Metabolism of Phospholipid in Mammary Gland: I. The Supply of Phospholipid for Milk Synthesis in the Rat and Goat

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ABSTRACT

Data presented in this study demonstrate that under normal physiological conditions, milk phospholipids in the rat and the goat originate predominantly, if not totally, by de novo synthesis within the mammary gland. Evidence to support this has been obtained for the goat by measurement of P^{32}-phosphate incorporation into milk phospholipids, and in the rat by measurement of P^{32}-phosphate incorporation and by feeding radioactive phospholipid to measure the incorporation of serum phospholipids into milk. The latter experiment showed that the fatty acid portion of the dietary phospholipid can readily be utilized by the mammary gland for triglyceride synthesis, but that the contribution of the serum phospholipid "backbone" to milk phospholipid is minimal.

INTRODUCTION

Previous data on the role of serum phospholipids in milk production by mammary gland have indicated that the circulating serum phospholipids play no direct part in the secretion of milk phospholipids by this tissue (1-4). Arteriovenous difference studies across mammary gland (1,2) indicate no significant uptake of phospholipid.

However a consideration of serum and milk phospholipid levels and of blood circulation time demonstrates that all milk phospholipids could be derived from blood supplies without a measurable drop in the level of serum phospholipids across the gland. For the lactating rat the levels of phospholipid in milk and serum were determined (5) as 130 mg/100 ml and 150 mg/100 ml respectively. If it is assumed that 10% of the total blood flow passes through the mammary gland, then from the cardiac output (35 ml/min [6]), total blood volume (6.7% body weight [7]), hematocrit of 0.45 (5), and the milk production rate (26 ml per day [8]), it can be calculated that the mean mammary serum flow to milk yield in a 300 g rat is approximately 87:1. From this datum, the arteriovenous difference across the mammary gland if the serum phospholipid provided all of the milk phospholipid would be 1.00%.

The corresponding data for the lactating goat are: serum phospholipid 160 mg/100 ml (9), milk phospholipid 37 mg/100 ml (10), and mean mammary plasma flow to milk yield 353:1 (9). By the same calculation as above, the corresponding arterio-venous difference if the serum provided all of the milk phospholipid would be 0.07%.

It has been suggested (3) that serum phospholipids are stabilizers of serum lipoproteins, but can also act as carriers of specific fatty acids to the mammary gland. However, experiments with P^{32}-labelled lipoproteins have demonstrated that the mammary glands of the cow (3) and rabbit (4) take up serum phospholipids. In the rabbit, no activity was recovered in the milk (4). Instead, the absorbed phospholipid was broken down in the tissue.

This study represents an attempt to measure the relative contributions of serum phospholipid and de novo synthesis within the mammary gland to milk phospholipid in the lactating rat and goat.

MATERIALS AND METHODS

Animals

All rats used were lactating females (250-400 g), of Sprague-Dawley strain, each nursing six pups, and experiments were performed between 10-18 days post partum. The rats were maintained on normal laboratory diet and water ad lib. The young were left with the mother and allowed to suckle at will for the duration of the experiment.

The goat used was from the department herd and was producing 1200 ml of milk daily at the start of the experiment. The animal was maintained on a normal hay-grain ration and water ad lib.

Radioactive Isotopes

Radioactive phosphorus (Carrier-free P^{32}-orthophosphoric acid in 0.02N HCl) was obtained from New England Nuclear, Boston, Mass. The acidity of the solution was neutralized before use. U-C^{14}-phosphatidyl-choline 1800 $\mu c/\mu$mole) was also obtained from New England Nuclear (Cat. No. 588, batch 526-061). The isotope contained 1.8% of the

activity as free fatty acid, and 2% as lysolecithin. It was used without further purification.

Administration of Isotope

Radioactive phosphorus was administered intravenously. In the experiment with rats the first animal was given 25 μc of the isotope and the second rat 10 μc. In each case the isotope was administered in 0.2 ml saline. In the goat experiment the animal was given 200 μc of the radioactive phosphorus injected in 4 ml of dilute phosphate buffer pH 7.

Uniformly labelled C^{14}-phosphatidyl choline (5 μc) was administered to one rat by gavage in 0.6 ml corn oil.

Collection of Samples

Blood samples were collected at various times after the administration of the isotopes—in the rat from the tail under ether anesthetic, and in the goat by venipuncture. Milk samples were collected from the goat by hand expression. In the rat, milk samples were obtained under slight suction after intramuscular injection of 0.1 USP unit oxytocin, usually at the same time as blood collection.

Extraction of Lipids

Rat Experiments: Cream and skim milk fractions were obtained from whole milk (0.5-2.0 ml) by centrifugation (2.0×10^4 x g min.). The cream samples were dissolved in 20 volumes of chloroform-methanol (2:1 v/v), and the skim-milk fractions added to 4 volumes of chloroform-methanol (1:3 v/v) to obtain a one-phase system. The skim-milk samples were then taken to dryness below 50 C under nitrogen (using acetone to remove water by co-evaporation), and the lipids dissolved in chloroform methanol (2:1 v/v).

Samples of blood serum, prepared from whole blood by centrifugation at 4.0×10^4 x g min, were treated in the same way as skim-milk fractions.

Goat experiment: The milk samples (40 ml) were cooled in ice, then centrifuged at 3.7×10^4 g/min, and the skim-milk drained from under the fat layer. Serum samples were prepared from whole blood as described above.

Skim-milk, cream and sera were extracted by the Roese-Gottlieb procedure (11). The extracts were evaporated to dryness under vacuum at 38 C, and the lipids taken up in a small quantity of chloroform.

Separation of Lipid Components

The various lipid classes in the extracts were separated in two ways. For the rat extracts,

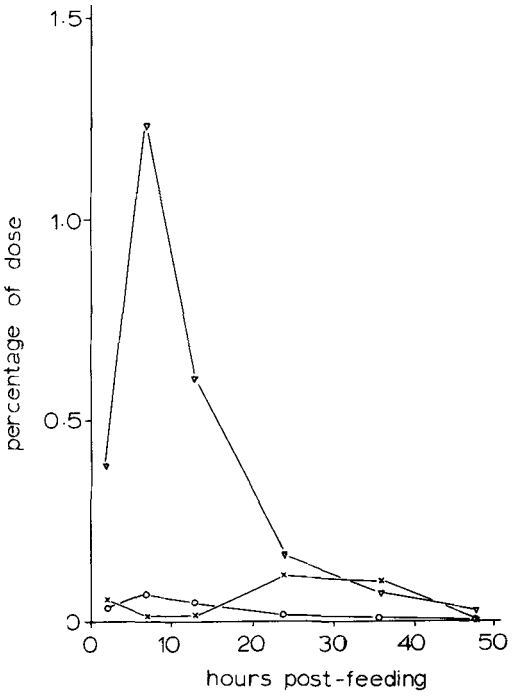

FIG. 1. Serum and milk radioactivity levels after $U^{14}C$-phosphatidyl choline feeding. The lactating rat was fed 5 μc of isotope in 0.6 ml of corn oil by gavage. Key: -▽- and -○- radioactivity equivalent to 1 ml of whole milk in cream and skim respectively, -x- total radioactivity per ml serum.

where the amount of lipid available was small, separation was effected by thin layer chromatography on Silica Gel F_{254} plates (E. Merck, Darmstadt, Germany), using for neutral lipids the solvent system: petroleum ether (boiling range 30-60 C): diethyl ether: acetic acid (70:30:1 v/v/v), and for polar lipids the two-dimensional system of Parsons and Patton (12). (First solvent, chloroform-methanol-water-ammonia [130:70:8:0.5 by vol]; second solvent, chloroform-acetone-methanol-acetic acid-water [100:40:20:20:10, v/v]). The lipids were extracted from the silicic acid after separation by scraping the gel off the plate and extracting several times with small quantities of polar solvents, e.g., diethyl ether for neutral lipids, methanol for polar lipids.

In some instances the location of the C^{14}-radioactivity in lipid classes was determined by autoradiography (13).

For the goat extracts, where larger quantities of material were available, extracts were chromatographed on silicic acid columns (14) to separate the phospholipids from neutral lipids. Portions of the phospholipid from the skim-milk and cream fractions at 31 hr were chro-

TABLE I

Radioactive Composition of Lipid Fractions -U-^{14}C-Phosphatidyl Choline Experiment

Sample	Percentage total radioactivity in each fraction				
	PL[a]	DG	Chol + FFA	TG	CE
Serum 2-7 hr	51.0	12.0	11.0	19.0	8.0
Serum 24-36 hr	44.0	10.0	35.0	3.0	7.0
Cream 7 hr	0.6	2.4	1.0	93.9	2.1
Skim 7 hr	2.2	2.5	1.9	92.4	0.7

[a]Abbreviations: PL, phospholipid; DG, diglyceride; Chol + FFA, cholesterol + free fatty acid; TG, triglyceride; CE, cholesterol ester.

matographed in the two-dimensional system described above and the distribution of P^{32} in the phospholipids was studied by autoradiography (13).

Radioactivity Determination

Samples of the various fractions from the rat experiments (C^{14} and P^{32}) were counted in methanolic Instagel scintillation fluid (Methanol: Instagel [Packard Instrument Co. Inc., Downers Grove, Ill. 60515] 1:3 v/v). This system was also used for determining the radioactivity in various lipid classes from thin layer chromatography by scraping the gel from the area and counting directly in scintillator. For colorless samples, chemical quenching was corrected by the channels-ratio method. For color-quenched samples (C^{14} only) internal standard (U-C^{14}-toluene [Packard]) was added, and the samples recounted to correct for this effect.

In the goat experiment (P^{32}) the results were not corrected for quenching and are expressed as counts/min.

To compensate for the decay of P^{32} all samples were either counted at the same time (goat experiment) or counted in conjunction with a P^{32} standard solution, and the results corrected to equivalent activity at the time of injection (rat experiments).

Hydrolysis Studies

In some instances lipids from the C^{14}-experiment were hydrolyzed by incubation in ethanolic KOH (4N KOH in 30% ethanol) at 80 C for 1 hr. After acidification with c.HCl the saponifiable material (fatty acids) was extracted with 3 x 5 volume petroleum ether. After evaporation of the solvent the fatty acids were counted in methanolic Instagel.

The aqueous residues (nonsaponifiable material) were counted whole in 5 volumes of methanolic Instagel, radioactivity due to K^{40} in the medium (from the ethanolic KOH used for saponification) being corrected by using the appropriate blanks.

Other Determinations

Phosphorus, total, inorganic and phospholipid, was assayed either by the method of Fiske and Subbarow (15) or that of Rouser et al. (16).

RESULTS AND DISCUSSION

Metabolism of Uniformly-labelled C^{14}-Phosphatidyl Choline by Lactating Rat Mammary Gland

Uniformly labeled phosphatidyl choline was administered by a dietary route into the serum of a lactating rat, and the radioactivity recovered in the serum and milk examined by the methods described above.

Practically all of the administered radioactivity was absorbed by the animal (total fecal activity 1%). Approximately 16% of the administered isotope was secreted into the milk over the 4 days of the experiment. At sacrifice the liver and mammary glands contained 0.05% and 0.15% of the administered dose respectively. The location of the rest of the activity was not determined.

The results (Fig. 1, Table I) show that the cream fraction contained the majority (93%) of the milk radioactivity, mainly in the form of triglyceride (94%). The activity in the skim-milk fraction also was mainly as triglyceride (92%). Analysis of these lipids by hydrolysis (see Materials and Methods) showed that the fatty acid part of the triglycerides contained 98% and 93% of the activity respectively. The origin of the radioactive fatty acids on these triglycerides is presumably the plasma lipids. Since all the serum lipid classes were radioactive (Table I) we cannot determine from this datum which lipid fraction of the serum was utilized by the mammary gland in this experiment.

Very little activity was recovered in the phospholipid fractions of the milk (Table I).

Analysis of this fraction from cream by hydrolysis showed all of the activity associated with the acyl residues on the lipid. In contrast the activity in the skim-milk phospholipid was distributed between the acyl (45%) and non-saponifiable (55%) portions of the molecule.

Bearing in mind that the original isotope (uniformly-labeled, acyl groups mainly C16 and C18 species) contained only 19-20% of its activity in the "backbone" of the phospholipid, the total amount of milk phospholipid which could have been derived from this portion of the administered isotope (by way of serum phospholipids) can be calculated. The total radioactivity associated with the skim-milk phospholipid over the experimental period was 1% x 2.2% of the original dose (from the text and Table I). Of this activity 55% was associated with the "backbone" of the phospholipid (see text). The contribution of the "backbone" of the dietary phospholipid to milk phospholipid was therefore approximately 0.05%. This demonstrates that the lactating rat mammary gland does not utilize pre-formed exogenous (serum) phospholipids to supplement its supply of phospholipid for milk production.

Metabolism of P^{32}-Phosphate by Lactating Rat Mammary Gland

Two experiments were performed in which P^{32} (as phosphate was injected intravenously into lactating rats, and the radioactivity in blood and milk determined at various times after injection. The results from one experiment are presented in Figure 2. Similar data were obtained in the second experiment (not shown).

In both experiments the administered isotope was rapidly taken up by the mammary gland and secreted into milk. Using the factor of 26 ml/day for milk production (8), then during the experimental period 32% (expt. 1) and 40% (expt. 2) of the administered radioactivity was recovered in the milk.

Most of the radioactivity in the milk was in the form of inorganic P^{32}-phosphate. However some radioactivity was detected in milk phospholipids: 1.9% and 2.5% of the total milk radioactivity in experiments 1 and 2 respectively. The maxima for radioactivity in milk phospholipid and total milk phosphorus occurred at approximately the same time (6 hr) after injection (Fig. 2).

If the inorganic phosphate in milk and the phosphorus necessary for milk phospholipid synthesis were provided from a single pool of phosphate in the tissue, then one would expect that the ratio of the activity in each fraction, i.e., 1.9% and 2.5% as above, would be similar

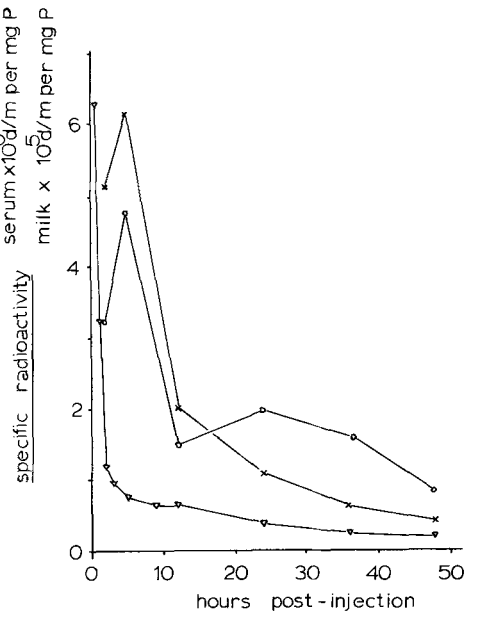

FIG. 2. Serum and milk radioactivity in the rat after P^{32}-phosphate injection. The rat received 25 μc sodium phosphate-P^{32} in saline (0.2 ml) by intravenous injection (caudal vein). Key: -x-, specific activity of non-lipid-P^{32} in milk; -○-, specific activity of lipid-P^{32} in milk; -▽-, specific activity of inorganic P^{32} in serum.

to the ratios of the amounts of phosphorus in each fraction. Values were determined therefore for inorganic phosphorus (2.1 mg/ml) and phospholipid phosphorus (0.04 mg/ml) in rat milk. The mass ratio of phospholipid phosphorus to total phosphorus in rat milk is therefore 1.9%, a value in close agreement to that obtained for the distribution of radioactivity.

Since serum phospholipids (or at least the "backbone" of the molecule) are not utilized to any great extent by the lactating rat mammary gland (demonstrated in Metabolism of Uniformly-labelled . . ., above) for milk phospholipid, these results on the incorporation of inorganic P^{32} into milk phospholipids show that milk phospholipids must be derived mainly (if not totally) by de novo synthesis from inorganic phosphate within the mammary gland.

Metabolism of P^{32}-Phosphate by Lactating Goat Mammary Gland

A lactating goat was injected intravenously with 200 μc inorganic phosphate-P^{32}. Milk and blood samples were collected and fractionated as described (see Materials and Methods).

As shown in Figure 3, in the first 12 hr of

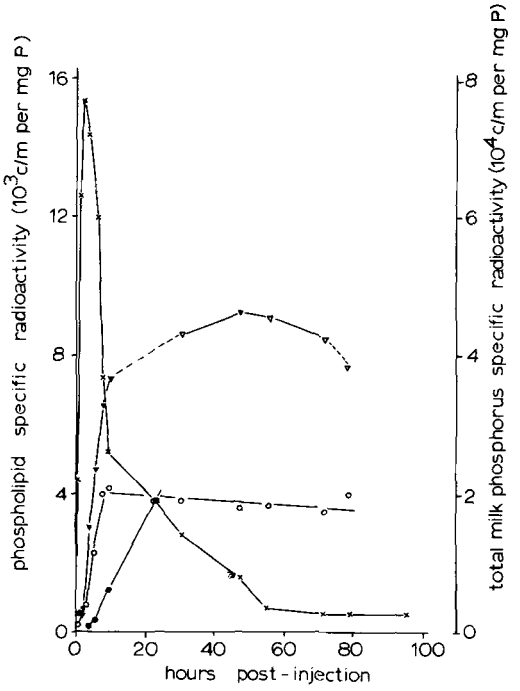

FIG. 3. Serum and milk specific radioactivity in the goat after P^{32}-phosphate injection. The goat received 200 μc orthophosphate-P^{32} in 4 ml of diluted phosphate buffer (pH 7) by intravenous injection (jugular vein). Key: -x-, total P^{32} in milk; -o-, skim-milk lipid P^{32}, -▽-, cream lipid P^{32}; -●-, serum lipid P^{32}.

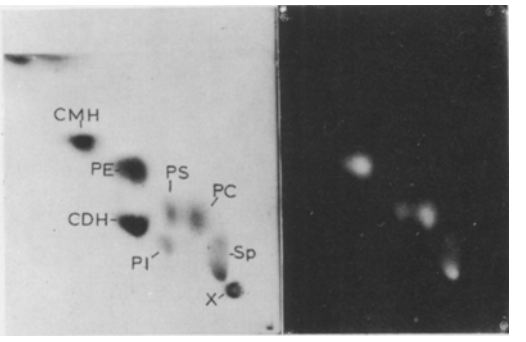

FIG. 4. Autoradiography of goat milk P^{32} phospholipids. Left: Two-dimensional thin layer chromatogram of polar lipids from milk fat globules 31 hr following intravenous injection of P^{32} (phosphate) into a goat. Right: Autoradiogram of the chromatogram. *Abbreviations:* CMH, cerebroside monohexoside; CDH, cerebroside dihexoside; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol Sp, sphingomyelin; X, lactose. Essentially identical chromatographic and autoradiographic data (not shown) were obtained for the skim-milk polar lipids.

the experiment the major form of milk radioactivity was as inorganic phosphate. However after the 12th hr, progressively more of the total milk radioactivity was associated with the skim-milk and cream phospholipids. Autoradiography of samples of cream (Fig. 4) and skim-milk phospholipids from the 31-hr milk indicated that all of the phospholipids normally present in goat milk were synthesized by the goat mammary gland from inorganic phosphate.

By extrapolation of the specific activity results for milk phospholipids past 96 hr, it was estimated that 2.1% of the total milk radioactivity would have been associated with the phospholipid fractions. Since total milk phosphorus is 685 μg/ml (17) and total milk phospholipid phosphorus is 14.7 μg/ml (10), then the corresponding mass ratio (phospholipid phosphorus-total phosphorus) is 2.1%, a value in excellent agreement with the ratio of radioactivity.

We can assume therefore that as with the lactating rat, a single pool of phosphate in the mammary gland is responsible both for supplying inorganic milk phosphate and for supplying the phosphate necessary for milk phospholipid synthesis. Since the specific activity of serum phospholipid is at all times lower than that of cream phospholipid, and for the first 15-20 hr lower than that of the skim-milk phospholipid, it is extremely unlikely for the milk phospholipid to be derived to any great extent from serum phospholipids transported across the mammary gland.

From the positioning of the maximum activities of total milk phosphorus (1.5-6 hr) and milk phospholipid phosphorus (8-80 hr) we must assume that in the goat (but not in the rat, see Metabolism of P^{32}-phosphate . . ., above), phospholipid synthesized within the mammary gland from inorganic phosphate is incorporated into a phospholipid pool within the tissue, and that the delay in buildup of milk phospholipid radioactivity (and slow turnover time) is caused by a gradual turnover of this phospholipid pool within the tissue.

The results from this experiment demonstrate that as with the lactating rat (Metabolism of Uniformly-labelled . . . and Metabolism of P^{32}-Phosphate . . ., above) the lactating goat mammary gland does not utilize circulating pre-formed serum phospholipids for milk production. Instead the gland utilizes a single pool of phosphate within the tissue both for the secretion of inorganic phosphate into the milk and for supplying the phosphorus necessary for milk phospholipid synthesis within the mammary gland.

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