V. Choline Exchange

In the previous experiments the relatively greater incorporation of labeled choline into PL could perhaps be attributed to active base exchange within the cells, especially in the PC molecules. However the importance of this mechanism became doubtful following a series of experiments in which cells that had been incubated for specific times with labeled choline to attain a determined PC specific activity were then reincubated in media containing an excess of cold choline (40 mM), and changes in SA of choline phospholipids were monitored (Table VII). As in previous experiments the ^{14}C-choline was incorporated linearly with time, mostly into the PC (A-D). Incubation of the cells containing ^{14}C-choline labeled PC with excess choline revealed little evidence of exchange in the PC except between the 6 and 12 hr interval, when SA decreased, i.e., C compared to C^1. The SA of the lysoPC from cellular lipids decreased after the 3 and 6 hr incubations, indicating some loss of labeled choline or acylation of lysoPC to PC. The small decline in SA of the cellular PC has been observed in subsequent experiments and indicates that, while choline exchange may occur, the magnitude of this exchange in bovine mammary cells does not explain the apparent preferential synthesis of PC. The data in Table VII indicated that labeled PL of high SA was secreted into the culture media in these experiments, especially in the initial periods. These data indicated that de novo PC synthesis was occurring in these cells, apparently via the classical enzymatic pathway (31).

TABLE V

Incorporation of Different Radioactive Precursors into Phospholipids by Bovine Mammary Tissue in Vitro (Experiment IV)[a]

	Radioactivity incorporated (cpm x 10^3)			
	Substrate			
Incubation, hr	1,2-^{14}C-Choline	1,2-^{14}C-Ethanolamine	Me-^{14}C-Methionine	3-^{14}C-Serine
1	21.5	3.1	0.40	1.8
3	48.1	10.2	0.60	3.9
6	78.3	18.9	0.89	6.2
12	194.6	65.7	2.20	14.6

[a]Each treatment contained ca. 5 x 10^7 cells and 50 μM labeled substrate. Specific activities of substrates were 1,2^{14}C-choline and 1,2^{14}C-ethanolamine 2μCi/μmol; Me-^{14}C-methionine and 3-^{14}C-serine; 5μCi/μmol. Average data from two experiments.

Analysis of the distribution of the radioactivity in the various molecular species of cell PC following incubation of mammary cells with choline showed that the PC molecules containing two double bonds contained the greatest quantity of incorporated choline (Table VIII). The labeling in the monounsaturated PC molecules decreased, whereas that in the triunsaturated species increased with incubation time.

DISCUSSION

The utilization of free fatty acids by lactating mammary tissue for synthesis of glycerolipids both in vitro and in vivo has been well documented (19,22,32-36). Most of the labeled fatty acids associated with the phospholipids were acylated in PC (18,19,22). Endogenous fatty acids were mostly acylated into secretory triglycerides (27,32-37); however those in the phospholipids, i.e., myristic and palmitic acid, are preponderantly found in the PC class (34,35). The preferential labeling of PC may be explained by very active acyl transferase enzyme(s), which have a high specificity for choline phosphatides. These are very active in the plasma membrane of liver cells (38). While these enzymes are active in mammary tissue (Gross and Kinsella, unpublished data), their class specificity has not been determined and the present data would preclude this mechanism as accounting for the greater labeling of PC in all experiments. The preferential incorporation of glycerol into PC compared to PE by bovine tissue has been reported and discussed (21).

The disparity between PC and PE labeling was also observed when labeled choline and ethanolamine were used as substrates. In the present studies stepwise methylation of PE to PC using the methyl group of methionine (39-44) was negligible. This is a major pathway in rat liver (42). The conversion of PS to PE and of some PE to PC has not been reported in ruminant mammary tissue. Presumably these interconversions occur via the mechanisms of decarboxylation and stepwise methylation (31,41,43). Direct base exchange of choline with the choline of PC was apparently quite limited, even in the presence of excess choline. This was consistent with reports for other mammalian tissues, e.g., liver (45), intestine (46) and cultured mast cells (47), and indicated that the CDP-choline pathway was operating (31.48).

The variation in distribution of the ^{14}choline among PC molecular species in mammary cells may indicate that these have different turnover rates, as shown for PC species in several animal tissues (43-45, 49-51). The mo-

TABLE VI

Percentage Distribution of Radioactivity in Phospholipids following Incubation of Bovine Mammary Tissue with Labeled Precursors (Experiment IV)

Lipid class[a]	Substrate: 1,2-^{14}C-Choline				Me^{14}C-Methionine				1-2-^{14}C-Ethanolamine				3-^{14}C-Serine			
	Incubation period, hr: 1	3	6	12	1	3	6	12	1	3	6	12	1	3	6	
LYL	2.3	2.6	3.5	2.3	3.3	2.8	2.9	1.9	0.6	0.2	0.3	0.3	---	0.4	0.4	
SPH	0.7	1.0	2.5	3.9	1.9	2.3	1.9	1.7	0.7	0.5	0.4	0.8	10.4	11.8	10.9	
PC	96.3	96.2	94.0	93.8	70.5	74.3	83.0	89.1	6.8	2.6	4.6	5.9	3.0	2.0	1.3	
PS	---	---	---	---	---	---	---	---	---	---	---	---	72.7	57.4	52.6	
PE	---	---	---	---	1.1	1.3	1.2	0.3	89.9	93.6	91.9	91.0	6.9	20.9	25.3	
MPE	---	---	---	---	23.2	19.3	11.2	7.0	1.0	0.6	0.8	0.9	---	---	---	
NL	0.7	0.2	---	---	---	---	---	---	1.0	1.5	2.0	1.1	7.0	7.5	9.5	

[a] Abbreviations: LYL, lysophosphatidylcholine; SPH, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; MPE, methylated phosphatidylethanolamine; NL, neutral lipids.

TABLE VII

Specific Activities of Phospholipids Isolated from Cells and Culture Medium following Incubation of Bovine Mammary with 1,2^{14}C-Choline and an Excess of Choline (Experiment V)[a]

	Specific activity (cpm x 10^3/μg lipid P.)						
	Incubation time, hr						
	1	3	6	12	3	6	12
	Flask						
Lipid class	A	B	C	D	A^1	B^1	C^1
Total phospholipids	0.67	1.83	2.67	3.98	0.95	1.90	2.55
Cell phospholipids	0.66	2.30	3.92	6.22	1.04	2.44	3.28
Medium phospholipids	0.68	1.36	1.43	1.75	0.87	1.39	1.82
Cell lipids							
Phosphatidylcholine	1.36	5.16	8.08	18.01	1.77	5.73	7.32
Lysophosphatidylcholine	0.25	0.53	1.10	1.15	0.29	0.37	0.57
Sphingomyelin	0.10	0.56	0.83	0.95	0.22	0.36	0.23

[a]All flasks, containing 5 x 10^7 cells and ca. 50μM 1,2-^{14}C-choline (2 mc/mM), were initiated simultaneously. Flasks denoted A, B, C, D were terminated at 1,3,6,12 hr, respectively. An excess of cold choline (40 mM) was aseptically added to flasks A^1, B^1 and C^1 at 1, 3 and 6 hr, respectively. These flasks were then sequentially terminated at 3, 6 and 12 after initiating the experiment. Lipids were extracted and analyzed as described in Methods. To detect choline exchange specific activities of phospholipids from flasks containing only radioactive choline were compared to those from those flasks containing excess choline that had been incubated for identical periods with labeled choline, i.e., A with A^1, B with B^1 and C with C^1, respectively.

lecular species containing the most radioactivity, i.e., diunsaturated PC, coincides with the preponderant species of PC found in milk (13) and this labeling pattern is similar to that found in other animal tissues (45). This may reflect the preponderant species of diglycerides available in ruminant mammary tissue, though available analytical data would suggest a preponderance of diglycerides with only one double bond. However the knowledge that CDP choline-diglyceride transferase is not markedly substrate specific (51) and the presence of acyl transferase(s) in mammary tissue may account for most of the radioactivity being in the PC species with two unsaturated bonds.

It is difficult to explain why more labeled choline was incorporated compared to ethanolamine, since lactating tissue secretes equal quantities of both PC and PE in milk (14,15). Possibly the entry rate of ethanolamine into the cells was much lower than that of choline, or the mammary cells had a high endogenous ethanolamine concentration compared to choline and a rapid dilution of labeled ethanolamine occurred. This is being studied. Low choline concentrations enhance the activity of the enzymes of PC synthesis in guinea pigs (52). Conceivably in lactating mammary tissue, choline is a limiting metabolite and hence used avidly when available, because the associated enzymes are very active. Choline kinase is moe active than ethanolamine kinase in bovine mammary tissue (Infante and Kinsella, unpublished, 1972). Enhanced PC synthesis is associated with intracellular membrane proliferation evoked by drugs and hormones (53-56), and possibly, in the actively secreting mammary cell, lecithin-rich intracellular membranes are

TABLE VIII

Percentage Distribution of Radioactivity in Different Phosphatidylcholine Molecules following Incubation of Bovine Mammary Cells with 1,2-^{14}C-Choline (Experiment V)

		Period of incubation, hr			
Molecular species of phosphatidylcholine	Wt %[a]	1	3	6	12
Saturated	10.2	0.4	0.1	—	0.1
Monounsaturated	44.1	31.9	35.7	20.0	14.0
Diunsaturated	27.3	48.2	51.0	50.0	48.0
Triunsaturated	10.4	15.0	8.6	27.0	20.0
Tetraunsaturated	4.4	2.5	2.8	10.0	14.0
Polyunsaturated	3.6	2.0	1.8	3.0	3.9

[a]Nutter and Privett (13).

being rapidly turned over with concomitant preferential synthesis of PC, because of their high PC content (15,57-60).

The bovine mammary gland at peak lactation secretes an average of 5% of its phospholipids into milk daily. This is comparable with turnover rates of 3.8 and 4.8% for bovine mammary protein and RNA, respectively (61). Based on concentrations in secretory tissue and in milk (14,15,19), the phospholipid classes have approximate turnover times of 29, 14 and 11 days for PC, PE and sphingomyelin, respectively. The apparent long turnover time of tissue PC compared to PE and SPH is noteworthy. Whether or not there is a relationship between the fact that significant quantities of the PE and SPH secreted in milk derived from the plasma membrane (in contrast to the PC) and their respective turnover times remains to be determined (15,19,57).

Milk phospholipids are secreted as phospholipoporteins surrounding the fat globule (1-20) and as lipopoprotein particles in milk serum (1,2,8,19,62). The latter may contain up to 30% of milk phospholipids, which are rich in PC (1,2,13,15,60). Conceivably the phospholipids of the fat globule membrane and the lipoprotein particles are secreted separately, with the latter having a much faster turnover rate. Keenan et al. (57,60) alluded to such a mechanism, suggesting that much of the water soluble components of milk are secreted in secretory vesicles derived from the endoplasmic reticulum via the membranes of the Golgi apparatus. These components, i.e., the lactose, proteins and possibly lipoprotein particles, are secreted more rapidly than milk fat globues (5,17,57,60). The secretion of milk lipoproteins via the Golgi may be analogous to the secretion of serum lipoproteins (63).

These speculations and the present results suggest that two discrete metabolic pools of PC may exist in the secreting mammary cell, i.e., a pool with a rapid turnover that is involved in intracellular metabolism, membrane replenishment and PC exchange reactions. PC molecules from this pool may behave as an interfacial surfactant for growing fat droplets (59) and may be involved in the formation of Golgi membranes and secretory vesicles, as suggested by Keenan et al. (57,60). Evidence for two pools of PC with different turnover rates has been reported in mast cells (47) and liver (44), and Gurr et al. (64) reported that during chylomicron formation in cat intestine (a process analogous to milk fat secretion) the rate of synthesis and perhaps turnover of PC is accentuated. Several workers have shown that active PC exchange occurs in cells between organelles (65-67).

Conceivably similar reactions are occurring in mammary cells. Following in vivo experiments, Patton and Keenan (19) showed that labeling of PC was much greater than PE. The PC secreted in milk lipoproteins had a 10-fold greater SA than those of the fat globule membrane and, significantly, the SA of PC in milk lipoproteins was six-fold greater than the SA of the corresponding PE. The data support previous in vitro studies showing that PC labeling exceeds that of PE (18,19,21,22), and the disparities of SA between PC and PE were of the same magnitude as observed in the present experiments.

Another pool of PC may be involved with replenishment of the plasma membrane (21) and be secreted more slowly as a component of the fat globule membrane (5,57,60). McCarthy (35) reported that mammary PL labeled with palmitic acid showed biphasic decrease in SA, i.e., a rapid phase of secretion into milk and a more prolonged phase of secretion during which SA decreased slowly. Further support for two discrete pools was recently provided by Patton et al. (68) from specific activity curves obtained following injection of 32P into lactating goat.

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