Intestinal Absorption of Retinol and Retinyl Palmitate in the Rat. Effects of Tetrahydrolipstatin

Ester Fernández[1,*] and **Bengt Borgström**
Department of Physiological Chemistry, University of Lund, S-221 00 Lund, Sweden

The aim of the present study was to characterize the intestinal absorption of retinol and retinyl palmitate in thoracic duct and bile duct fistulated rats and to investigate the effect of a simultaneously administered lipase inhibitor, tetrahydrolipstatin (THL). Absorption was determined as lymphatic recovery over a 24-hr period, including an initial 12-hr continuous intraduodenal infusion of either [11,12-^{3}H]retinol or [11,12-^{3}H]retinyl palmitate given in emulsified glyceryl trioleate or in mixed micellar solution of monoolein and oleic acid. From micellar dispersion, labeled retinol and retinyl palmitate were recovered in the lymph to 50–60% and both to the same extent. Administered in emulsified form, labeled retinol from fed retinyl palmitate was recovered to 47%, but retinol from fed retinol to only 18%. THL (10^{-4} M) in the infusate had no significant effect on the recovery of ^{14}C-labeled oleic acid. The recovery of label from emulsified glyceryl tri[1-^{14}C]oleate was significantly decreased at this concentration of THL (76.5% vs 19.6% recovery). When administered in emulsified form, retinol absorption was not significantly affected by THL at 10^{-4} M, while retinyl palmitate absorption was very significantly decreased (5.0% compared to 47.8%). In the presence of THL, retinol absorption from retinyl palmitate in micellar solution was decreased (from 58% to 17%). Most of the retinol in the lymph extracts (72.2 to 91.3) was present as retinyl ester, regardless of the chemical and physical form of administration. Furthermore, THL did not induce any change in this pattern.
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The intestinal absorption of nonpolar fats, such as cholesterol, is dependent on a mixed bile salt micellar system, normally generated from the hydrolysis of dietary triglycerides by the action of gastrointestinal lipases. The absorption of highly nonpolar long-chain cholesterol esters is also directly dependent on the lipolytic process as the esters must be hydrolyzed to free cholesterol prior to absorption (1).

It has recently been shown that a lipase inhibitor, tetrahydrolipstatin (THL), almost completely prevents the intestinal absorption of cholesterol from cholesteryl oleate in micellar as well as emulsified dispersions, while free cholesterol is not affected (1). In the present study we have extended these experiments to include the effect of THL on the intestinal absorption of the analogous pair retinol/retinyl palmitate.

[1]Present address: Department of Physiology and Cell Biology, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.
*To whom correspondence should be addressed.

Abbreviations: DMSO, dimethylsulfoxide; TLC, thin-layer chromatography; THL, tetrahydrolipstatin; EDTA, ethylenediaminetetraacetic acid.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Oleic acid, [11,12-^{3}H]retinol and glyceryl tri[^{14}C]oleate were purchased from Amersham International, Buckinghamshire, U.K. [11,12-^{3}H]Retinyl palmitate was synthesized from free labeled retinol according to the method described by Baldwin and Daubert (2) as modified in our laboratory for use with light-sensitive retinol derivatives. The radiopurity was better than 92% as determined by thin-layer chromatography (TLC) radioactivity. Sodium taurocholate and sodium taurodeoxycholate were synthesized according to Norman (3). 1-Monoolein was prepared according to Mattson and Volpenheim (4). Lecithin was purified as described by Singleton *et al.* (5).

THL [(S)-1-(2S,3S)-3-hexyl-4-oxo-2-oxetanyl)methyldodecyl(S)-2-formamido-3-methyl-valerate] was obtained from F. Hoffmann La Roche Ltd. (Basel, Switzerland). All solvents were redistilled before use and all other reagents were of analytical grade.

Animals. Male Sprague-Dawley rats weighing 275–325 g were obtained from ALAB (Stockholm, Sweden), fed with a standard rat diet and kept in a room with controlled environmental temperature, humidity and lighting cycle (12 hr light/12 hr dark).

Surgery. After an overnight fast, the rats were anesthetized by inhalation of diethyl ether. The thoracic duct was cannulated according to the method described by Bollman *et al.* (6) and modified later by Lindström (7). The bile duct was cannulated allowing intact pancreatic flow to the intestinal lumen. A duodenal fistula was made inserting a silicone-tipped vinyl catheter in the duodenum through the gastric fundus. Immediately after surgery, 1 mg/kg of diazepam was given intraperitoneally.

A glucosaline solution (2.5% glucose, 0.5% NaCl and 0.05% KCl) was infused through the duodenal catheter at a rate of 3 mL/hr for 18–24 hr. The animals were allowed to drink *ad libitum* the same glucosaline solution. After this recovery period, the experimental infusates containing labelled lipids were given intraduodenally at the same rate for 12 hr. After this period, the lipid-containing infusates were substituted again by the glucosaline solution which was infused for another 12-hr period. Drinking was not allowed during this period. Lymph was collected hourly for 24 hr in tubes moistened with ethylenediaminetetraacetic acid (EDTA) solution.

This experiment has been approved by the Ethical Committee of the University of Lund.

Micellar infusates. Suitable amounts of either unlabelled or radioactive lipids were taken from organic stock solutions and placed into a round flask. The solvents were evaporated under nitrogen at room temperature and protected from light. Total bile salts were dissolved in 1/5 volume (i.e., 10 mL) of buffer (12 mM Na_2HPO_4; 8 mM NaH_2PO_4; 113 mM NaCl; 5 mM KCl; 1 mM $CaCl_2$ and 10 mM glucose, pH 6.5). This mixture was stirred with an egg-shaped magnetic bar at 500 rpm till there was no evidence of floating fat droplets. The

remaining 4/5 volume of buffer (i.e., 40 mL) was added with continuous stirring. A suitable amount of THL was taken from a stock dimethyl sulfoxide (DMSO) solution (25 mg THL/mL DMSO) and added to the whole volume to reach a final concentration of 10^{-4} M. The same amount of DMSO (100 μL) was added to the control infusates. The final concentration of DMSO in all the infusates was approximately 2.2 mg/mL. The pH of the infusate was set at 6.5 before infusion.

Infusate A: 10 mM [1-^{14}C]oleic acid; 5mM 1-monoolein; 2 μM [11,12-^{3}H]retinol; 20 mM sodium taurocholate and 5 mM sodium taurodeoxycholate in buffer solution. This was a clear solution. Slight turbidity appeared when THL 10^{-4}M was added.

Infusate B: 10 mM [1-^{14}C]oleic acid; 5 mM 1-monoolein; 2 μM [11,12-^{3}H]retinyl palmitate; 20 mM sodium taurocholate and 5 mM sodium taurodeoxycholate in buffer solution. This was a clear solution. Slight turbidity appeared when 10^{-4}M THL was added.

Emulsified infusates. Suitable amounts of either unlabelled or radioactive lipids were taken from organic stock solutions and put in 20-mL glass vials. The solvents were evaporated under nitrogen at room temperature and protected from light. Total bile salts were dissolved in 1/5 volume (i.e., 10 mL) of buffer (12 mM Na_2HPO_4; 8 mM NaH_2PO_4; 113 mM NaCl; 5 mM KCl; 1 mM $CaCl_2$ and 10 mM glucose, ph 6.5) and added to the vial. A sufficient amount (100 μL) of THL dissolved in DMSO (25 mg THL/mL DMSO) or 100 μL of DMSO (control) was also added to the vial. This mixture was sonicated 4 × 30″ (Branson sonifier). After sonication the whole was transferred to a beaker and the remaining 4/5 volume of buffer (i.e., 40 mL) was added. The mixture was sonicated again (4 × 30″). The final concentration of DMSO in all the emulsions was 2.2 mg/mL. In emulsions containing THL, the final concentration was always 10^{-4} M. The pH of the infusate was set at 6.5 before infusion.

Infusate C: 5mM glyceryl tri[^{14}C]oleate; 0.286 mM phosphatidylcholine; 2μM [11,12-^{3}H]retinol; 20 mM sodium taurocholate and 5 mM sodium taurodeoxycholate in buffer solution.

Infusate D: 5mM glyceryl tri[^{14}C]oleate; 0.286 mM phosphatidylcholine; 2μM [11,12-^{3}H]palmitate; 20 mM sodium taurocholate and 5 mM sodium taurodeoxycholate in buffer solution.

Micellar solutions were infused using a syringe infusion pump. Emulsions were infused with a peristaltic pump (Microperpex®, LKB, Bromma, Sweden) with continuous magnetic stirring of the reservoir flask. The infusates were protected from light during infusion.

Radioactivity determinations. Two hundred mL aliquots of the hourly lymph samples and 800 μL of distilled water were mixed with 10 mL of emulsifying scintillant (Ready Safe, Beckman, Fullerton, CA). The radioactivity was counted in a Packard Tri-Carb scintillation counter equipped with an automatic quench correction program.

After extraction of the pooled lymph samples with chloroform/methanol (2:1, v/v) and splitting of the phase with water, the relative amounts of free and esterified retinol were determined in the chloroform phase. To separate retinol and retinyl esters, aliquots of the chloroform phase were evaporated, redissolved in a small volume of chloroform and run on TLC plates (Alufolien, Merck, Darmstadt, Germany) using a solvent system consisting of light petroleum ether/diethyl ether/acetic acid (79:20:1, v/v/v).

RESULTS

Lymphatic transport of retinol and retinyl palmitate. The lymphatic output of ^{3}H-activity derived from the infused [11,12-^{3}H]retinol increased during the first 3 hr and reached a steady value 4–5 hr after the start of the intraduodenal infusion of the probe. The kinetic profile was similar regardless of the physicochemical state of the infusate (i.e., micellar solution or emulsion). Despite the similarity of the kinetic profile, the steady-state level reached was different after infusing emulsions and micellar solutions (approximately 45% and 20% of the infused dose/hr for emulsions and micellar solutions, respectively). When [11,12-^{3}H]retinyl palmitate was infused either in micellar or emulsified form, the steady-state level reached was around 50–60% for both systems. These results are depicted in Figures 1 and 2.

After the infusion of micellar solutions containing tritium-labeled retinol or retinyl palmitate, the total recoveries of ^{3}H-activity in lymph were 49.4% and 58.0% respectively. The difference was probably significant ($P = 0.055$). Table 1 summarizes these results.

Following the infusion of emulsions containing tritium-labeled retinol, the total percentage of ^{3}H-activity recovered in lymph was 19.1%. In contrast, the recovery of ^{3}H-activity derived from the infusion of [^{3}H]retinyl palmitate was 47.8%. The difference between these values is highly significant ($P < 0.0001$). Table 1 summarizes the recovery values under the different experimental conditions.

Effects of tetrahydrolipstatin on retinol and retinyl palmitate lymphatic transport. The presence of THL at a final concentration of 10^{-4}M in the micellar solution decreased to a high extent the total lymphatic output of ^{3}H-radioactivity derived from [^{3}H]retinyl palmitate

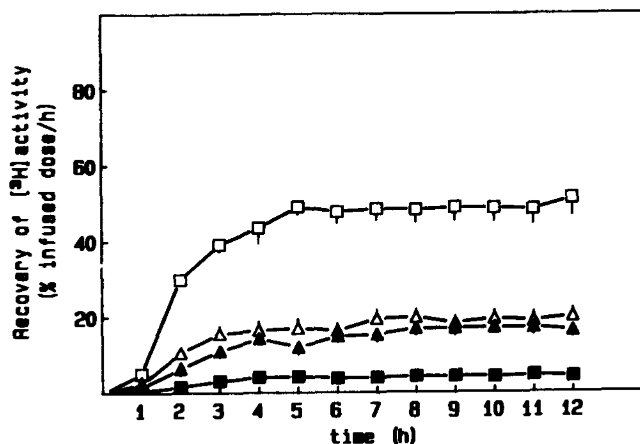

FIG. 1. Hourly recovery (% of the infused dose/hr) of ^{3}H-activity in lymph during a 12-hr infusion of emulsions containing: [^{3}H]retinol + [^{14}C]triolein + phosphatidylcholine + bile salts with (▲) or without (△) THL; [^{3}H]retinyl palmitate + [^{14}C]triolein + phosphatidylcholine + bile salts with (■) or without (□) THL. Data points represent mean ± SEM.

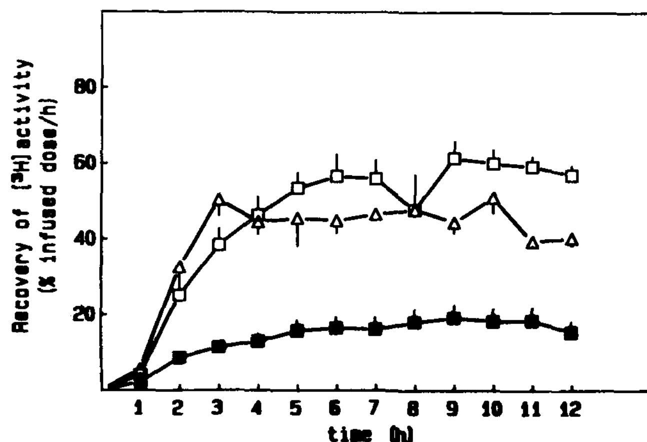

FIG. 2. Hourly % recovery (% of the infused dose/hr) of ^{3}H activity in lymph during a 12-hr infusion of micellar solutions containing: [^{3}H]retinol + [^{14}C]oleic acid + monoolein + bile salts (△); [^{3}H]retinyl palmitate + [^{14}C]oleic acid + monoolein + bile salts with (■) or without (□) THL. Data points represent mean ± SEM.

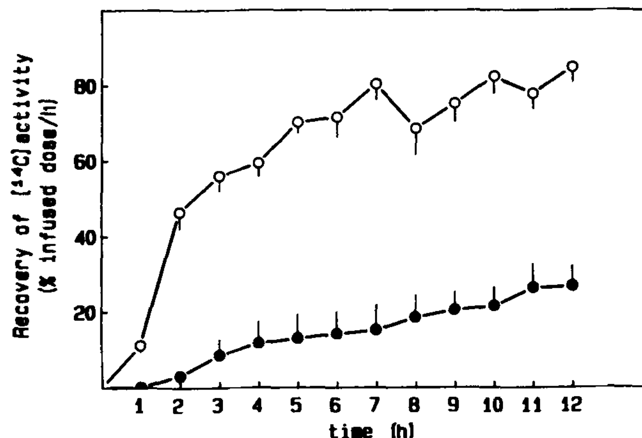

FIG. 3. Hourly % recovery of ^{14}C activity in lymph after a 12-hr infusion of emulsions containing: [^{3}H]retinol + [^{14}C]triolein + phosphatidylcholine + bile salts with (●) or without (○) THL. Data points represent mean ± SEM.

TABLE 1

Total 24-hr Lymphatic Recovery of [^{3}H]Activity from Infused [^{3}H]Retinol and [^{3}H]Retinyl Palmitate[a]

	% recovery in 24-hr lymph
Micellar infusates	
Retinol	49.4 ± 3.8[b]
Retinyl palmitate	58.0 ± 8.2
Retinyl palmitate + THL 10^{-4}M	17.4 ± 8.7[c]
Emulsified infusates	
Retinol	18.0 ± 6.0[d]
Retinol + THL 10^{-4}M	15.7 ± 2.1
Retinyl palmitate	47.8 ± 7.8[e]
Retinyl palmitate + THL 10^{-4}M	5.0 ± 2.4[f]

[a]Data are presented as mean % recovery ± standard deviation.
[b]Probably significantly different compared to micellar retinyl palmitate ($P < 0.055$).
[c]Significantly lower than micellar retinyl palmitate ($P < 0.0001$).
[d]Significantly lower than micellar retinol ($P < 0.0001$).
[e]Significantly higher than emulsified retinol ($P < 0.0001$).
[f]Significantly lower than micellar retinyl palmitate ($P < 0.0001$).

(control 58.0% recovery; 10^{-4}M THL 17.4% recovery, inhibition = 70%, $P < 0.0001$).

The presence of 10^{-4}M THL in emulsions containing [11,12-^{3}H]retinyl palmitate led to a more pronounced decrease in the total lymphatic output of ^{3}H-radioactivity (5.0% recovery vs 47.8% in controls). In this case the percentage inhibition was 90%.

The total lymphatic recovery of ^{3}H-radioactivity from emulsions containing [^{3}H]retinol was slightly lower in the presence of 10^{-4}M THL, although the difference with respect to controls was not significant (15.7% vs 18.0%, $P = 0.34$, Table 1).

The TLC analysis of the pooled lymph extracts showed that most of the ^{3}H-radioactivity (72.2 to 91.3%) is found in the retinyl palmitate fraction regardless of the chemical form of retinol infused. THL did not induce any significant difference in the distribution pattern of retinol/retinyl palmitate in the lymph extracts (77.0% to 95.6% of the radioactivity was found in the retinyl palmitate fraction).

The lymphatic output of ^{14}C-radioactivity after infusing emulsified glyceryl tri[1-^{14}C]oleate is significantly decreased (76.5% vs 19.6% recovery in controls and THL 10^{-4}M; $P = 0.0001$, 74.4% inhibition). The kinetics of this process is presented in Figure 3. THL had no effect on the incorporation of ^{14}C-radioactivity derived from micellar infusates containing ^{14}C-labeled oleic acid.

DISCUSSION

Most reports in the literature focus on the intestinal absorption of free retinol and its provitamin forms (8–11), whereas little is known about the relative bioavailability of the esterified form. Adhikari *et al.* (12) studied the absorption of [^{3}H]retinol and unlabeled retinyl palmitate after oral administration to normal and protein-deficient rats. The experimental design did not allow a comparison of the extent of absorption, as only the amount found in the intestinal contents, small intestine and muscles were accounted for. To our knowledge, the present study is the first to report the relative lymphatic transport of [^{3}H]retinol and [^{3}H]retinyl palmitate in the rat. In our experiments, the concentrations of retinol and retinyl palmitate in the infusions were adjusted to give approximately the recommended daily intake of vitamin A during the 12-hr intraduodenal infusion (13). This is particularly relevant considering the results of Hollander and Muralidhara (11) showing that the kinetics of the absorptive process for vitamin A is different when given at physiological and pharmacological levels.

In the present investigation, labeled retinol and retinyl palmitate have been infused intraduodenally in micellar and emulsified form. In the former dispersion, the fat should be in a chemical and physical form available for uptake by the intestinal mucosa, although the retinyl ester would be expected to have to undergo hydrolysis prior to absorption. In the emulsified form, micelles

should be expected to be generated by hydrolysis of the triglycerides (and the retinyl ester bond should be hydrolyzed).

If the transport to the lymph of labeled retinol infused in micellar form (49.4%) is compared to the emulsified (18.0%) form, a significant difference is seen. Such a difference was not seen in a similar study in which cholesterol was the nonpolar lipid (1). So far this difference is unexplained.

The transport of retinol to the lymph from micellar retinol and retinyl palmitate is similar, as is the transport from emulsified retinyl ester. This agrees with the findings for cholesterol and cholesteryl oleate in a previous study (1), although there is a general tendency to a higher absorption from micellar compared to emulsified probes. Such a difference may be explained by the immediate availability of the lipids in micellar form when the infusate reaches the intestine.

The presence of 10^{-4}M THL in the infusate significantly reduces the recovery of triglyceride in the lymph while it does not significantly affect the recovery of fatty acid from micellar infusates (this study and ref. 1). This difference is most likely explained by an inhibition of hydrolysis of triglycerides by THL in the lumen. This effect, however, does not seem strong enough to significantly inhibit retinol absorption.

The lymphatic transport of radioactive retinol from retinyl palmitate is significantly inhibited by THL from micellar as well as emulsified substrates indicating the importance of inhibition of the enzyme hydrolyzing retinyl palmitate. The enzyme most probably responsible for the hydrolysis of retinyl esters is the carboxyl ester lipase (cholesterol ester hydrolase) of pancreatic origin (14). This enzyme has been ascribed importance in the absorption of cholesterol and cholesteryl esters by Bhat and Brockman (15). Although THL at the concentration used in this study had no significant effect on the absorption of free cholesterol from micellar or emulsified dispersions, the inhibition of cholesteryl oleate absorption in both administration forms was much more marked (0.13% and 0.09% transport to the lymph) as compared to the absorption of retinol from retinyl palmitate (17.4 and 5.0%, respectively). A difference in the experimental design between this and the previous study is the difference in concentration of the probes in the infusates (2 μM for retinol and retinyl ester and 150 μM for cholesterol and its ester). How this difference affects the kinetics of digestion and absorption is not known.

The results of this investigation support the idea that retinyl esters have to be hydrolyzed before absorption, and that the enzyme which hydrolyzes retinyl esters is of no importance for the absorption of retinol from the intestine.

TLC analysis of the lymph extracts indicates that retinyl ester is the major form of vitamin A secreted by the enterocyte to the chyle. There was no difference in the distribution pattern of free and esterified retinol in lymph after the administration of retinol or its ester. Furthermore, THL did not induce any significant change in this pattern. This indicates that THL does not modify the reesterification process that takes place in the enterocyte. Similarly, we have previously shown that THL does not affect reesterification of cholesterol. This process, therefore, is not dependent on enzymes which can be efficiently inhibited by THL, or, alternatively, that THL, if absorbed into the enterocyte is metabolized, losing its inhibitory properties.

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