# Retinyl ester secretion by intestinal cells: a specific and regulated process dependent on assembly and secretion of chylomicrons

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**Abstract** **Retinyl esters (RE) have been used extensively as markers to study chylomicron (CM) catabolism because they are secreted in the postprandial state with CM and do not exchange with other lipoproteins in the plasma. To understand the mechanism of secretion of RE by the intestine under the fasting and postprandial states, differentiated Caco-2 cells were supplemented with radiolabeled retinol under conditions that support or do not support CM secretion. We observed that these cells assimilate vitamin A by a rapid uptake mechanism. After uptake, cells store retinol in both esterified and unesterified forms. Under fasting conditions, cells do not secrete RE but secrete free retinol unassociated with lipoproteins. Under postprandial conditions, cells secrete significant amounts of RE only with CM. The secretion of RE with CM was independent of the rate of uptake of retinol and intracellular free and esterified retinol levels, and was absolutely dependent on the assembly and secretion of CM. The secretion of RE was correlated with the secretion of CM and not with the secretion of total apolipoprotein B. Inhibition of CM secretion by Pluronic L81 decreased the secretion of RE and did not result in their increased secretion with smaller lipoproteins. These data strongly suggest that RE secretion by the intestinal cells is a specific and regulated process that occurs in the postprandial state and is dependent on the assembly and secretion of CM. We propose that RE are added to CM during final stages of lipoprotein assembly and may serve as signposts for these steps.**—Nayak, N., E. H. Harrison, and M. M. Hussain. **Retinyl ester secretion by intestinal cells: a specific and regulated process dependent on assembly and secretion of chylomicrons. *J. Lipid Res.* 2001.** 42: **272–280.**

**Supplementary key words** lipoproteins • vitamin A • absorption • Caco-2 cells • retinol • retinyl esters • fatty acids • Pluronic L81

Chylomicrons (CM) are large (diameter, 75–450 nm), spherical lipoprotein particles secreted by the intestinal cells in the postprandial state. In addition to CM, intestinal cells secrete very large density lipoprotein (VLDL)-size (diameter, 30–80 nm) particles (1–3). VLDL are the major particles secreted by these cells during the fasting state (4), under experimental infusion of lecithin or palmitate (5), and during inhibition of CM assembly (6).

Despite the physiologic importance of CM assembly and secretion in the transport of dietary fat and fat-soluble vitamins (7) and in the growth and development of neonates (8), the molecular assembly of these particles is poorly understood. We presented a model suggesting that CM assembly may involve three distinct events: assembly of primordial lipoprotein particles, formation of triglyceride-rich droplets, and core expansion (2). Furthermore, evidence has been presented to suggest that preformed phospholipids are signposts for the first step in the assembly of lipoproteins, that is, synthesis of primordial lipoproteins (2, 3, 9). In contrast to the preformed phospholipids, large amounts of newly synthesized triglycerides were found associated with CM, indicating that significant amounts of nascent triglycerides are preferentially used for CM assembly (2, 3, 9). In the present study, we made an effort to identify markers for the final stages of CM assembly. We concentrated on retinyl esters (RE) because they have been used as markers to study CM catabolism (10–12). Little is known about the biochemical and molecular mechanisms involved in the secretion of RE with CM. It is generally believed that RE secretion with CM is a passive process. The perception exists, probably because most of the vitamin A and CM remnant metabolism studies are performed in the postprandial state.

In the plasma, RE remain associated with CM because plasma does not contain a protein that can transfer RE between lipoproteins. After the hydrolysis of CM triglycerides by the endothelial cell-bound lipoprotein lipase, RE are delivered to liver and other tissues as a component of

Abbreviations: apo, apolipoprotein; CM, chylomicrons; FBS, fetal bovine serum; Fx, fraction; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; OA, oleic acid; PA, palmitic acid; RE, retinyl esters; TC, taurocholate; VLDL, very low density lipoproteins.

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CM remnants (1, 13–16). In the liver, RE are hydrolyzed and re-esterified, and stored largely in hepatic stellate cells. Depending on the need and physiologic conditions, RE stored in the liver are hydrolyzed and free retinol is secreted bound to retinol binding protein, and delivered to other tissues (10, 17–23). Thus, the intestine is the only organ that secretes RE, whereas other cells secrete free retinol bound to retinol-binding protein. However, it is not known why only the intestine secretes RE. Does the intestine secrete RE with intestinal VLDL? What is the fate of dietary retinoids in the absence of CM assembly and secretion?

There were two aims of the present study: to identify the biochemical markers for the final stages of CM assembly and to understand vitamin A absorption and transport under fasting and postprandial conditions. For these purposes, we used Caco-2 (human colon carcinoma) cells. Caco-2 cells have been used to study intestinal vitamin A metabolism (24, 25). Studies concerning the uptake of retinol by Caco-2 cells indicated that retinol at physiologic and pharmacological concentrations was taken up by saturable, carrier-mediated process and nonsaturable, diffusion-dependent processes, respectively (24). The retinol taken up by these cells was esterified and the RE mainly contained palmitic and oleic acids (24, 25). We have shown that these cells can be induced to secrete triglyceride-rich lipoproteins of different sizes under different experimental conditions. They secrete VLDL/intermediate density lipoprotein (IDL) under fasting conditions and secrete CM when postprandial conditions are simulated in cell culture (9, 26).

## MATERIALS AND METHODS

### Materials

Oleic acid (OA), taurocholate (TC), hexane, diethyl ether, and other chemicals were obtained from Sigma (St. Louis, MO). Pluronic L81 was kindly provided by the BASF Corporation (Washington, NJ), and was freshly prepared in medium for each experiment. [11,12-$^3$H(N)]retinol (specific activity, 30–60 Ci/mmol) was from NEN Life Science Products (Boston, MA).

### Cell cultures

Caco-2 cells were allowed to differentiate on Transwells (24-mm diameter, 3-μm pore size; Corning Costar, Cambridge, MA) for 3 weeks. Differentiation of Caco-2 cells was determined by studying the induction of apolipoprotein B (apoB) secretion and increase in sucrase activity (9). Unless otherwise stated, cells received 1.5 ml of Dulbecco's modified minimum essential medium containing 20% fetal bovine serum (FBS), [$^3$H]retinol, 1.6 mM OA, and 0.5 mM TC on the apical side and 2 ml of Dulbecco's modified minimum essential medium containing 0.1% FBS on the basolateral side. Basolateral-conditioned medium from two Transwells was pooled and subjected to a sequential density gradient ultracentrifugation to obtain large CM ($S_f$ > 400), small CM ($S_f$ 60–400), VLDL (d < 1.006 g/ml, $S_f$ 20–60), and other lipoprotein fractions as described earlier (9). Aliquots

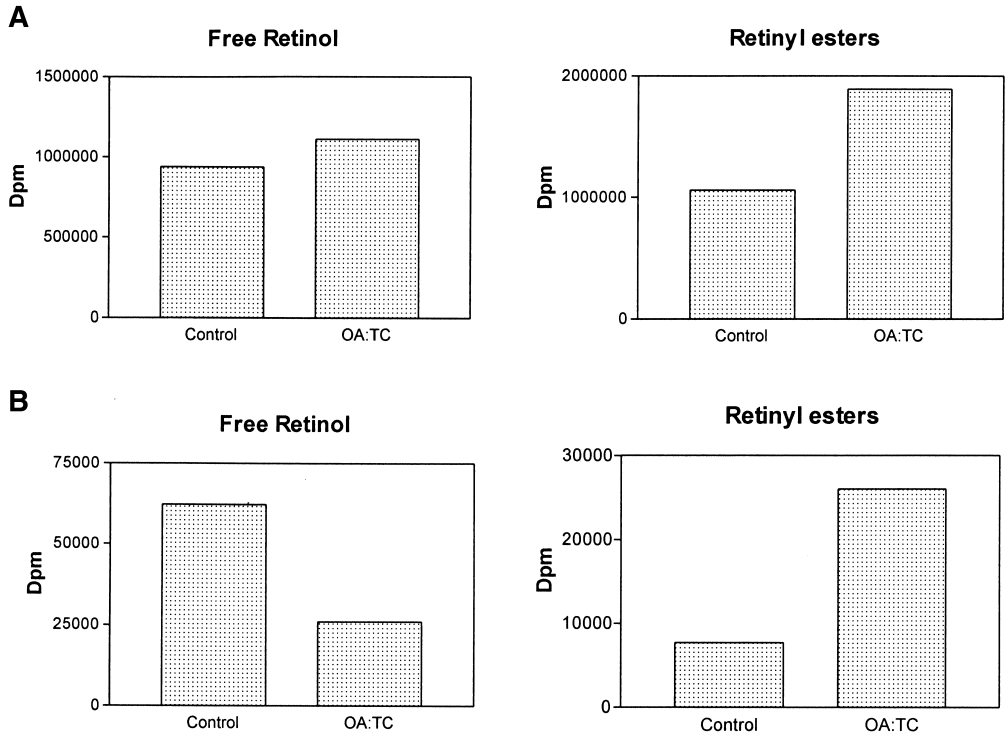


**Fig. 1.** Intracellular (A) and secreted (B) amounts of free and esterified retinol. Differentiated Caco-2 cells were supplemented with medium containing 20% FBS, 5 μCi/ml of [$^3$H]retinol in the absence (control) or presence of OA:TC (1.6:0.5 mM) and incubated for 17 h. A: At the end of the experiment, cells were washed and lipids were extracted in isopropanol. Free and esterified retinol were separated with alumina columns as described in Materials and Methods. B: Lipids were extracted from medium in duplicate, using ethanol-hexane as described in Materials and Methods. Retinol and RE were separated with alumina columns and counted. The average values are plotted. The data are representative of six independent experiments.

($3 \times 100$ μl) from each fraction were used to quantify apoB by a sandwich enzyme-linked immunosorbent assay (ELISA) (27, 28). The remaining portions were used for the total extraction of free retinol and RE.

### Extraction and analysis of free and esterified retinol

We added 2 ml of ethanol and 5 ml of hexane to 500 μl of the conditioned medium or different lipoprotein fractions. After mixing and centrifugation (tabletop centrifuge, 1,500 rpm, 4°C, 20 min), the top hexane layer was collected, and dried under nitrogen. For extraction from cells, adherent cells were washed twice by the addition and aspiration of ice-cold phosphate-buffered saline, and incubated overnight with 1 ml of isopropanol at 4°C. The isopropanol was collected into glass tubes, and cells were washed with another 500 μl of isopropanol and combined with the earlier extract, and dried under nitrogen. The dried lipid extracts were dissolved in 1 ml of hexane, applied on small columns containing ~1.5 g of deactivated alumina (100 g of dried aluminum oxide mixed with 10 ml of distilled water) equilibrated with hexane, and washed three times with 1.5 ml of hexane. RE and free retinol were eluted with hexane ($3 \times 1.5$ ml) containing either 3% or 50% diethyl ether, respectively. The fractions were dried, resuspended in liquid scintillation cocktail, and counted (29). The qualitative and quantitative aspects of the separation of RE and free retinol, using alumina columns, were validated with radiolabeled retinyl palmitate in each experiment. The majority (~90–95%) of the retinyl palmitate was eluted with 3% diethyl ether and ~5–10% was eluted with 50% ether. This spillover was determined in each extraction and corrected.

### Retinol uptake experiments

Uptake experiments were performed essentially as described by Compassi et al. (30). Differentiated Caco-2 cells received 1.5 ml of medium containing 20% FBS, 0.5 mM TC, [$^3$H]retinol (1 μCi/ml), and different OA concentrations. At each time point, 100 μl of medium was removed and replenished with the same medium devoid of radiolabeled retinol. The medium was counted in triplicate and the fraction remaining at each time point was calculated. The graphs were analyzed by nonlinear regression analysis using Prism (GraphPad, San Diego, CA) to determine the half-life.

## RESULTS

### Secretion of RE by Caco-2 cells as part of CM

To study RE secretion, differentiated Caco-2 cells were supplemented with [$^3$H]retinol in the absence (control) and presence of OA:TC on the apical side for 17 h (**Fig. 1**). At the end of the experiments, control and OA:TC-treated cells contained similar amounts of radiolabeled free retinol (Fig. 1A). In contrast, cells treated with OA:TC contained 56% higher amounts of RE compared with control cells (Fig. 1A). During the same period, control and OA:TC-treated cells secreted similar amounts ($70 \times 10^3$ and $52 \times 10^3$ dpm, respectively) of [$^3$H]retinol. However, these cells secreted different forms of retinol. Control cells mainly secreted free retinol, whereas cells treated with OA:TC secreted RE (Fig. 1B). Cells supplemented with OA:TC secreted 3-fold higher amounts of RE than control cells. These studies indicate that cells treated with OA:TC secrete significantly higher amounts of RE.

Next, we looked at the distribution of apoB, and of free and esterified retinol, in different lipoprotein fractions (**Fig. 2**). The majority of apoB secreted by control cells was in the VLDL fraction (Fig. 2A). In contrast, cells incubated with OA:TC secreted apoB in CM and VLDL fractions, as is consistent with our earlier study (9). In the absence of OA:TC, small amounts of RE were secreted and

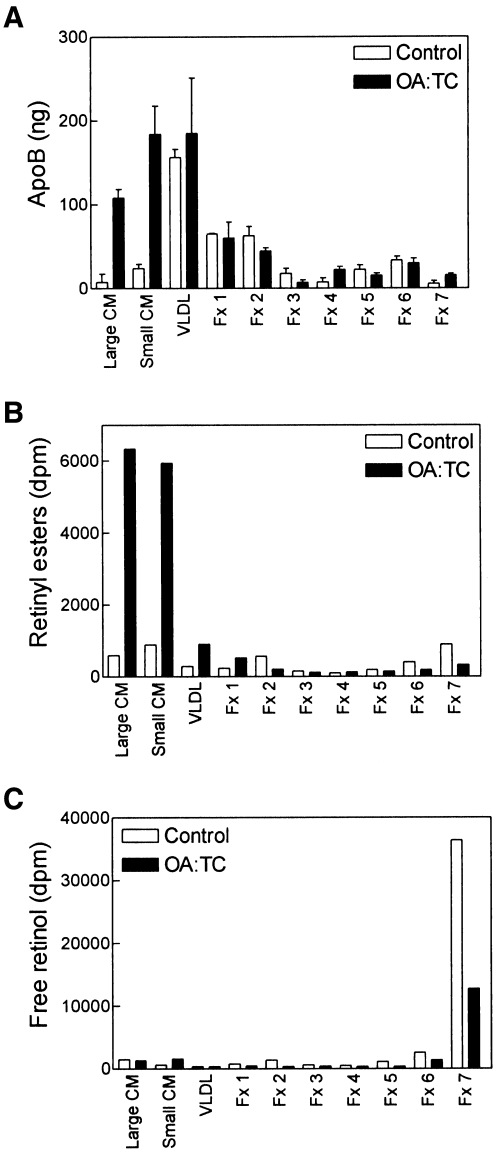


**Fig. 2.** Distribution of apoB, RE, and free retinol in different lipoproteins secreted by differentiated Caco-2 cells. Caco-2 cells were allowed to differentiate for 3 weeks on Transwells (9). Cells were supplemented with medium containing 20% FBS, [$^3$H]retinol (5 μCi/ml) in the absence (control) or presence of OA:TC (1.6:0.5 mM) and incubated for 17 h. Basolateral medium was subjected to a sequential density gradient ultracentrifugation for the isolation of different lipoprotein fractions. A: ApoB in each fraction was measured in triplicate ($3 \times 100$ μl) by enzyme-linked immunosorbent assay. Average and standard deviations are plotted as bar graphs and error bars, respectively. Total free retinol and RE were extracted from each fraction into hexane, applied onto alumina columns, eluted with different concentrations of diethyl ether as described in Materials and Methods, and counted. RE (B) and free retinol (C) present in different lipoprotein fractions are plotted as bar graphs. The data are representative of six independent experiments.

these were almost evenly distributed in all fractions (Fig. 2B, control). Cells incubated with OA:TC, however, secreted 86% of RE in CM fractions (Fig. 2B). In this experiment, the largest percentage of esters was in the large CM fraction. However, in other experiments, the largest percentage of RE was present in small CM. In contrast to CM, VLDL and IDL [fraction (Fx) 1]-size lipoproteins contained only small amounts (6% and 2%, respectively) of RE even though these fractions contain 43% of secreted apoB. The other fractions contained ⩽2% of the counts each. In contrast to RE, significant amounts of free retinol were secreted under both conditions (Fig. 2C); the amounts of free retinol secreted by OA:TC-treated cells were significantly lower than were secreted by control cells. In both conditions, the majority of the secreted free retinol was present in the nonlipoprotein fraction. The secretion and distribution of free and esterified [$^3$H]retinol was not affected by the addition of 700 pg of unlabeled retinol (data not shown), an amount simulating the normal recommended daily allowance of retinol for humans (31). Thus, OA:TC treatment induces secretion of RE but not of free retinol. Furthermore, RE display a marked preference for CM, whereas free retinol is mainly secreted unassociated with lipoproteins.

### Secretion of RE is independent of the rate of uptake of retinol

Attempts were then made to understand the reasons for the secretion of RE with only CM by cells supplemented with OA:TC. Consideration was given to the possibility that OA:TC might have facilitated the initial uptake of retinol and thus stimulated the incorporation of RE into CM. It is conceivable that in the postprandial state, because there is an enormous flux of free fatty acids into the enterocytes while the amounts of vitamin A are considerably lower, fatty acids may facilitate the uptake of vitamin A. To test the hypothesis, we studied the disappearance of [$^3$H]retinol from the apical medium in the presence of different concentrations of OA (**Fig. 3**). Studying the disappearance from the medium is a commonly used method for cellular uptake (32, 33). Inclusion of OA had no effect on the rate of disappearance of the labeled retinol from the apical medium (Fig. 3A). The nonlinear regression analysis using one exponential decay equation showed that the half-life of retinol under these conditions ranged between 20 and 26 min. This indicates that retinol uptake by Caco-2 cells is a fast process. Surprisingly, only ~50% of the added radiolabeled retinol was taken up by Caco-2 cells at the end of 6 h. To determine whether poor uptake of retinol by cells was due to serum, we studied the uptake of retinol in the presence and absence of serum (Fig. 3B). Indeed, retinol was removed significantly faster in the absence of serum, and up to 80% of the retinol was taken up by cells within 2 h. The uptake of and intracellular retinol levels were not affected by the addition of unlabeled retinol (data not shown). Thus, these studies suggest that one fraction of the added retinol is rapidly removed and another fraction may bind to serum proteins, most likely retinol-binding protein, and is not immediately available for cellular uptake. More importantly, OA does not affect the kinetics of retinol uptake. Thus, secretion of RE with CM is independent of the rate of uptake of retinol.

### Dependence of retinyl ester secretion on the assembly and secretion of CM

Previously, we showed that the assembly and secretion of CM was dependent on the presence of high concentrations of OA in the medium (9). When supplemented with 0.4 mM OA, Caco-2 cells secrete apoB as small CM and VLDL-size particles. At ⩾0.8 mM OA, Caco-2 cells secrete large CM. To determine whether RE could be secreted with VLDL, Caco-2 cells were incubated with different concentrations of OA and [$^3$H]retinol (**Fig. 4**). At 0.8 mM

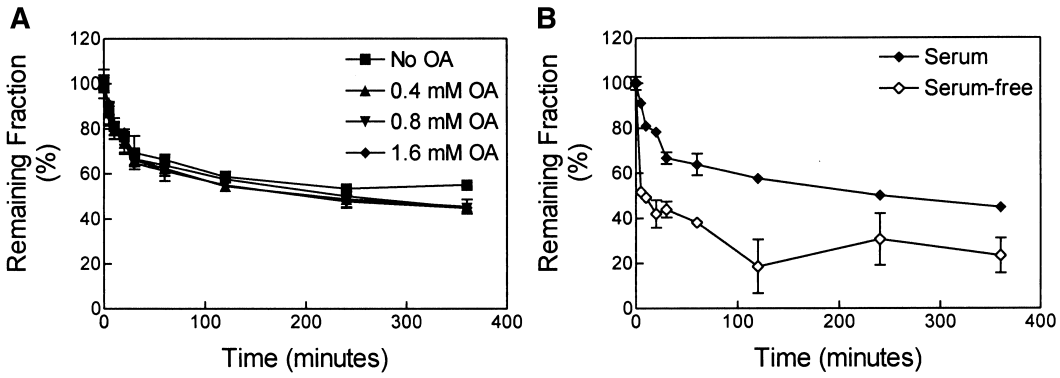


**Fig. 3.** Uptake of retinol by differentiated Caco-2 cells. A: Effect of oleic acid. Caco-2 cells were supplemented on the apical side with medium containing 20% FBS, 0.5 mM taurocholate, and [$^3$H]retinol (1 μCi/ml) in the presence of different concentrations of OA for the indicated times. At each time point 100 μl of the medium was removed from the apical sides and replenished with 100 μl of the same medium without retinol. B: Effect of serum. Caco-2 cells were supplemented on the apical side with medium containing 0.5 mM TC, 1.6 mM OA, and [$^3$H]retinol (1 μCi/ml) in the presence and absence of 20% FBS for the indicated times. To determine the disappearance from the apical side 3 × 30 μl of medium were counted. The percentage (average ± standard deviation) of the original [$^3$H]retinol present at a given time point is plotted.

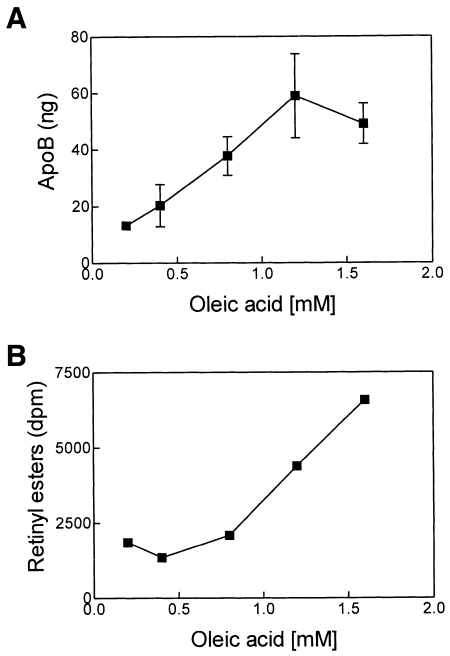


**Fig. 4.** Effect of different concentrations of oleic acid on the secretion of RE with CM. Differentiated Caco-2 cells were incubated with medium containing 20% FBS, 0.5 mM TC, and [$^3$H]retinol (5 μCi/ml) in the presence of different indicated concentrations of OA for 6 h. Basolateral medium were subjected to density gradient ultracentrifugation. Large and small CM fractions were combined. Aliquots (3 × 100 μl) were used to quantify apoB (A) and the remaining fractions were used for the determination of RE (B).

OA, apoB began to appear as CM in the basolateral medium and is thus consistent with an earlier study (9). The secretion of apoB as CM was saturated at 1.2 mM OA (Fig. 4A). In contrast to apoB, significant amounts of RE were secreted only at 1.2 mM OA and the secretion was not saturated at 1.6 mM OA. These data indicate that RE secretion is dependent on the presence of high concentrations of OA, and does not occur until cells are optimally secreting apoB as CM.

To study further the importance of CM assembly, we studied the effect of palmitic acid (PA) on the secretion of RE (**Fig. 5**). It is known that the supplementation of OA or PA results in the secretion of CM/VLDL- and IDL/low density lipoprotein (LDL)-size particles, respectively (34, 35). Incubation of cells with either OA or PA had no significant effect on the intracellular levels of RE (Fig. 5A). However, cells incubated with PA:TC secreted 4.5-fold less amounts of RE (Fig. 5B). The data indicate that secretion of CM-size particles is probably critical for the secretion of RE.

### Intracellular retinol metabolism under the conditions that simulate postprandial state

To study retinol metabolism under the conditions that simulate postprandial conditions, Caco-2 cells were incubated with OA:TC and [$^3$H]retinol for different time periods and the amounts of free and esterified retinol were determined in cells (**Fig. 6**). At 2 h, significant amounts (~75%) of retinol were present as free retinol. At later time points, there were variations in the amounts of cellular free retinol, but the data indicate that free retinol levels remain generally constant throughout the experiment. In contrast, RE levels were low at 2 h, increased significantly with time, reached a maximum at 6 h, and decreased progressively. It appears that the rates of increase and decrease of intracellular RE were similar. Between 4 and 10 h, equal amounts of free and esterified retinol were present in the cells. These data suggest that Caco-2 cells tightly control intracellular free retinol levels. When the influx of free retinol is increased, most of the retinol is stored as RE.

To determine the time course of RE secretion, differentiated Caco-2 cells were incubated with OA:TC and [$^3$H]retinol for different times (**Fig. 7**). The amount of protein per filter was 621, 625, 607, 635, 642, and 635 μg (average of two filters) at 2, 4, 6, 8, 10, and 12 h, respectively, indicating that cellular protein levels did not change during the course of the experiment. However, the amount of apoB (Fig. 7A) and RE (Fig. 7B) in the secreted CM increased steadily. There was a significant correlation ($r^2$ = 0.887, $P$ = 0.005) between the amount of apoB and RE secreted at different times as CM (Fig. 7C). However, there was no correlation ($r^2$ = 0.2118, $P$ = 0.3584) between total RE and total apoB secreted by these cells. These studies indicate that the secretion of RE with CM by these cells continues for a long time after the addition of OA:TC. Furthermore, the RE secretion is correlated significantly with CM secretion.

### Retinol metabolism under conditions that simulate fasting conditions

Retinol transport experiments in animals are usually performed in the postprandial state. However, the fate of vitamin A ingested during fasting conditions, in which the intestine does not secrete CM but secretes VLDL, is not

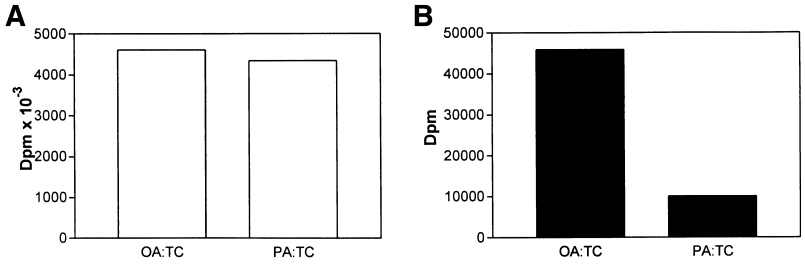


**Fig. 5.** Effect of supplementation of palmitic acid on the secretion of RE. Caco-2 cells were incubated with media containing 20% FBS, 6 mM TC, and [$^3$H]retinol (5 μCi), with either OA or PA (1.6 mM). After 18 h, intracellular (A) and secreted (B) amounts of RE were quantified as described in Materials and Methods.

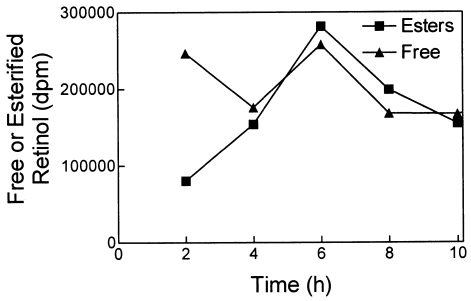


**Fig. 6.** Intracellular metabolism of retinol. Two Transwells of differentiated Caco-2 cells were incubated with OA:TC (1.6:0.5 mM) and [$^3$H]retinol (1 μCi/ml). At the indicated times, cells from two wells were washed and incubated with isopropanol to extract cellular lipids. Free and esterified retinol were separated with an alumina column as described in Materials and Methods. Values from both wells were averaged and plotted as line graphs.

known. We hypothesized that under fasting conditions retinol is stored as RE and under postprandial conditions it is secreted with CM, and evaluated this by performing pulse-chase studies. Differentiated Caco-2 cells were pulsed with [$^3$H]retinol for 6 h in the absence of OA:TC. Next, cells were washed extensively and supplemented with new medium containing either TC (simulating fasting state) or OA:TC. After 17 h, cells supplemented with TC and OA:TC contained $57 \times 10^3$ and $71 \times 10^3$ dpm as RE and $18 \times 10^3$ and $19 \times 10^3$ dpm as free retinol, respectively. Thus, cells supplemented with or without OA retained similar amounts of free and esterified retinol. Next, the distribution of secreted apoB, RE, and free retinol in different lipoprotein fractions was determined (**Fig. 8A**). Cells supplemented with TC secreted apoB as VLDL, IDL (Fx 1), and LDL (Fx 2)-size particles (Fig. 8A, open columns). The maximum apoB was in fraction 2. However, these cells did not secrete any significant amounts of RE (Fig. 8B). On the other hand, cells supplemented with OA:TC secreted significant amounts of RE, and secreted esters were present mainly in CM. Note that under these conditions only 30% of apoB was in large and small CM fractions, but these particles contained 79% of the secreted esters. Furthermore, the majority (26%) of apoB was in fraction 1, and this fraction contained small amounts (5%) of RE. Analysis of the free retinol revealed that the secretion of free retinol was considerably reduced in these experiments compared with that observed during the continuous pulse labeling of cells in Fig. 2 (note different y scales). Free retinol was secreted whether or not cells secreted CM (Fig. 7C). In the absence of OA, free retinol was secreted unassociated with lipoproteins. The free retinol secreted by cells supplemented with OA:TC was present in both lipoprotein and nonlipoprotein fractions. Within lipoprotein fractions, free retinol was mainly present with CM. These data indicate that cells store significant amounts of RE in the presence and absence of CM assembly. The RE are secreted only when cells assemble and secrete CM. The secretion of the stored RE does not parallel apoB but parallels the secretion of CM.

### Effect of inhibition of CM secretion on retinyl ester secretion

Next, we determined the fate of RE under conditions in which cells can assemble CM but do not secrete them. The aim was to distinguish whether conditions that support CM assembly are sufficient or whether the assembly and secretion of CM are important for RE secretion. When Caco-2 cells are supplemented with OA, CM assembly and secretion is induced. Pluronic L81 inhibits the secretion of CM but does not affect VLDL secretion at low concentrations (9). If the conditions that support CM assembly, but not its secretion, are important, then we hypothesized that Pluronic L81-treated cells might secrete RE with VLDL. For this purpose, we studied the effect of different concentrations of Pluronic L81 on RE secretion. Total apoB secretion was not affected in the presence of a 0–12 μg/ml concentration of Pluronic L81 (data not shown). Similarly, intracellular levels of esterified and free retinol were not affected by these concentrations of the surfactant (**Fig. 9A**). In contrast, increasing concentrations of Pluronic L81 significantly inhibited the secretion of RE as part of CM (Fig. 9B). The amounts of free retinol secreted with CM were low and were not affected by different concentrations of Pluronic L81. Subsequently, we determined whether Pluronic L81 affects the secretion of intracellular RE in different lipoproteins to a similar extent.

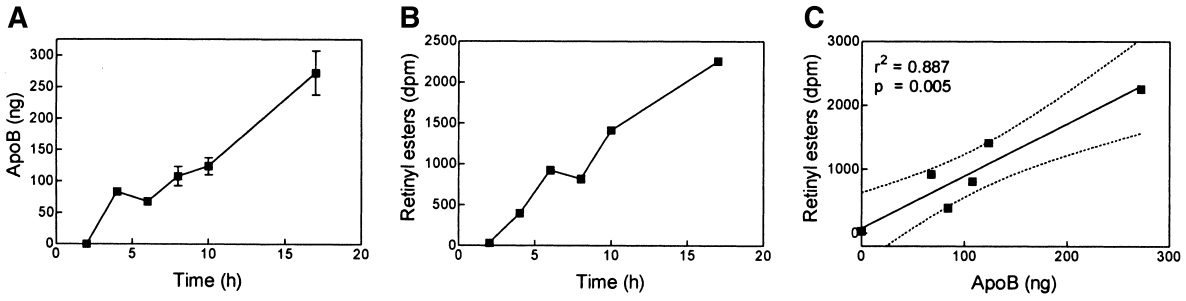


**Fig. 7.** Time course of the secretion of RE and apoB as CM. Two Transwells of differentiated Caco-2 cells were incubated with OA:TC (1.6:0.5 mM) and [$^3$H]retinol (1 μCi/ml) for different indicated times. Basolateral condition medium was pooled and subjected to density gradient ultracentrifugation to obtain different lipoprotein fractions. Large and small CM were pooled and used to determine the amounts of apoB and RE. The apoB (A) and RE (B) secreted as CM are plotted against time. For correlation (C), RE and apoB present in CM at different times were plotted, and subjected to linear regression analysis. Dotted curves represent the 95% confidence intervals.

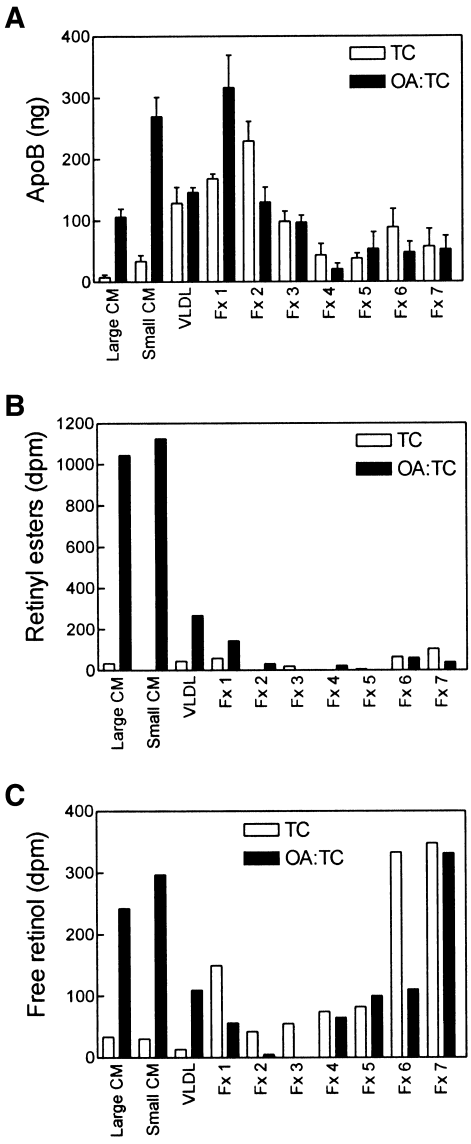


**Fig. 8.** Secretion of intracellular RE by Caco-2 cells. Cells were incubated with [$^3$H]retinol for 6 h, washed, and supplemented with medium containing either TC (0.5 mM) or OA:TC (1.6:0.5 mM). After 17 h, basolateral medium was subjected to density gradient ultracentrifugation, and distribution of apoB (A), RE (B), and free retinol (C) was determined in different fractions. The data are representative of three independent experiments.

Cells were pulse labeled with retinol for 6 h and then exposed to different concentrations of Pluronic L81 for 18 h in the presence of OA:TC (**Fig. 10**). Pluronic L81 at 3 μg/ml had no significant effect on the secretion of apoB in different lipoproteins compared with control. However, at 6 μg/ml it significantly inhibited the secretion of apoB in large CM but not in small CM and other smaller lipoproteins (Fig. 10A). Similar to apoB, Pluronic L81 at 3 μg/ml had no significant effect on the secretion of RE in different lipoprotein fractions, and at 6 μg/ml Pluronic L81 significantly inhibited the secretion of RE (Fig. 10B). However, in contrast to apoB, RE levels were decreased in all the lipoproteins. These data indicate that Pluronic L81 is a more potent inhibitor of the secretion of RE than it is of

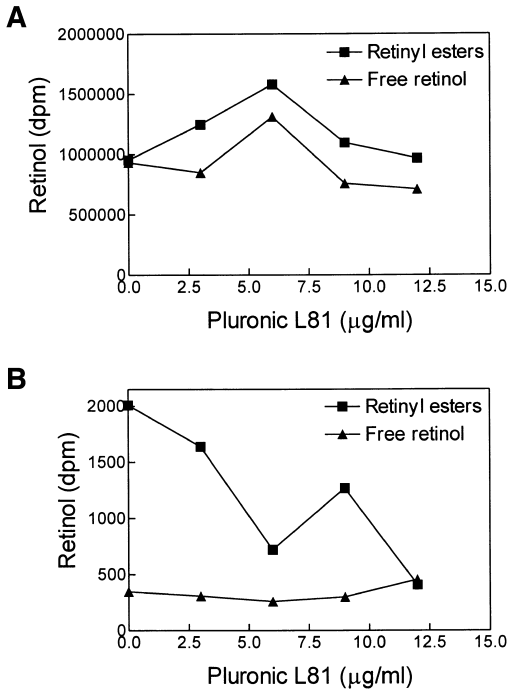


**Fig. 9.** Effect of Pluronic L81 on intracellular levels and secretion of RE. Differentiated Caco-2 cells were incubated with [$^3$H]retinol (5 μCi/ml) and OA:TC with different indicated concentrations of Pluronic L81 for 6 h. A: Cells were washed, lipids were extracted in isopropanol, and free retinol and RE were separated by alumina column chromatography and counted. Average values from duplicate determinations are presented. B: The basolateral-conditioned medium from two wells was pooled, and subjected to ultracentrifuge. RE and free retinol values in large CM and small CM were pooled and plotted against different concentrations of Pluronic L81.

CM secretion. It is possible that RE are added after the assembly of CM and Pluronic L81 may inhibit the incorporation of RE into CM.

## DISCUSSION

### CM assembly is essential for the secretion of RE

Our studies indicate that RE secretion follows CM secretion and not the secretion of apoB-containing lipoproteins. First, Caco-2 cells supplemented with TC and [$^3$H]retinol do not secrete RE even though they secrete apoB-containing VLDL, IDL, and LDL (Figs. 2, 7, and 9). Second, cells supplemented with OA:TC secrete CM and also secrete RE (Figs. 2, 5, 7, and 9). In contrast, cells supplemented with PA:TC secrete IDL/LDL-size particles (34, 35) and do not secrete RE (Fig. 5). Third, RE secretion appears to begin after cells are maximally primed for CM secretion (Fig. 4). Fourth, rates of secretion of RE and CM show significant correlation (Fig. 6). Fifth, inhibition of CM secretion by Pluronic L81 results in decreased secretion of RE (Figs. 8 and 9). Thus, the unique ability of Caco-2 cells to secrete apoB-containing lipoproteins of various sizes allows us to conclude that CM assembly is essential for the secretion of RE.

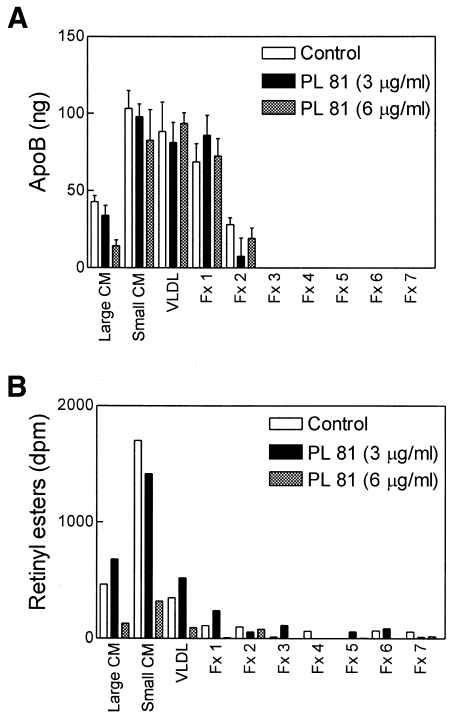


**Fig. 10.** Effect of Pluronic L81 on the secretion of intracellular RE in different lipoprotein fractions. Caco-2 cells were pulsed with [$^3$H]retinol for 6 h, washed, and then exposed to 20% FBS, OA:TC (1.6:0.5 mM) medium containing no (control), 3 μg/ml, or 6 μg/ml Pluronic L81 (PL 81) for 18 h. Basolateral medium was subjected to ultracentrifugation and the amounts of apoB (A) and RE (B) present in different lipoprotein fractions were determined.

Secretion of RE showed a remarkable specificity for the assembly and secretion of CM. The secretion of esters was not linked to retinol uptake, because the uptake of retinol was not affected by OA concentrations (Fig. 2). Furthermore, it was not linked to intracellular storage levels of RE because intracellular RE were similar when cells were supplemented either with TC alone or with OA:TC. However, the RE secretion was inhibited when CM secretion was inhibited by Pluronic L81 (Figs. 8 and 9). Thus, retinol secretion is dependent on the assembly and secretion of CM and is independent of the rate of retinol uptake and intracellular retinol ester levels.

In contrast to the amounts of triglycerides, RE constitute a small fraction of secreted lipoproteins. Our data indicate that the incorporation of RE into CM is not a passive process but is an exquisitely orchestrated and highly regulated event. RE secretion does not occur at all times. It is induced when cells can assemble and secrete CM. Secretion of RE does not follow the secretion of apoB, but follows the size of the particles. The need for CM may be related to the larger hydrophobic core available in these lipoproteins for the solubilization of RE. However, we observed that RE could be easily incorporated into phospholipid vesicles containing no triglycerides prepared by sonication (data not shown). Thus, it appears that intestinal cells have a specific recognition/delivery system for the incorporation of RE into CM and that these cells wait for CM assembly to secrete RE.

### RE: markers for the final stages of CM assembly?

Because of the remarkable specificity of RE with CM, we propose that RE may serve as markers with which to study the final stages of CM assembly. Two different models for the assembly of CM have been presented (2). In the independent model, CM and VLDL assembly is hypothesized to occur by two independent pathways (5, 36). On the basis of the data presented here, intracellular lipoproteins of various sizes that contain RE may represent intermediates in the CM assembly pathway. In contrast, apoB-containing lipoproteins of various sizes that lack RE may represent intermediates in VLDL assembly pathway.

In the sequential assembly model, CM assembly has been proposed to involve three distinct events: synthesis of primordial lipoproteins, formation of triglyceride-rich lipid droplets, and core expansion (2). We postulate that RE are added at the end of CM assembly and may serve as signposts for the final stages of CM assembly. If RE are specific markers for the final stages of CM assembly, then intracellular RE are not expected to associate with primordial lipoproteins. However, RE are expected to associate with intracellular CM and lipid droplets. Thus, RE may help distinguish between two models and help characterize various intermediates in the biosynthesis of CM.

### Cellular retinol metabolism under fasting and postprandial conditions

To address whether RE can be secreted during the fasting state, we took advantage of the observation that Caco-2 cells secrete apoB-containing lipoproteins of different sizes under different conditions (9, 26). Present studies show that Caco-2 cells do not secrete RE even when they secrete VLDL-, IDL-, and LDL-size apoB-containing, triglyceride-rich lipoproteins (Figs. 7 and 9). An important corollary of the observations is that RE are not transported across the intestinal epithelium in the fasting state. During the fasting state intestinal cells may absorb and store retinol both as free and in esterified forms but may not secrete them. In the postprandial state these cells take up retinol and esterify, store, and secrete RE. Thus, it is possible that ingestion of retinol in the fasting state may result in delayed bioavailability. In contrast, consumption of vitamin A with a fatty meal may result in immediate bioavailability. It is difficult to predict the ultimate bioavailability of retinol ingested in fasting and feasting conditions.

In contrast to the secretion of RE, secretion of free retinol was independent of the assembly and secretion of lipoproteins. During continuous pulse experiments, significant amounts of free retinol were secreted toward the basolateral sides (Fig. 2). The transport of retinol across the cells may occur in the direction of the concentration gradient due to transcytosis. In pulse-chase experiments smaller amounts of free retinol were secreted toward the basolateral side (Fig. 8), and might represent an active uptake and subsequent regulated secretion bound to retinol-binding protein. This suggestion is in agreement with the observations that, besides the lymphatic route, some of the retinol is transported directly to the portal circulation (37).

In summary, we have shown that retinol is taken up rap-

idly by differentiated Caco-2 cells, esterified, and stored in the cells. A portion of the intracellular free retinol is secreted associated and unassociated with lipoproteins, whereas a portion of intracellular RE is secreted with lipoproteins. Secretion of RE was dependent on the assembly and secretion of CM. It is proposed that RE secretion with CM is a specific and regulated process and that the association of RE with CM may be used as a signpost to characterize final stages in the molecular assembly of CM. Understanding this process may unravel novel mechanisms controlling the intracellular handling of fat-soluble vitamins and other micronutrients by intestinal cells.

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