Chylomicron Margination, Lipolysis, and Vitamin A Uptake in the Lactating Rat Mammary Gland: Implications for Milk Retinoid Content

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We have reported previously that the concentration of vitamin A (VA) in the milk of lactating rats varies with dietary VA intake, even when plasma retinol concentration is unaffected. In the current study, we investigated the role of lipolysis in the uptake of chylomicron (CM) VA into mammary tissue of lactating rats and estimated the proportion of CM-VA that is associated with the mammary gland during CM clearance. Chylomicrons containing [^{3}H]VA, mainly as retinyl esters, were prepared in donor rats and administered intravenously to lactating recipient rats. Chylomicron VA rapidly disappeared from plasma and appeared in mammary tissue (maximum within 2–3 mins), followed by a decline. Concomitantly, uptake by liver increased continuously, reaching a plateau within 20–30 mins. Active lipolysis in mammary tissue was necessary for rapid VA uptake, as significantly less CM-VA was recovered in mammary tissue of postlactating rats than of lactating rats, after heparin treatment in lactating rats, or after injection of preformed CM remnants in lactating rats. [^{3}H]Vitamin A uptake by mammary tissue increased linearly with CM-VA dose over a 150-fold dose range (R^2 = 0.972, P = 0.0001), suggesting a high capacity for uptake and apparent first-order assimilation of CM-VA during CM remnant formation *in situ*. Model-based compartmental analysis using WinSAAM predicted that ~42% of CM-VA marginated, that is, were temporarily removed, from plasma to the mammary glands during lipolysis and that a total of 3.8% of CM-VA was transferred to mammary tissue. The model-predicted $t_{1/2}$ for CM remnants was 3.04 mins. The metabolism of CM-VA in the lactating mammary gland, in proportion to VA absorption and CM-VA contents, may explain how milk VA concentration varies even when plasma retinol levels are unchanged. The mechanism of CM margination and mammary gland uptake described here for VA may be similar for other lipophilic substances. Exp Biol Med 229:46–55, 2004

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It has long been known that the placental transfer of vitamin A (VA) from mother to developing young is quite limited (1). As a result, an adequate transfer of VA from mother to young during lactation is needed to increase the neonate's VA reserves prior to weaning. The mother's ability to secrete an adequate amount of VA in her milk depends on several factors, including her own VA status (2–7 and references therein). Vitamin A status is closely related to liver VA concentration (8), although VA is contained in many tissues. Vitamin A is released from liver into the circulation as retinol bound to its specific transport protein, retinol-binding protein (RBP) (9). Numerous studies have shown that the concentration of retinol-RBP is maintained within a narrow range, independent of liver VA concentration, until liver VA stores become severely depleted and thereby limit the availability of retinol for secretion as holo-RBP (reviewed in Refs. 8, 10).

Epidemiological and clinical studies in lactating women (4–7) and experimental studies in rats (2, 3) have shown that dietary VA influences the quantity of VA present in breast milk. In previous studies conducted in lactating rats, we reported that milk VA concentration was significantly higher in rats fed a VA-supplemented diet even though plasma retinol concentrations were not affected (2, 3).

At present, the physiological mechanisms responsible for the transfer of VA into the lactating mammary gland and its secretion into milk are not fully understood. It is

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likely that both chylomicrons (CM), intestinal lipoproteins formed during lipid absorption that contain VA mainly as retinyl esters (11, 12), and circulating holo-RBP (9) contribute VA to milk (13–15). The proportion of mammary gland and milk VA that is contributed by each of these sources is still uncertain, and the importance of long-term VA status (e.g., maternal liver VA reserves) compared with recent dietary intake (CM-VA content) has still not been defined. However, recently, it was estimated that the proportion of milk VA contributed by CM versus holo-RBP was 32% in rats fed a low VA diet and 52% at a higher intake (15).

The lactating mammary gland would seem ideally suited to obtain VA from CM because mammary tissue is a major site of lipoprotein lipase (LPL) activity during lactation (16, 17). After CMs enter the circulation from the intestinal lymph ducts, their triglycerides (TG) are rapidly metabolized by LPL that is present primarily on the capillary endothelium of many tissues, including cardiac and skeletal muscle and adipose tissue (18–20). Shortly before the onset of lactation, LPL activity is induced in the mammary gland (21, 22) and activity remains high as long as suckling continues (16, 23). Chylomicrons are bound through their constituent apolipoproteins to LPL during lipolysis, after which most of the newly formed CM remnants are released back into plasma for uptake by liver and, to a lesser extent, by extrahepatic tissues (24). Although the regulation of LPL expression in the lactating mammary gland and its role in CM triglycerides metabolism have been investigated extensively (reviewed in Refs. 16, 17, 20), little is known concerning the role of this enzyme in the metabolism and uptake of VA into mammary tissue and milk.

In the present studies, we studied the uptake of CM-VA in the mammary gland of lactating rats and the role of lipolysis in uptake by this tissue. The results showed that CM-VA is associated with mammary tissue within a few minutes of iv administration but that only a fraction is retained at later times. Both the initial association and the longer term retention are dependent on CM lipolysis within lactating mammary tissue. Based on these observations, we then conducted an exploratory analysis using model-based compartmental analysis to estimate the fraction of CM-VA that rapidly disappears from plasma by margination in mammary tissue (i.e., presumably by rapidly binding to mammary gland LPL) and the fraction that remains resident in mammary tissue after the formation of CM remnants. An important result of this model is its potential to explain how variations in milk VA concentration may be related to differences in the VA contents of the CM that are metabolized within mammary tissue. Moreover, an improved understanding of CM-VA metabolism by the lactating mammary glands may help to predict the metabolism and uptake of other fat-soluble dietary compounds, which may be assimilated in a manner similar to VA, during lactation.

Materials and Methods

Preparation of [^{3}H]VA-labeled CM. For injection into recipient rats, CM labeled with [^{3}H]VA were prepared in donor, mesenteric lymph duct-cannulated rats following procedures described previously (25). Briefly, donor rats were prepared with an intragastric fistula and cannula directed into the upper duodenum (0.03 in. i.d. Silastic tubing; Dow Chemical Co., Midland, MI), and a mesenteric lymph duct cannula (0.025 in. i.d. Silastic tubing). Typically, a mixture of [^{3}H]retinol (approximately 10 μCi; New England Nuclear, Boston, MA) was mixed with 20 μg (70 nmole) unlabeled retinol (Sigma, St. Louis, MO) and dissolved in 0.5 ml of vegetable oil, which was then infused over a period of 1–2 hrs into the duodenum. In one experiment, 0.1 mCi of [^{3}H]retinol and 0.5 mCi of [1-^{14}C] palmitic acid (53.0 mCi/mmol, New England Nuclear) was infused simultaneously. For the dose-response experiments, donor rats were infused with increasing amounts of [^{3}H]retinol in a constant rate of oil to obtain different ratios of VA per TG. In another experiment, unlabeled CMs were collected for administration after injection of [^{3}H]VA-labeled CM. In all cases, chyle was collected on ice in aluminum-foil-wrapped centrifuge tubes, which were changed every 15–30 mins, and small aliquots of chyle were counted to select those fractions containing the peak of newly absorbed VA. These fractions were pooled, passed through sterile gauze, and subjected to ultracentrifugation to obtain the $S_f > 400$ fraction CM (26). The CM preparations thus obtained were resuspended by passage through a 23-g needle and polyethylene (PE-20) tubing prior to injection into recipient rats.

Chylomicron TG was quantified by the method of Sardesai and Manning (27), and CM-VA contents were measured after saponification by high-performance liquid chromatography (HPLC) analysis (28). The percent of CM-VA as retinyl ester was assessed by liquid scintillation counting after separation of retinyl esters and retinol on columns of aluminum oxide (26); 94%–95% of the CM [^{3}H]retinol was esterified.

Animals and CM Administration. The experimental protocols were approved by the Institutional Animal Use and Care Committee of the Medical College of Pennsylvania. Untimed pregnant virus-antibody-free Sprague-Dawley CD rats or virgin rats, or untimed pregnant Lewis rats, according to availability, were purchased from Charles River Laboratories (Kingston, NY) and fed a stock rodent diet (RMH 3000; Agway Inc., Syracuse, NY). To standardize metabolic demands (23, 29), litter sizes were adjusted to 7–8 pups on the day after birth. Lactating rats were studied in mid lactation, 14–16 days after delivery. Immediately before each experiment, the dam was removed from her pups, lightly anesthetized by diethyl ether inhalation, and a small incision was made in the skin of the right thigh to expose the saphenous vein. A dose, described below, of VA-labeled CM or CM remnants (0.5–1 ml)

was injected through a 25-g needle into the vein and a stopwatch was started immediately.

To collect tissue samples at exact time points from 2 to 30 mins after CM injection, the abdomen was opened 45 secs before the sampling time, when rats at early times were still under anesthesia or rats at later times had been reanesthetized. At the designated time, a 1.5–2 ml blood sample was drawn from the vena cava into a heparinized syringe and immediately placed on ice. The entire liver and spleen were removed and placed in cold saline. One half of the abdominal skin and mammary tissue (i.e., the entire left side) was dissected from the underlying muscle, quickly chilled in crushed ice, and then pinned to a dissecting board, rapidly freed of skin, muscle, and fascia, and placed in cold saline. The mammary tissue was then blotted and weighed, and samples of ~2 g each were weighed for extraction, taking care to obtain tissue samples from both the inguinal-abdominal and cervical mammary glands. It was assumed that total mammary tissue weighed twice the amount that was dissected. For analysis of liver, all of the major lobes were sampled. Tissue collection was completed within 5 mins of the time point indicated.

Mammary tissue, liver, and spleen samples were either placed immediately in 20 volumes of chloroform to methanol, 2:1 (v/v), in foil-wrapped flasks for lipid extraction (see below), or dissected tissues were frozen in dry ice and extracted later. Plasma was isolated by centrifugation, and aliquots of plasma and the injected doses were extracted as described below. The plasma volume of lactating rats was assumed to be 4.02% of body weight (29).

In some experiments, rats designated as postlactating were separated from their pups 24 hrs before receiving the same dose of CM as administered to lactating rats. Their mammary tissue was notably white and appeared engorged with milk. Additionally, five virgin control rats were injected with CM and sacrificed 2–15 mins later to determine the rate of disappearance of CM-VA from plasma and its appearance in liver; their mammary tissue was not developed and therefore was not analyzed.

Treatment with Heparin *in vivo*. To modify LPL activity in the mammary glands, lactating rats (n = 4) received an iv injection of 50 U/kg body weight of sodium heparin (porcine Grade III; Sigma) dissolved in sterile saline (1 ml/kg) 10 mins before the injection of the CM-VA dose.

Preparation of CM Remnants *in vitro*. As a source of lipoprotein lipase activity, postheparin plasma was collected from control rats that were injected iv with heparin (500 U/kg body weight). After 12 mins, the rats were euthanized and blood was collected from the vena cava. Postheparin plasma was stored in aliquots at –20°C. To prepare CM remnants, postheparin plasma was incubated with CM (approximately 25 mg TG), bovine serum albumin, and 1 mg/ml gentamicin at 37°C for 2 hrs, essentially as described previously (25). After visual clearing had occurred, the incubation mixture was chilled to 4°C, diluted in phosphate-buffered saline of density 1.006 g/ml, and subjected to ultracentrifugation for 20 hrs in an SW27 swinging bucket rotor (Beckman Instruments, Palo Alto, CA). The CM remnant fraction of density <1.006 g/ml was resuspended in phosphate-buffered saline containing gentamicin. Remnant preparations were stored at 4°C in the dark and under nitrogen and used within 8 days of preparation (25). Triglyceride analysis indicated that an average of 78% and 83% of CM TG was removed by *in vitro* lipolysis in two experiments.

Extraction of Tissues, Chromatography, and Liquid Scintillation Counting. Liver, spleen, and mammary tissues were minced finely in chloroform to methanol, 2:1 (v/v), and lipids were extracted overnight and then washed by the method of Folch *et al.* (30). Vitamin A and TGs from plasma and CM lipids were extracted into hexanes as described previously (12). For analysis of the composition of VA as retinyl esters and retinol, samples of the Folch extracts or hexanes were evaporated to dryness under nitrogen, redissolved in hexanes, and subjected to alumina column chromatography (12). Aliquots of the Folch extract or alumina-column eluates were dried, dissolved in liquid scintillation fluid (Scinti-Lene; Fisher Scientific Co., King of Prussia, PA), and counted in a liquid scintillation spectrometer. Counting efficiencies and channel overlaps were determined using calibrated internal standards of [^{3}H]- and [^{14}C]toluene (New England Nuclear). The weight of the organ or total plasma volume was used to estimate the fraction of the injected dose in each organ.

Statistics. Group data are presented as the mean ± SE. Multiple group comparisons were made by one-factor analysis of variance (ANOVA) (SuperAnova; Abacus Concepts, Berkeley, CA) followed by Fisher's protected least significant difference test, and two-group comparisons were made using a two-tailed t test (InStat; GraphPad Software, San Diego, CA). Data points from individual rats were used for determination of half-life, regression analysis, and kinetic modeling. Simple regression analysis was performed using SuperAnova.

Kinetic Analysis. Data from CM injection studies were analyzed retrospectively by model-based compartmental analysis (31, 32). Group mean data on fraction of the injected dose in plasma, liver, and mammary glands at each time were analyzed using WinSAAM, the Windows version of the Simulation, Analysis, and Modeling computer program (33, 34). First, a starting model (see Results) was postulated based on current knowledge and assumptions about CM and VA metabolism during lactation. The model structure and parameters (fractional transfer coefficients or L(I, J)s) were then iteratively adjusted until a good fit between observed and model-predicted data was obtained. Weighted, nonlinear regression analysis was used to determine the reported values for L(I, J)s or the fraction of compartment J's tracer transferred to compartment I each minute. For weighting purposes, a fractional standard deviation (FSD) of 0.075 was assigned to each datum.

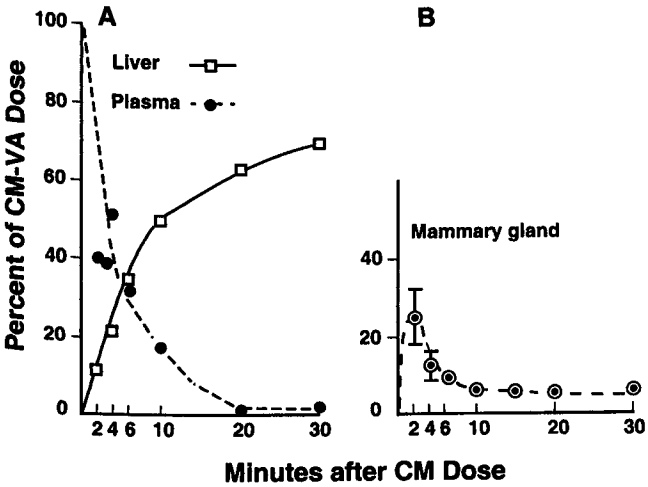

Figure 1. Kinetics of chylomicron (CM) [^{3}H]vitamin A (VA) disappearance from plasma (A), and appearance of [^{3}H]VA in liver (A) and mammary tissue (B) of lactating Sprague-Dawley rats. Each point in A is an individual rat. In B, n = 11 at 2 mins (mean ± SE), n = 4 at 4 mins (mean ± SE), or n = 2 (mean) for all other times.

Goodness of fit of the model was determined by visual inspection of model-predicted versus observed data and by calculation of the estimated FSD for each L(I, J). Parameters were considered well identified if their estimated variability was less than 0.5.

Results

Chylomicron Metabolism in Lactating, Postlactating, and Heparin-Treated Rats. We first asked whether CM-VA is taken up by the mammary tissue of lactating rats. Based on previous studies of CM-VA removal from plasma and the appearance of VA in liver, we anticipated that VA uptake, if it occurred, would be rapid and thus we focused initially on early time points, beginning at 2 mins after CM injection. In each animal, the disappearance of CM-VA from plasma and its appearance in liver, mammary gland, and spleen were determined. As shown in Figure 1A, CM-VA disappeared very rapidly from the circulation of lactating rats. The half-time of disappearance of the CM [^{3}H]VA dose from the plasma of lactating rats was estimated to be 3.3 mins. In comparison, the half-time of CM [^{3}H]VA in five virgin rats was 11 mins (data not shown). In the liver of lactating rats, CM [^{3}H]VA increased in a hyperbolic fashion, reaching ~70% of the injected dose by 30 mins (Fig. 1A). In lactating mammary tissue, CM-VA content increased rapidly, reaching a maximum of 26 ± 6% (n = 11) of the dose at 2 mins (Fig. 1B). Thereafter, CM-VA content declined to a nearly steady level of approximately 6%–8% of the injected dose between 10 and 30 mins. The spleen was also analyzed because it is known to rapidly clear materials that are removed from plasma by phagocytosis. The percentage of CM [^{3}H]VA in the spleen of lactating rats never exceeded 1.05% and averaged only 0.43 ± 0.03% (n = 29 rats in experiments with four different CM preparations), indicating that uptake by phagocytosis was insignificant.

In a second experiment, CM labeled with both [^{3}H]VA and [^{14}C]fatty acid (palmitic acid) were administered to lactating Lewis rats. Tissue contents of labels were studied over an 8-hr time course. The tissue distribution of CM [^{3}H]VA was very similar in this study (Fig. 2) compared with the previous study in lactating Sprague-Dawley rats (Fig. 1). Both ^{3}H and ^{14}C disappeared rapidly from plasma (Fig. 2A and 2B). However, the percent of ^{14}C in plasma at 2 mins was half that of ^{3}H, consistent with a very rapid hydrolysis of CM [^{14}C]TG and the removal of [^{14}C] fatty acids from plasma into tissues. In liver, [^{3}H]VA increased between 2 and 30 mins to >60% of the injected dose and remained relatively constant thereafter, while ^{14}C did not exceed 10% of the injected dose at any time and declined significantly between 30 mins and 8 hrs (Fig. 2C and 2D). In lactating mammary tissue, [^{3}H]VA was highest (21% of dose) at 2 mins and declined thereafter, similar to the results in Figure 1, while [^{14}C]fatty acid was higher than [^{3}H]VA at each time. [^{14}C]Fatty acid increased slightly from ~40% at 2 mins to ~50% at 30 mins. The ^{14}C content in tissues declined by 8 hrs (Fig. 2E and 2F) and [^{14}C]fatty acid could have undergone metabolism to non-fatty acids products by this time.

Modification of Factors Affecting CM Lipolysis. Because the results described above imply that the binding of CM to mammary gland LPL may be important for the uptake of CM-VA by the lactating mammary gland, we next explored the effects of three conditions that are known to modify the activity of mammary gland LPL. First, it is known that suckling and prolactin secretion are necessary for the maintenance of LPL activity in lactating mammary glands (21, 22). Therefore, we compared CM-VA metabolism in lactating rats and in postlactating Sprague-Dawley rats whose pups were removed 24 hrs before the administration of the CM [^{3}H]VA dose. Table 1 shows a comparison of the [^{3}H]VA contents 30 mins after CM injection in plasma, liver, and mammary tissue of normal lactating rats and postlactating rats. By 30 mins, more than 95% of CM [^{3}H]VA had been cleared from plasma in both lactating and postlactating rats. The percentage of the dose present in liver at this time did not differ between lactating and postlactating rats. However, the percentage of the CM-VA dose in mammary tissue of postlactating rats was lower, only one fifth of that present in mammary tissue of lactating rats (P = 0.0001). In another experiment, two postlactating rats were sacrificed 6 and 15 mins after administration of the CM dose. The estimated fraction of the dose associated with postlactating mammary tissue was also much lower (6 mins: 0.66% in postlactating versus 8.69% and 8.46% in lactating mammary tissue; 15 mins: 0.65% in postlactating versus 4.97% in lactating mammary tissue).

In other experiments, we modified mammary gland LPL activity by administering heparin. It is known that the intravenous administration of heparin results in the rapid

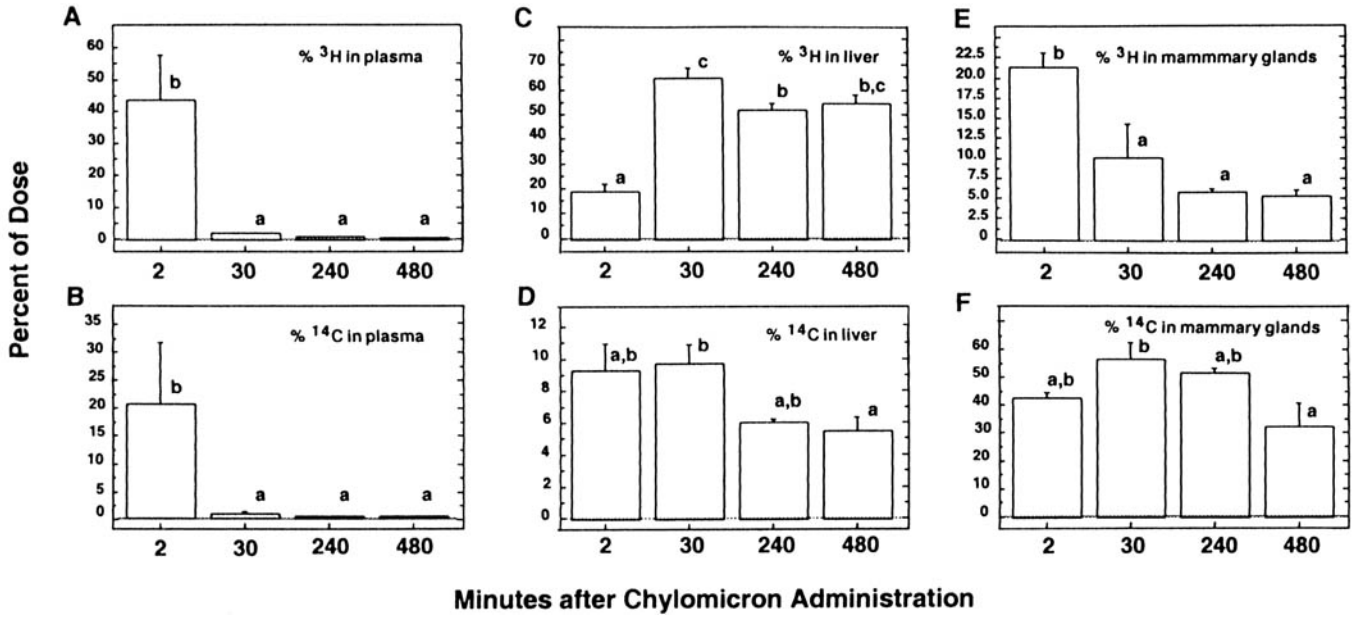

Figure 2. Comparison of the percent of injected dose of [^{3}H]vitamin A (VA) and [^{14}C]palmitate from doubly labeled CM in the plasma (A and B), liver (C and D), and mammary glands (E and F) of lactating Lewis rats from 2 mins to 8 hrs after CM administration. Results are the mean ± SE; a < b < c, $P \leq 0.05$, Fisher's protected least significant difference test for time. Comparing ^{3}H to ^{14}C, differences were significant, $P < 0.001$, for liver and for mammary glands at each time.

displacement of LPL from its binding sites on endothelial surfaces (35). Although the LPL that is released into plasma is enzymatically active and CM remnants are produced, they are formed in the plasma bulk phase rather than on the endothelial surface proximal to tissues. We predicted, therefore, that CM-VA uptake (margination at early times) would be significantly lower in the mammary tissue of heparin-treated lactating rats compared with lactating rats without heparin treatment. Lactating rats were treated 10 mins before CM administration with 50 U/kg of sodium heparin; 2 mins after CM or CM remnant administration, plasma, liver, spleen, and mammary tissue were collected. As shown in Table 2, whereas heparin treatment accelerated CM clearance, as implied by the higher uptake of CM [^{3}H]VA into the liver, this treatment essentially prevented the early association (margination) of CM [^{3}H]VA with lactating mammary tissue.

As a third condition, we studied plasma clearance of preformed CM remnants. It is known that CM remnants have a reduced affinity for LPL (36). Therefore, we predicted that CM remnants formed *ex vivo* and then injected into the plasma of lactating rats would contribute less VA to mammary tissue than would native, nonlipolyzed CM. Remnants were generated by incubating [^{3}H]VA-labeled CM with rat postheparin plasma, a source of LPL. After lipolysis, which removed 78% and 83% of CM TG in two experiments, CM remnants were isolated, and an equivalent number of either CM or CM remnant particles, based on an equivalent content of [^{3}H]VA, were injected into the circulation of lactating rats; plasma, liver, spleen, and mammary tissue were collected 2 min later. In comparison with the uptake of intact CM, the uptake of preformed CM remnants was significantly lower in lactating mammary tissue and, conversely, significantly greater in liver (Table 2).

Dose Response. We next asked whether the quantitative uptake of CM-VA is directly related to the VA content of the CM particle. We reasoned that, if

Table 1. Chylomicron Vitamin A in Plasma, Liver, and Mammary Tissue of Lactating and Postlactating Rats

	% chylomicron [^{3}H]vitamin A in tissues 30 min after iv injection[a]		
	Plasma	Liver	Mammary tissue
Lactating	2.00 ± 0.14 (n = 14)	68.51 ± 1.31 (n = 14)	8.38 ± 0.59 (n = 14)
Postlactating	4.89 ± 1.02 (n = 4) P = 0.0001	67.63 ± 4.89 (n = 4) P = 0.804	1.73 ± 0.25 (n = 3) P = 0.0001

[a] Mean ± SE for the number (n) of Sprague-Dawley rats shown; exact P values are for two-tailed t tests.

Table 2. Heparin Treatment *In Vivo* or Prior Chylomicron Remnant Formation *In Vitro* Reduce the Early Association of Chylomicron [^{3}H]Vitamin A with Mammary Glands of Lactating Rats

Dose or treatment	% of chylomicron vitamin A in tissues 2 min after iv injection[a]		
	Plasma	Liver	Mammary tissue
Intact chylomicrons	46.18 ± 4.10	14.06 ± 1.85	24.53 ± 3.68
Heparin pretreated[b]	42.82 ± 6.39	45.68 ± 8.19	4.97 ± 1.13
	$P = 0.6735$	$P = 0.0093$	$P = 0.0023$
Chylomicron remnants[c]	11.37 ± 3.37	62.70 ± 8.99	9.75 ± 3.77
	$P = 0.0077$	$P = 0.0018$	$P = 0.0309$

[a] Mean ± SE, n = 4/group. P values are for two-tailed t test, compared with intact chylomicrons (CM).
[b] Sodium heparin was administered iv 10 mins prior to injection of CM in lactating Sprague-Dawley rats.
[c] Chylomicron remnants were formed by *in vitro* lipolysis of [^{3}H]vitamin A (VA)-labeled CM prior to injection (see Materials and Methods); the content of VA was equal in the remnants and the intact CM from which they were prepared.

a constant fraction of CM-VA is transferred into the lactating mammary glands during the lipolytic process, then the mass of VA transferred should increase linearly with the amount of VA that CMs contain. Two experiments were performed, which, together, spanned a 17-fold range of CM TG and a 150-fold range of CM-VA. For the first experiment, we collected a series of CM preparations from a donor rat that was given increasing intraduodenal doses of [^{3}H]retinol along with a steady infusion of oil for lipid absorption. Four CM-VA doses were collected, each with nearly equal TG contents (3.1–3.3 mg/dose) but differing ~8-fold in their VA content (0.285–2.0 μg) and VA to TG ratio. To administer higher doses of CM-VA, it was necessary to increase the TG dose as well. Thus, in part of this experiment and in a second experiment, the CM doses contained from 3.1 to 56.1 mg of TG and 2.6–42 μg of VA/dose. Overall, for 14 lactating Lewis rats that received the same amount of TG (3.1–3.3 mg) with [^{3}H]VA ranging from 0.285 to 8 μg, the percent of [^{3}H]VA in tissues at 30 mins was almost constant and equaled 2.00% ± 0.14% in plasma, 8.38% ± 0.59% in mammary tissue, 68.58% ± 1.30% in liver, and 0.44% ± 0.06% in spleen. For 16 lactating rats that received CM doses in which the TG content ranged from 3.7 to 56.1 mg and the VA contents from 2.6 to 42 μg, the fractional uptake of CM [^{3}H]VA by mammary tissue at 30 mins was similar to that in the first experiment. The fractional uptake determined at 2 mins was also independent of the CM TG or VA dose. In Figure 3A and 3B, data from these experiments are plotted together as the mass of mammary tissue VA in comparison with the mass of VA in the CM doses. Over the 150-fold range of CM-VA mass tested in these two experiments, there was a significant linear relationship between the dose of CM-VA and the VA contents of lactating mammary tissue (R^2 = 0.979 at 2 mins and R^2 = 0.972 at 30 mins; P = 0.0001 for both times). Based on the difference in the slopes of the regression lines in Figure 3A and 3B, the percentage of the dose associated with lactating mammary tissue was approximately 4.3 times higher at 2 mins than at 30 mins.

In additional rats, we determined whether [^{3}H]VA-labeled CM bound to the mammary tissue could be displaced by a second dose of unlabeled CM injected 2 mins after the first dose of [^{3}H]VA-labeled CM. However, there was no change in the percentage of [^{3}H]VA present in mammary tissue (data not shown), indicating that CM in the second dose did not displace the [^{3}H]VA-labeled CM that were already undergoing metabolism and providing further evidence that CM metabolism and VA uptake into lactating mammary tissue are not saturated at the CM doses used in our studies.

Compartmental Model. To better understand the metabolism of CMs during active lactation, we retrospectively applied model-based compartmental analysis to tracer response data from plasma, liver, and mammary tissue after administration of [^{3}H]VA-labeled CM to lactating Sprague-Dawley rats (times from 2 to 30 mins). Later times were excluded because, beyond 30 mins, the contribution of [^{3}H]retinol recycled from the liver (presumably as RBP-bound retinol) after CM-VA uptake could not be excluded (37). To begin the modeling process, a nine-compartment starting model was postulated (Fig. 4). In the proposed model, VA-labeled CMs were injected into plasma Compartment 1, delipidated by LPL to remnants (Compartments 2–5), marginated in mammary tissue (Compartment 6), or taken up by liver (Compartment 8). The label that marginated in mammary tissue either remained there (Compartment 7) or returned to plasma (Compartment 5). Chylomicron remnants (Compartment 5) were taken up either by liver (Compartment 8) or other tissues (Compartment 9).

The proposed model was compared with the observed data using WinSAAM (34). For regression purposes, the plasma data point at 30 mins was unweighted because there was visual evidence (the upswing in the plasma tracer response profile; Fig. 5) of [^{3}H]VA recycling to plasma as holo-RBP. As shown in Figure 5, the proposed model was robust enough to fit tracer data for plasma, liver, and mammary tissue. Parameters (fractional transfer coefficients or L(I, J)s) for the final fit are shown in Figure 4. CMs

appear to marginate rapidly compared with the times chosen for plasma sampling. Thus, there is uncertainty in the values of L(2, 1), L(6, 1), and L(8, 1), with FSDs of 1.7, but a consistent ratio between them. The other five parameters in the model (L(5, 4), L(7, 6), L(5, 6), L(8, 5), and L(9, 5)) were well identified, with FSDs of 0.06–0.15.

The model predicts that 6.6% of the dose was rapidly cleared by the liver (L(8, 1)). This most likely corresponds to a nonphysiological component in the administered dose (e.g., clumped CM [38]). This fraction was not considered in subsequent calculations. The model predicts that 42% of the injected label marginated to mammary tissue during lipolysis (L(6, 1)/[L(6, 1) + L(2, 1)]; Fig. 4) and 9.1% of the [^{3}H]VA CM dose (L(7, 6)/[L(7, 6) + L(5, 6)]) (3.8% of the initial CM-VA dose) remained in mammary tissue. The model-predicted $t_{1/2}$ for CM remnants was 3.04 mins.

Discussion

Our experiments provide physiological evidence that the lactating mammary gland, through the mediation of the binding of CM to LPL and the lipolytic process, takes up and retains newly absorbed VA from CM. Chylomicron VA uptake required binding of CM to LPL, as shown by the reduction in mammary gland-associated [^{3}H]VA when CM injection was preceded by injection of heparin, a treatment known to displace LPL from the endothelium (Table 2). VA uptake also required active lipolysis of CM [^{3}H]VA as shown by the higher percentage of [^{3}H]VA associated with mammary tissue in actively lactating rats compared with postlactating rats (Table 1). Moreover, it was necessary that lipolysis occurred *in situ* within mammary tissue as shown by the significant reduction in [^{3}H]VA present in mammary tissue after administration of preformed CM remnants, which had been depleted of the majority of their TG prior to injection, as compared with unmetabolized intact CM. The lipolysis of CM TG was rapid in all animals studied but was especially rapid in lactating rats in which the plasma half-life for CM [^{3}H]VA was 3.04 mins (compartmental analysis) in lactating rats, compared with 11 min in virgin rats. Despite the significant differences between lactating and postlactating rats in the rate of disappearance of CM from plasma and the uptake of CM [^{3}H]VA into mammary tissue, there was no significant difference in the percentage of CM [^{3}H]VA recovered in liver. These data imply that the formation of CM remnants was similar in both situations, and that the liver was the major site of CM [^{3}H]VA uptake in both lactating and postlactating rats. Although we studied lactating rats only in midlactation, it is likely that these results are applicable to earlier and later stages of lactation because LPL activity is induced near parturition and remains induced while suckling continues.

As noted above, the metabolism of CM in the lactating mammary gland requires active, local lipolysis. Because LPL is bound to cell surface heparan proteoglycans (39), the process we observed as CM [^{3}H]VA margination is likely

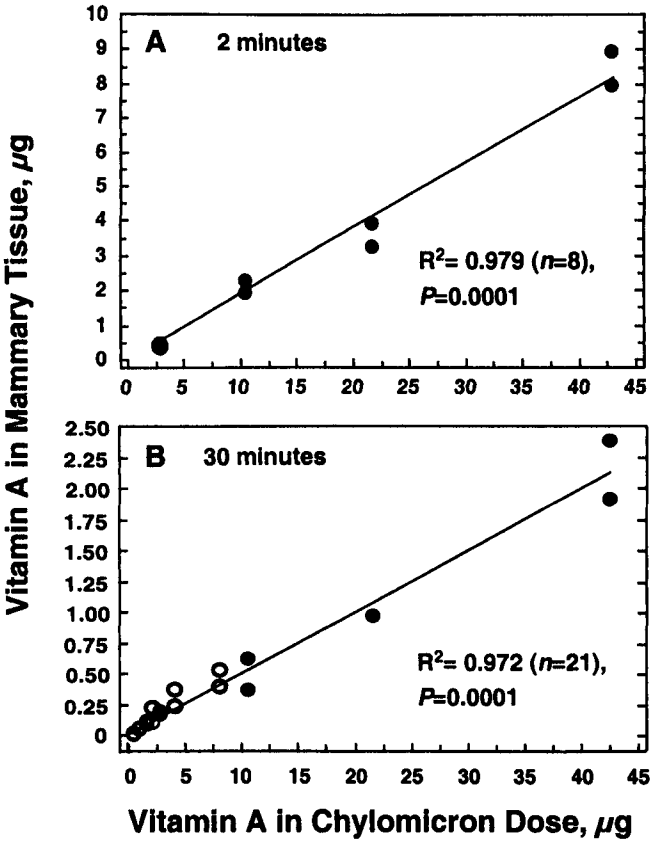

Figure 3. Regression analysis of mammary tissue vitamin A (VA) content as a function of chylomicron (CM) VA dose. Solid circles represent results from 16 Lewis rats (n = 8 at 2 mins [A] and n = 8 at 30 mins [B]) in which VA mass varied from 2.6 to 42 μg while CM triglyceride (TG) mass varied from 3.1 to 56.1 mg; open circles in B represent results from 14 Sprague-Dawley rats in which CM TG mass was held nearly constant (3.1–3.3 mg) while CM-VA mass, and therefore the VA to TG ratio per CM particle, varied. Each point represents an individual rat.

due to the binding of CM particles to endothelial-bound LPL during the initial phase of CM metabolism. It appears that transient tethering to LPL and subsequent TG lipolysis play important roles in determining the amount of CM-VA that is taken into the mammary gland. The estimated fraction of CM [^{3}H]VA initially (2 mins) bound to mammary tissue (Table 2) was significantly reduced when lipolysis proceeded in the bulk phase of plasma rather than on the endothelium (after heparin injection) or when the injected dose consisted of CM remnants instead of intact CM. Under the conditions studied, which included a single bolus dose of CM TG in amounts as high as 56 mg TG per lactating rat, the estimated fraction of CM [^{3}H]VA that was taken up by mammary tissue was nearly constant (Fig. 3). Thus, the mass of VA taken into mammary tissue increased linearly with the CM-VA dose. As the VA content per CM particle increased, so did the VA content of lactating mammary tissue. Regression analysis showed that the proportion of CM-VA initially associated with mammary tissue (2 mins) and the proportion taken up by the lactating mammary glands (30 mins) could be reliably predicted from the VA

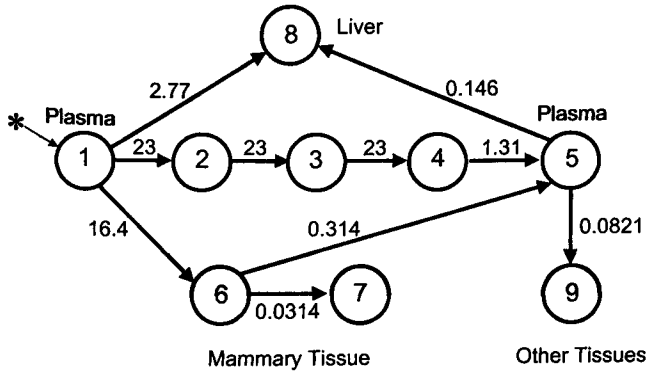

Figure 4. Working hypothesis compartmental model for metabolism of chylomicron (CM) [^{3}H]vitamin A (VA) in lactating rats. Circles represent compartments and interconnectivities are fractional transfer coefficients (L(I, J)s or the fraction of compartment J's tracer transferred to compartment I each minute). Model-predicted L(I, J)s are shown above each arrow. The asterisk indicates that [^{3}H]VA-labeled CM were injected into plasma (Compartment 1). Compartments 2, 3, 4, and 5 are marginated CM and delipidated CM remnants in plasma; Compartments 6 and 7 are both mammary gland compartments (see text); Compartment 8 is liver; and Compartment 9 represents other tissues.

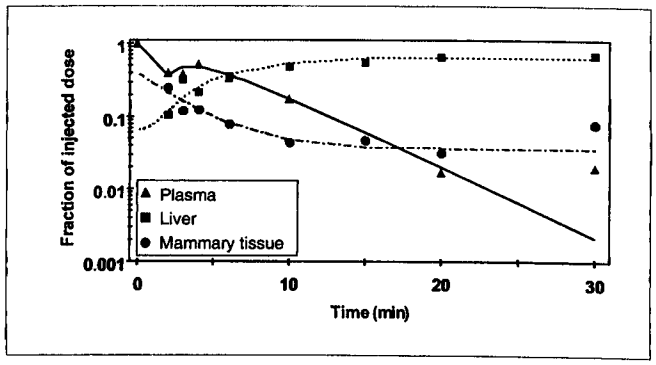

Figure 5. Tracer response profiles in plasma, liver, and mammary gland of lactating rats. Lactating Sprague-Dawley rats were injected with [^{3}H]Vitamin A (VA)-labeled chylomicrons (CM) on Day 16 of lactation and sacrificed from 2 to 30 mins after dose administration. Points represent individual rats (3 and 20 mins) or means for 2–15 rats per time for plasma, liver, and mammary tissue; lines are values predicted for the compartmental model shown in Figure 4.

content per CM TG. These results imply, first, that the process by which VA is taken up by mammary tissue is not readily saturated and, second, that the uptake of VA is a passive process resulting from the association of CM with LPL in the lactating mammary gland. Our results for VA are consistent with the results of earlier studies in which CM fatty acids and cholesterol were monitored. Those experiments showed that a fraction of CM remnant lipids generated during lipolysis is retained within the lactating mammary gland either by engulfment of remnant particles or by diffusion of lipid products into adjacent plasma membranes (40).

To obtain more quantitative insights into the metabolism of CM during active lactation, we retrospectively applied model-based compartmental analysis to data on [^{3}H]VA kinetics in plasma, mammary tissue, and liver of lactating Sprague-Dawley rats. Our model (Fig. 4) predicts that ~42% of the injected CM marginated into mammary tissue, where they were presumably acted on by LPL. Further, the model predicts that ~9% of the dose, which was sequestered in mammary tissue (or ~4% of the injected dose), was cleared into the tissue while the rest returned to circulation for clearance by the liver or other tissues. The prediction that such a large proportion of CMs are metabolized in mammary tissue is presumably related to the high LPL activity in the gland to support milk production. Future studies using model-based compartmental analysis should include sampling at earlier time points to better define the early components of the model.

Presumably, the VA that remains in lactating mammary tissue after lipolysis of CM TG will either be stored in the tissue (3) or incorporated into milk fat droplets and secreted into milk, as has been demonstrated for CM cholesterol and fatty acids (41, 42). Vitamin A in milk is derived from two sources, CM and holo-RBP (14). Even when lactating rats are fed a VA-free diet, there is a basal level of the vitamin in milk (3), presumably from the uptake of retinol transported by RBP (Fig. 6). Thus, as long as maternal liver VA is sufficient to maintain the secretion of holo-RBP, VA will be present in milk. Because the plasma concentration of holo-RBP is homeostatically regulated and is relatively constant over a wide range of VA intake as long as liver VA stores are adequate, we would expect that the contribution (mass) of VA delivered by RBP is relatively constant, independent of maternal VA status as long as status is adequate. However, milk VA concentration has been shown to be significantly higher in lactating rats fed high versus low VA diets, even when their plasma retinol concentrations are not different (2, 3). It was recently estimated that CM contribute 32% of the VA in milk when lactating rats are fed a maintenance level of VA and 52% at a higher intake. From the linearity of VA uptake into lactating mammary tissue with CM-VA dose (Fig. 3), it can be predicted that the contribution of newly absorbed CM-VA to milk VA contents will increase proportionately when VA intake is increased by either an increase in dietary VA or by VA supplementation (Fig. 6).

Understanding the basic mechanisms of CM-VA metabolism in the lactating mammary gland may have practical implications for maternal and child health. Breast feeding of infants is strongly encouraged as one means to improve the VA status of young children and thereby prevent the development of VA deficiency in the post-weaning period, the time at which clinical deficiency is most likely to be apparent (43). An implication of the present study is that the mother's recent intake of VA makes an important contribution to her milk VA levels and would therefore be expected to be important in establishing VA reserves in the nursing young. Our data imply that VA in the maternal diet will have a nearly immediate impact on the VA contents of breast milk due to the postprandial metabolism (margination and lipolysis) and partial retention

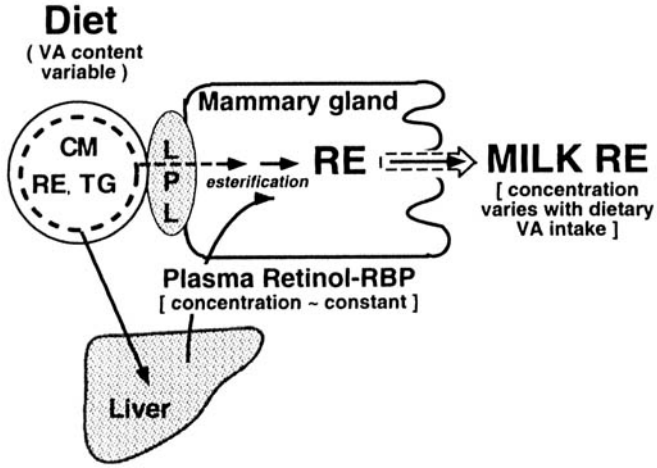

Figure 6. Schematic model of the predicted effect of variations in dietary vitamin A (VA) on chylomicron (CM) retinyl ester (RE) content and, hence, the uptake of VA into the lactating mammary glands and milk VA (RE) concentration. When maternal VA status is adequate, plasma retinol-RBP is relatively constant and RBP contributes a relatively constant amount of VA to the lactating mammary gland. Chylomicron VA varies directly with recent VA intake. Thus, the metabolism of CM-VA in the lactating mammary glands is proposed to provide VA in proportion to the mother's recent consumption of VA. Abbreviations: LPL, lipoprotein lipase; RBP, retinol-binding protein; TG, triglyceride.

of CM-VA in the lactating mammary gland. Moreover, because the majority of CM-VA is taken up by liver, an increase in maternal dietary VA should also provide reserves to maintain the secretion of holo-RBP, even if dietary VA is limited. A recent study of VA supplementation in lactating sows, designed to model the mammary gland response of lactating women and the hepatic VA response of their infants, showed a rapid uptake of VA into the mammary glands and milk, and increased liver VA in the offspring (44).

Lactation is known to reduce the risk of breast cancer (45, 46). While many mechanisms contribute to this association, it is possible that one mechanism by which lactation may reduce breast cancer risk involves the delivery by CM of lipophilic compounds with anticarcinogenic activity to the mammary gland during the lactation period, similar to that observed here for VA. The role of CM in the delivery of other compounds to the lactating mammary gland merits further study.

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