Rapid transient absorption and biliary secretion of enantiomeric cholesterol in hamsters

Emily J. Westover,[1,*] Xiaobo Lin,[1,†] Terrence E. Riehl,[§] Lina Ma,[†] William F. Stenson,[§] Douglas F. Covey,[*] and Richard E. Ostlund, Jr.[2,†]

Department of Molecular Biology and Pharmacology,* Division of Endocrinology, Metabolism, and Lipid Research,† and Division of Gastroenterology,§ Department of Medicine, Washington University School of Medicine, St. Louis, MO 63110

Abstract **To probe the pathway and specificity of cholesterol absorption, the synthetic enantiomer of cholesterol (*ent*-cholesterol) and cholesterol were labeled with deuterium, gavaged into hamsters, and measured by negative ion mass spectrometry. Initial uptake of both tracers into the intestinal mucosa at 30 min was similar but cholesterol was temporarily retained there, whereas mucosal *ent*-cholesterol declined rapidly with concomitantly increased enrichment in both the systemic circulation and the gut lumen. In a 3 day fecal recovery study, *ent*-cholesterol was quantitatively recovered in the stool, whereas cholesterol absorption was 53.2%. *ent*-Cholesterol given by intracardiac injection was selectively secreted into bile, and the ratio of *ent*-cholesterol to cholesterol tracers in the gut lumen increased down the length of the small bowel, with the largest value being found in stool. *ent*-Cholesterol is efficiently taken up by the intestinal mucosa and undergoes transient enterohepatic recirculation, but it is quantitatively eliminated over 3 days as a result of selective secretion into bile and selective enrichment within the lumen of the intestine. These findings suggest that cholesterol absorption is structurally specific and likely to be mediated by enantiospecific cellular proteins.—**Westover, E. J., X. Lin, T. E. Riehl, L. Ma, W. F. Stenson, D. F. Covey, and R. E. Ostlund, Jr. **Rapid transient absorption and biliary secretion of enantiomeric cholesterol in hamsters.** ***J. Lipid Res.*** **2006.** 47: **2374–2381.**

Supplementary key words cholesterol absorption • enantiomer • deuterium • mass spectrometry • animal studies

The intestinal absorption of dietary and endogenous biliary cholesterol is a key regulatory control point for cholesterol homeostasis. In contrast to other nutrients, only approximately half of intestinal cholesterol is normally absorbed, and excretion of the unabsorbed fraction constitutes the principal pathway for cholesterol catabolism (1). Reducing cholesterol absorption is a potentially effective way to reduce LDL-cholesterol levels and cardiovascular risk. However, the molecular mechanisms underlying cholesterol absorption are complex and not yet fully understood.

Although cholesterol absorption can be completely blocked by biliary diversion or bile salt deficiency, attempts to disrupt cholesterol absorption selectively while maintaining the absorption of other nutrients have been only moderately successful. Phytosterols reduce cholesterol absorption by ~35% when added to the diet (2), whereas the new drug ezetimibe displays maximum inhibition of 54% (3). Many of the proteins that may play a role in cholesterol absorption have been identified, including ABCA1, ABCG5, and ABCG8 (sterol efflux pumps), Niemann-Pick C1-Like 1 (NPC1L1) (a target of ezetimibe), and ACAT-2 (the intestinal form). However, single-gene knockout of each of these candidates has produced incomplete reduction in cholesterol absorption efficiency (4–8). Likewise, overexpression of NPC1L1 in nonenterocyte cells failed to generate active cholesterol transport, suggesting that additional subunits or cofactors may be required (9). Therefore, although the hypothesis that cholesterol is absorbed by a specific protein or proteins is attractive, the evidence to support it is still incomplete.

Cholesterol absorption is inherently a low-affinity process acting on millimolar amounts of cholesterol in the intestine. Thus, the specificity of cholesterol absorption cannot be studied effectively by traditional kinetic methods using a tracer and its competing unlabeled ligand. An alternative approach is to use the unnatural enantiomer of cholesterol (*ent*-cholesterol) in metabolic studies. As shown in **Fig. 1**, *ent*-cholesterol is the mirror image of cholesterol and is chemically synthesized with the opposite configuration at each of the eight chiral centers. *ent*-Cholesterol has physical properties identical to those of cholesterol with a different three-dimensional configuration, making it likely to be recognized differently by cell

Manuscript received 11 April 2006 and in revised form 1 August 2006 and in re-revised form 25 August 2006.

Published, JLR Papers in Press, August 26, 2006.
DOI 10.1194/jlr.M600165-JLR200

Abbreviations: NPC1L1, Niemann-Pick C1-Like 1.

[1] E. J. Westover and X. Lin contributed equally to this work.

[2] To whom correspondence should be addressed.
e-mail: rostlund@im.wustl.edu

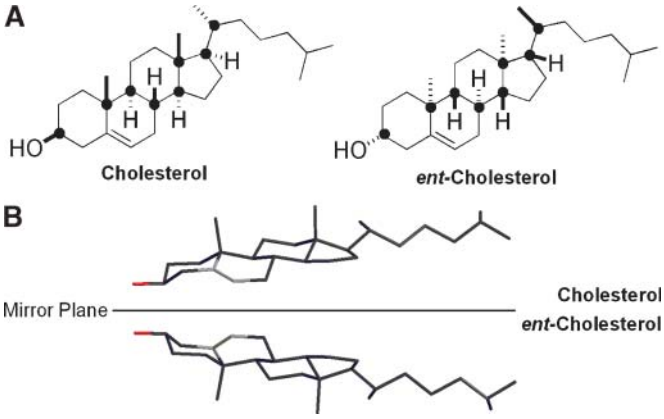

Fig. 1. Structures of cholesterol and *ent*-cholesterol. Cholesterol and *ent*-cholesterol are shown in two- and three-dimensional representations, highlighting the mirror image relationship between the molecules. In the two-dimensional representation, all eight stereocenters are noted with black circles. In the three-dimensional representation, all hydrogens have been removed, the 3-hydroxyl group is shown in red, and the double bond at C5-C6 is shown in light gray. At a glance, the enantiomers appear quite similar, consistent with their identical chemical constitution and physical properties. However, a closer look shows the difference in absolute configuration or three-dimensional shape. By virtue of their different shapes, cholesterol and *ent*-cholesterol may be distinguished by proteins. It is useful to note that although *ent*-cholesterol has a 3α-hydroxyl group, this group is equatorial (i.e., in the plane of the rings) as it is in cholesterol, because all of the stereocenters in the molecule are inverted.

proteins. By contrast, the interactions of cholesterol and *ent*-cholesterol with other lipids are identical (10, 11). Because enantiomers are not separable by conventional, nonchiral chromatographic methods and have identical mass spectra, the use of differential deuterium labeling and mass spectrometry for their detection is required.

In this work, we gavaged hamsters with a mixture of cholesterol labeled with seven deuterium atoms and *ent*-cholesterol labeled with two deuterium atoms and measured tracer enrichment with negative ion mass spectrometry. *ent*-Cholesterol was recognized differently from cholesterol and, despite rapid transient absorption into the systemic circulation, was quantitatively excreted in the stool over 3 days. Moreover, *ent*-Cholesterol tracer given by intracardiac injection was selectively secreted into bile, enriched in the intestinal lumen, and eliminated in the stool.

MATERIALS AND METHODS

Materials

[25,26,26,26,27,27,27-$^{2}H_7$]cholesterol was synthesized by CDN Isotopes (Montreal, Quebec, Canada). Deuterated *ent*-cholesterol was synthesized by reduction of *ent*-desmosterol (*ent*-Δ^{24}-cholesterol) using a platinum/carbon catalyst and deuterium gas as described (12). The actual distribution of deuterium incorporation was 38.2% none, 35.7% one, 16.2% two, 6.4% three, 2.3% four, 0.9% five, and 0.3% six, similar to previous reports of sterol side chain labeling reactions (13). [5,6,22,23-$^{2}H_4$]sitostanol was purchased from Medical Isotopes (Pelham, NH).

Animals

F1B male hamsters weighing ~120 g were obtained from Charles River Breeders (Boston, MA) and housed individually in a room maintained at 24°C with a 12 h light/12 h dark cycle. Animals were fed Purina Rodent Chow 5053 containing 4.5% fat, 20.0% protein, and 54.8% carbohydrate. Procedures were approved by the Washington University Animal Studies Committee.

Animal protocols

ent-Cholesterol and cholesterol tracers were mixed in ethanol, dried under vacuum, and redissolved in soybean oil. For time-course experiments, food was removed at the beginning of the light cycle at 6 AM; the animals were fasted for 2 h and then gavaged with 0.6 ml of soybean oil only (for time 0 controls) or containing 0.34 mg of cholesterol-d_7 and 1.06 mg of *ent*-cholesterol-d_2. Hamsters (three for each time point) were euthanized by carbon dioxide asphyxia at 0, 0.5, 1, 2, and 4 h afterward. Blood was collected by heart puncture, and the liver and small intestine were removed. The small intestine was divided into four equal segments that were analyzed separately. Lumenal contents were collected by washing with ice-cold PBS, and then the mucosa was separated from the bowel wall and extruded by exerting pressure with a microscope slide. Lipids were extracted by the method of Bligh and Dyer (14) and saponified. The neutral sterols were extracted into petroleum ether, dried, and converted to pentafluorobenzoyl esters (15).

In some experiments, mucosal free and esterified cholesterol were analyzed separately. To aliquots of the unsaponified total lipid extracts were added 3 μg of sitostanol oleate and 3 μg of sitostanol-d_4 as internal standards. The mixture was taken up in 0.5 ml of hexane and applied to 3 ml silica solid-phase extraction columns (LC-Si; Sigma, St. Louis, MO) preequilibrated with hexane. Sterol esters were eluted with 6 ml of 2% ethyl acetate in hexane, and free sterols were then collected in 6 ml of 25% ethyl acetate in hexane. The fractions were then saponified and prepared for GC-MS.

For 3 day cholesterol absorption measurements, five hamsters were gavaged with 0.9 ml of soybean oil containing 2.390 mg of *ent*-cholesterol-d_2, 0.787 mg of cholesterol-d_7, and 0.0787 mg of sitostanol-d_4. Three control hamsters received 0.9 ml of soybean oil alone. Stools were collected from wire-bottomed cages for a 3 day period, after which blood was obtained by cardiac puncture. Serum and stool samples were processed as described above.

For the intracardiac injection experiment, *ent*-cholesterol and cholesterol tracers were dissolved in ethanol and then mixed with Intralipid (16). Either Intralipid only (0.25 ml for time 0 controls) or Intralipid containing 0.160 mg of cholesterol-d_7 and 0.499 mg of *ent*-cholesterol-d_2 was administered to hamsters by intracardiac injection after 2 h of fasting, and animals (n = 3 for each time point) were euthanized at time 0 and after 4 or 24 h. Tissues were collected as for the gavage experiment. Food was only returned to animals for the 24 h time point.

GC-MS

Steryl pentafluorobenzoates, which give intense molecular anions (15), were separated on a 15 m × 0.25 mm inner diameter RTX-200 trifluoropropylmethyl polysiloxane column with 0.5 μm film thickness (Restek, Bellefone, PA). The oven temperature was kept at 250°C for 2 min, increased at a rate of 20°C/min to a final temperature of 320°C, and held for 4.5 min. The effluent was analyzed in an Agilent Technologies 5972 quadrupole mass spectrometer (Palo Alto, CA) using negative ion chemical ionization mode. Selected ion monitoring was performed at *m/z* 580 (M^- for cholesteryl pentafluorobenzoate), 581, 584, and

587 for cholesterol and *ent*-cholesterol and at *m/z* 610 and 614 for sitostanol.

Calculations

The enrichment of deuterated tracers was determined from ion areas measured at *m/z* 587 (cholesterol) and 584 (*ent*-cholesterol). The latter mass was chosen because it resulted in optimum discrimination between *ent*-cholesterol tracer and the large background of unlabeled natural cholesterol. Using a system of simultaneous equations, the tracer peaks at *m/z* 587 and 584 were corrected for the contribution of unlabeled natural cholesterol and for contributions from each other. Note that more *ent*-cholesterol than cholesterol tracer was given to improve its detection but that enrichments are reported as the mole ratio of tracer with respect to natural cholesterol per milligram of administered tracer [(mol tracer/mol cholesterol-d_0) × 100/mg tracer administered]. The percentages of *ent*-cholesterol-d_2 and cholesterol-d_7 in the liver and stool were calculated from each tracer recovered relative to its respective total amount administered. Results presented are means ± SEM. The independent effects of time, intestinal quarter, and type of tracer were analyzed using a general linear statistical model (SAS Institute, Cary, NC). A two-sample *t*-test was used to compare tracer enrichments whenever appropriate.

RESULTS

Oral time-course study

Cholesterol absorption is a multistep process that includes uptake into the mucosa from the intestinal lumen, transit across the enterocyte, and delivery into the systemic circulation. To examine the initial phase of absorption, hamsters were gavaged with a mixture of cholesterol-d_7 and *ent*-cholesterol-d_2. Tracer levels in the intestinal lumen and mucosa, serum, and liver were then determined by GC-MS at 0.5, 1, 2, and 4 h. Each quarter of the small bowel was analyzed separately. Data are presented as tracer enrichment (defined in Materials and Methods), values analogous to specific activity or cpm/mg cholesterol in experiments using radioactivity. **Figure 2A, B** show that mucosal uptake of both tracers was similar at early time

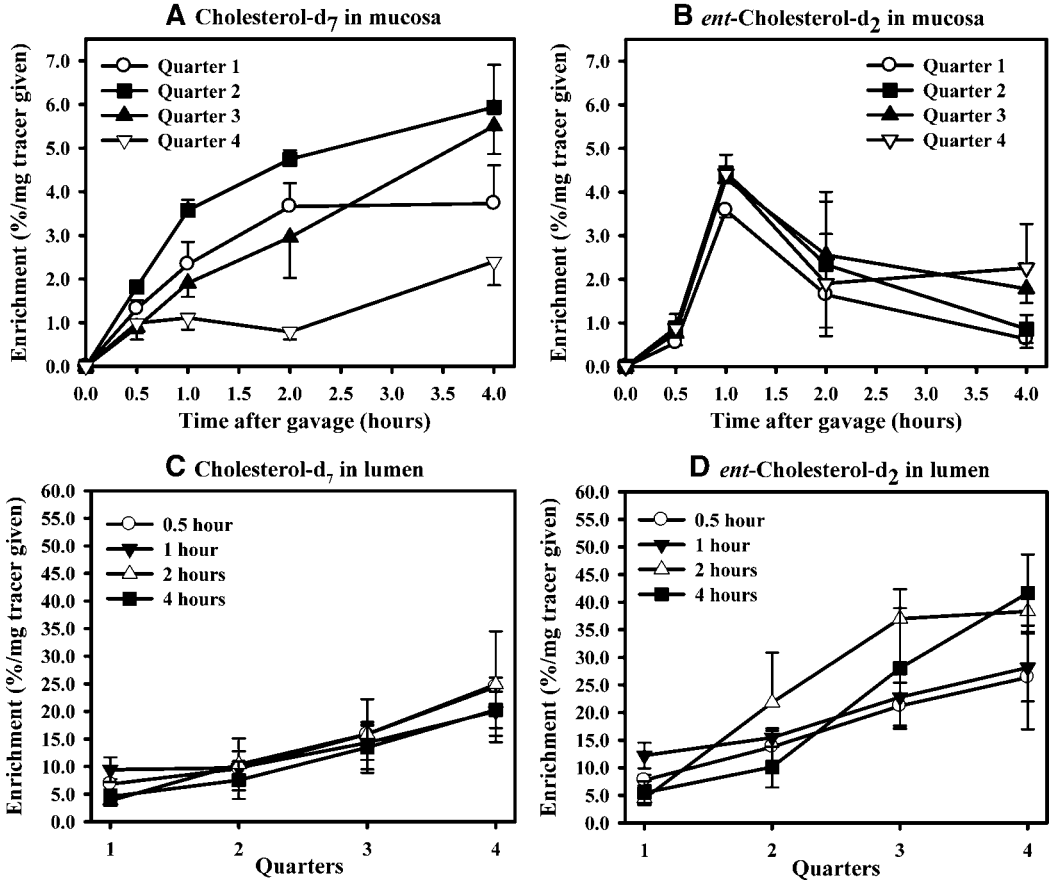

Fig. 2. Oral time-course study: mucosal enrichment of cholesterol-d_7 (A) and *ent*-cholesterol-d_2 (B), and lumenal enrichment of cholesterol-d_7 (C) and *ent*-cholesterol-d_2 (D). Hamsters (n = 3 for each time point) were fasted for 2 h before gavage of cholesterol-d_7 and *ent*-cholesterol-d_2 in soybean oil. At the indicated times, the small intestine was cut into four equal quarters. Mucosa was scraped after removal of the lumenal contents. The levels of cholesterol and *ent*-cholesterol tracers in the intestinal mucosa and lumen were determined by GC-MS as described in Materials and Methods. The enrichment of each tracer was calculated relative to cholesterol-d_0 in the sample and normalized to its respective amount administered. Values are means ± SEM.

TABLE 1. Oral time-course study: percentage esterification of cholesterol-d_7 and *ent*-cholesterol-d_2 in the first mucosal quarter

	Percentage Esterification		
Tracer Administered	1 h	2 h	4 h
Cholesterol-d_7	4.3 ± 1.5	13.2 ± 1.0	7.4 ± 2.9
ent-Cholesterol-d_2	1.3 ± 0.3	6.8 ± 0.6	4.8 ± 1.3

Male hamsters (n = 3) were gavaged with a mixture of cholesterol-d_7 and *ent*-cholesterol-d_2 as described in Materials and Methods. Mucosa was collected and analyzed for free and esterified sterol content by mass spectrometry. Esterification of each of the cholesterol enantiomers was calculated as a percentage of the total (esterified and free forms). By ANOVA, there were significant overall effects of time ($P = 0.001$) and tracer type ($P = 0.007$). Values are means ± SEM.

points. The cholesterol tracer level increased in general over time across the intestinal segments (Fig. 2A). In contrast, the level of *ent*-cholesterol tracer peaked at ~1 h and then declined; this pattern was similar for all four quarters of the small intestine (Fig. 2B). These data suggest that *ent*-cholesterol is rapidly taken up and then eliminated from the enterocyte, either by efflux back into the intestinal lumen or by delivery across the basolateral side of the enterocyte, or both.

The potential effect of sterol esterification was studied by determining the percentage of each tracer that was esterified at 1, 2, and 4 h after tracer administration in the first quarter of the intestinal mucosa (**Table 1**). By ANOVA, there were independent statistically significant effects of both time ($P = 0.001$) and tracer type ($P = 0.007$). However, the esterification of both tracers was relatively small.

Upon harvesting the small intestine, the contents of the lumen were collected using a PBS wash. Sterols were subsequently extracted from the lumenal contents and analyzed by GC-MS. Figure 2C shows that the level of cholesterol tracer was relatively steady over 4 h in each region of the intestinal lumen. However, for any given time, there was more cholesterol tracer in more distal sections of the bowel. Similarly, *ent*-cholesterol tracer levels increased with distance down the bowel (Fig. 2D). Furthermore, the ratio of *ent*-cholesterol to cholesterol tracer increased with distance down the intestine ($P = 0.01$); for example, the ratio was 2.2 in quarter 4 at 4 h, indicating some differential recognition of the sterol tracers by the mucosa.

To assess transit across the basolateral side of the enterocyte, the tracer content of the serum was measured. Only small amounts of the cholesterol tracer were found in the serum over the 4 h time course (**Fig. 3A**, open circles). The accumulation of the cholesterol tracer in the mucosa and the small enrichment in serum indicated a delay in the systemic absorption of cholesterol tracer. In contrast, significant amounts of *ent*-cholesterol tracer (Fig. 3A, closed circles) were found in the serum as early as 30 min. By 4 h, there was a 48.3-fold enrichment of *ent*-cholesterol tracer compared with cholesterol tracer in the serum.

Only small amounts of the cholesterol tracer were found in the liver (Fig. 3B); for example, <1% of the administered cholesterol tracer was in the liver at 4 h. By contrast, a significant portion of the *ent*-cholesterol tracer (14% of the administered tracer) was detected in the liver at 4 h after administration. The enrichment of the *ent*-cholesterol tracer was 21.2-fold that of the cholesterol tracer in the liver at 4 h. Gallbladder bile was aspirated and analyzed for sterol tracers, but neither cholesterol nor *ent*-cholesterol tracer was found in significant amounts within the time frame of these experiments (data not shown).

Cholesterol and *ent*-cholesterol absorption over a 3 day period

A traditional measurement of sterol absorption was made by gavaging the tracers and collecting stools from the animals for 72 h. At the end of the experiment, serum samples were also analyzed for tracer levels. Based on anal-

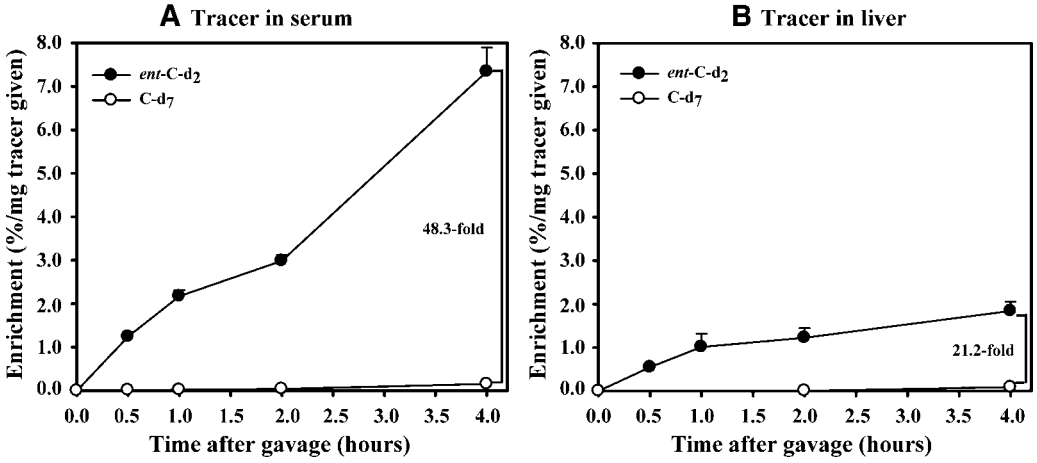

Fig. 3. Oral time-course study: enrichment of cholesterol-d_7 (C-d_7) and *ent*-cholesterol-d_2 (*ent*-C-d_2) in serum (A) and liver (B). Hamsters (n = 3 for each time point) were fasted for 2 h before gavage of cholesterol-d_7 and *ent*-cholesterol-d_2 in soybean oil. The levels of cholesterol and *ent*-cholesterol tracers in serum and liver were determined as described in Materials and Methods. The enrichment of each tracer was calculated relative to cholesterol-d_0 in the sample and normalized to its respective amount administered. Values are means ± SEM.

TABLE 2. Three day study: absorption of cholesterol and *ent*-cholesterol by fecal recovery

Tracer Administered	Amount Administered	Amount in Stool	Absorption
	mg		%
Cholesterol	0.79	0.37 ± 0.03	53.2 ± 3.9
ent-Cholesterol	2.39	2.57 ± 0.17	N.D.

Male hamsters (n = 5) were gavaged with a mixture of cholesterol-d_7 and *ent*-cholesterol-d_2 as described in Materials and Methods. Stools were collected for 72 h and analyzed for sterol content by mass spectrometry. Absorption of each of the cholesterol enantiomers was calculated by reference to unabsorbed sitostanol-d_4. N.D., not detected.

ysis of the 72 h stool samples, cholesterol absorption was calculated as 53.2% (**Table 2**), similar to the mean of 56% found in humans (16). In contrast, all of the administered *ent*-cholesterol was recovered in the stool. At 72 h, cholesterol-d_7 was readily detectable in serum but *ent*-cholesterol tracer was not (data not shown).

Intracardiac injection of cholesterol enantiomers

To explore the mechanism(s) by which *ent*-cholesterol-d_2 was differentiated from cholesterol and excreted, a mixture of cholesterol and *ent*-cholesterol tracers was delivered directly to the systemic circulation by intracardiac injection. Tracer levels in various compartments were then determined by GC-MS at 4 or 24 h.

The serum levels of cholesterol and *ent*-cholesterol were similar at 4 h after intracardiac injection (**Fig. 4A**). However, serum enrichment of both tracers decreased at 24 h (overall time effect; $P = 0.0004$), with the cholesterol-d_7 being 2-fold higher relative to *ent*-cholesterol-d_2 ($P = 0.025$), suggesting more rapid clearance of *ent*-cholesterol-d_2. Compared with cholesterol-d_7, more *ent*-cholesterol-d_2 was found in the liver ($P < 0.0001$), and liver enrichment of both tracers decreased over time ($P < 0.0001$) (Fig. 4B). Approximately 82.9% of the injected *ent*-cholesterol-d_2 was found in the liver at 4 h versus 51.1% of cholesterol-d_7; at 24 h, the corresponding values were 28.8% versus 18.4%. Figure 4C shows even higher *ent*-cholesterol-d_2 enrichment relative to cholesterol-d_7 in the bile ($P = 0.0002$ for the tracer effect), indicating a selective excretion of *ent*-cholesterol-d_2 through the bile.

The enrichment of *ent*-cholesterol-d_2 in the stool was ~26-fold that of cholesterol-d_7 at 4 h after injection ($P = 0.03$) and 27-fold at 24 h ($P = 0.02$) (Fig. 4D). As a percent-

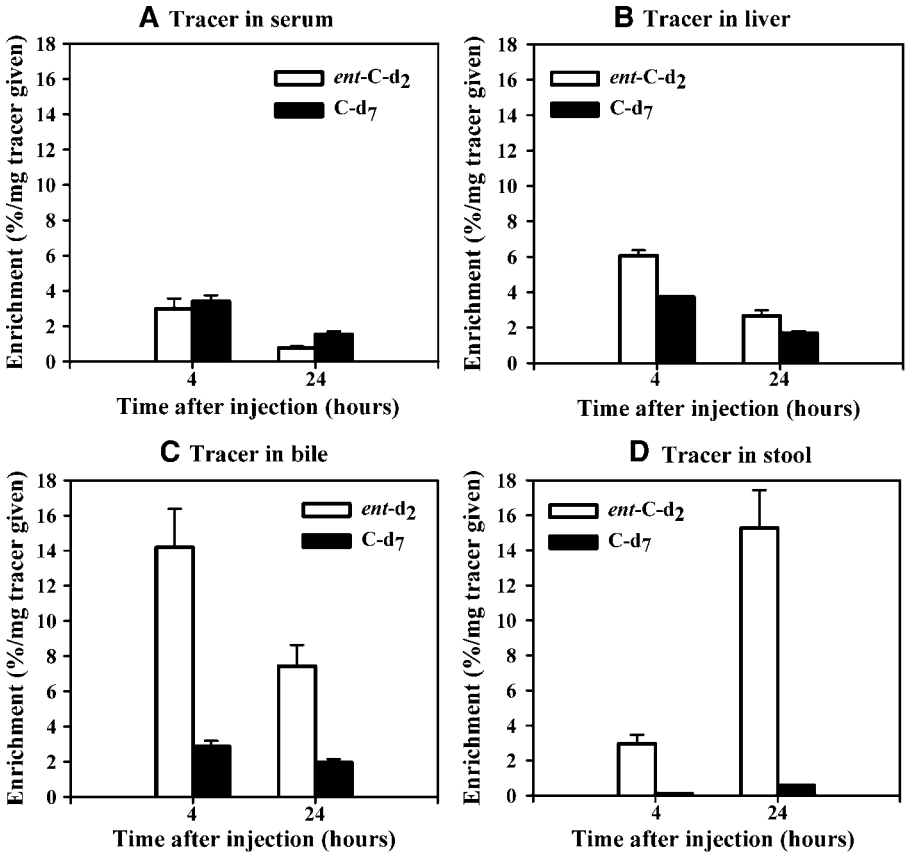

Fig. 4. Intracardiac injection study: enrichment of cholesterol-d_7 (C-d_7) and *ent*-cholesterol-d_2 (*ent*-C-d_2) in serum (A), liver (B), bile (C), and stool (D). Hamsters (n = 3 for each time point) were fasted for 2 h before intracardiac administration of cholesterol-d_7 and *ent*-cholesterol-d_2 in Intralipid. The levels of cholesterol and *ent*-cholesterol tracers were determined as described in Materials and Methods. The enrichment of each tracer was calculated relative to cholesterol-d_0 in the sample and normalized to its respective amount administered. Values are means ± SEM.

age of the amount injected, the amounts of *ent*-cholesterol-d_2 increased from 2.7% at 4 h to 24.1% at 24 h, whereas the amounts of cholesterol-d_7 were 0.11% and 0.97%, respectively. These results indicated that *ent*-cholesterol-d_2 was selectively excreted over the 24 h of this experiment.

After intracardiac injection, there were significant effects of time ($P < 0.0001$) and quarter ($P = 0.0009$) in the enrichment of cholesterol-d_7 (**Fig. 5A**) but none in *ent*-cholesterol-d_2 in the mucosa (Fig. 5B). The ratio of *ent*-cholesterol to cholesterol tracers in the mucosa changed across all quarters ($P = 0.0007$) and over time ($P < 0.001$). In the intestinal lumen, there were significant effects of time ($P = 0.0009$ for cholesterol-d_7 enrichment and $P < 0.0001$ for *ent*-cholesterol-d_2) and quarter ($P = 0.0181$ for cholesterol-d_7 and $P < 0.0001$ for *ent*-cholesterol-d_2) (Fig. 5C, D). Significantly, the ratio of *ent*-cholesterol to cholesterol tracers in the lumen increased across all quarters ($P < 0.0001$) but remained unchanged over time.

DISCUSSION

Approximately 53% of the deuterated cholesterol tracer administered as a single oral dose was absorbed over 3 days. This result is consistent with previous reports in humans (16). In contrast, after 3 days, the *ent*-cholesterol tracer was not detectable in the serum. Rather, it was quantitatively recovered in the stool. *ent*-Cholesterol was thus considered not "absorbed" in this classical cholesterol absorption experiment.

Previous studies of orally administered tracers have shown that <2% of phytosterols are absorbed over several days (17). Because phytosterols were not included in this study for direct comparison, whether *ent*-cholesterol was handled similarly to phytosterols by the absorptive process remains unknown. Nonetheless, it is clear that the overall process of cholesterol absorption not only distinguishes cholesterol from phytosterols, which differ from cholesterol physically and structurally, but also distinguishes cholesterol from its enantiomer, which differs only in absolute configuration. Because cholesterol and *ent*-cholesterol differ in three-dimensional configuration, chiral molecules such as proteins have the potential to distinguish between the two sterols. The finding that hamsters discriminate completely between intragastrically delivered cholesterol and *ent*-cholesterol provides firm evidence that overall cholesterol absorption is highly stereospecific and likely to be protein-mediated, with little reliance on diffusion or nonspecific pathways.

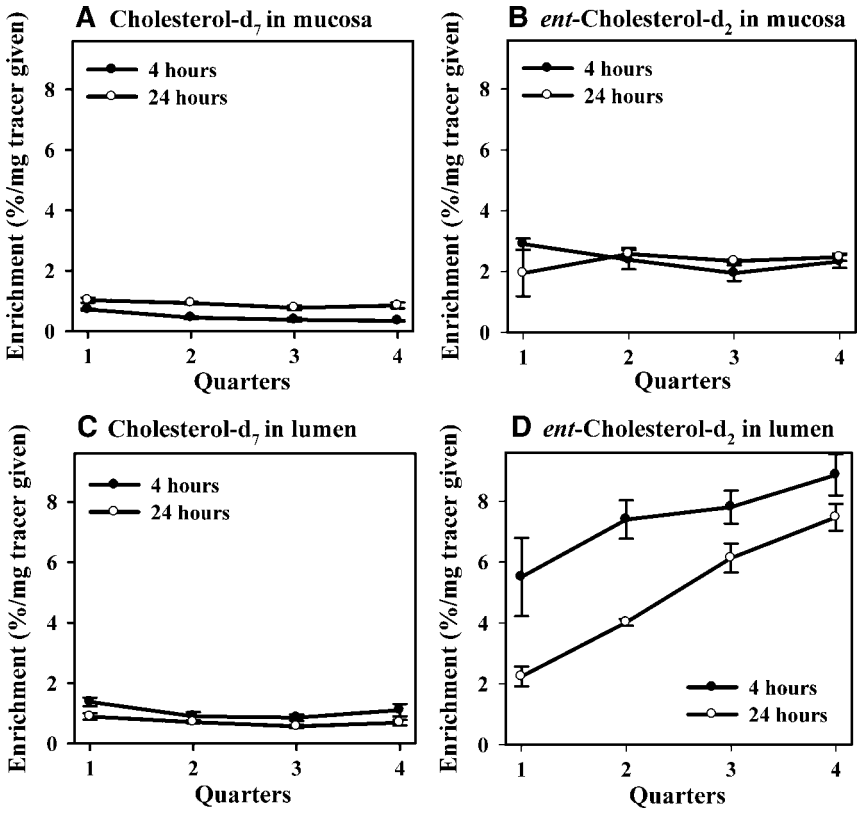

Fig. 5. Intracardiac injection study: mucosal enrichment of cholesterol-d_7 (A) and *ent*-cholesterol-d_2 (B), and lumenal enrichment of cholesterol-d_7 (C) and *ent*-cholesterol-d_2 (D). Hamsters (n = 3 for each time point) were fasted for 2 h before intracardiac administration of cholesterol-d_7 and *ent*-cholesterol-d_2 in Intralipid. The small intestine was cut into four equal quarters. Mucosa was scraped after removal of the lumenal contents. The levels of cholesterol and *ent*-cholesterol tracers in the intestinal mucosa and lumen were determined as described in Materials and Methods. The enrichment of each tracer was calculated relative to cholesterol-d_0 in the sample and normalized to its respective amount administered. Values are means ± SEM.

Despite the lack of systemic retention after several days, the intragastric time-course studies showed that *ent*-cholesterol was taken up by the enterocyte and transiently absorbed. The mucosal levels of cholesterol and *ent*-cholesterol tracers were similar through the first 30 min across all quarters. Over the first 4 h after administration, cholesterol tended to accumulate in the mucosa. However, this tracer was found only in small amounts in the serum or liver by 4 h after oral administration, indicating slow systemic absorption of cholesterol. We cannot exclude the possibility that cholesterol could be delivered efficiently to peripheral tissues. Yet, our results are consistent with other reports that cholesterol tracers given orally may take several days to reach a peak in plasma (16, 18, 19). By contrast, mucosal levels of *ent*-cholesterol peaked across all four quarters at 1 h and then declined rapidly afterward, indicating a rapid uptake of *ent*-cholesterol across all quarters of the small intestine at 1 h followed by export. Other studies have shown that phytosterols also show rapid but transient accumulation in enterocytes (20). Although its exact role is still unclear, NPC1L1 appears to play an important role in the uptake of both cholesterol and phytosterols from the intestinal lumen into the enterocyte brush border (5, 9), and our results suggest that NPC1L1 may not discriminate between enantiomers.

In the serum and liver, a significant enrichment of *ent*-cholesterol over cholesterol was observed as early as 30 min after oral administration. The ratio of *ent*-cholesterol to cholesterol enrichment reached 48.3- and 21.2-fold in the serum and liver, respectively, at 4 h after oral administration. These results indicated that *ent*-cholesterol was indeed rapidly transported across the basolateral membrane of enterocytes, whereas part of it may have been resecreted back into the intestinal lumen. It is likely that the majority of *ent*-cholesterol was transported in its unesterified form, because only a small portion was found to be esterified in the mucosa (Table 1). Because free cholesterol is transported by apolipoprotein B-independent pathways, such as efflux mediated by ABCA1 or HDL assembly (19), these pathways may be responsible for the rapid absorption of *ent*-cholesterol from the enterocyte to the systemic circulation (19, 21). Alternatively, other pathways may be involved.

Despite rapid uptake and delivery to the systemic circulation, the net absorption of *ent*-cholesterol is apparently incomplete. Of the *ent*-cholesterol-d_2 given by intracardiac injection, 2.7% of this tracer was lost in the stool at 4 h, and this loss increased to 24.1% at 24 h (Fig. 4D). In contrast, loss of cholesterol-d_7 accounted for $<1\%$ of the intracardiac administered tracer at both time points. Consistent with the selective resecretion of *ent*-cholesterol tracer, its serum enrichment in the intracardiac injection study was similar to that of cholesterol tracer at 4 h but was lower than that of cholesterol at 24 h (Fig. 4A). The intracardiac injection study directly showed a preferential accumulation of *ent*-cholesterol-d_2 over cholesterol-d_7 in liver and bile at 4 h (Fig. 4B, C). The enrichment of both tracers in liver and bile decreased at 24 h, which is consistent with continued clearance over time. Nonetheless, at 24 h, *ent*-cholesterol tracer was still 1.6- and 3.8-fold that of cholesterol tracer in liver and bile, respectively. These results indicate that the liver contributes to the specificity of cholesterol absorption by selectively secreting *ent*-cholesterol-d_2 into bile for elimination.

Lumenal levels of both *ent*-cholesterol and cholesterol in the intragastric experiment increased with distance down the small intestine. There was, however, a 2.2-fold enrichment of *ent*-cholesterol compared with cholesterol in quarter 4 at 4 h. Because only negligible amounts of *ent*-cholesterol were found in the bile within 4 h of oral tracer administration, there should be no significant biliary *ent*-cholesterol contribution to its lumenal enrichment within this short time in the oral study. Similarly, *ent*-cholesterol enrichment in the lumen increased steadily across quarters at both 4 and 24 h after intracardiac injection (Fig. 5D). In fact, the ratio of *ent*-cholesterol to cholesterol tracers increased 2.2-fold from quarters 1 to 4 at 4 h and 4.3-fold at 24 h. In contrast, the ratio of *ent*-cholesterol to cholesterol tracers in the mucosa was reduced at 24 h compared with 4 h. The sustained increase of *ent*-cholesterol-to-cholesterol ratio in the lumen is consistent with the preferential elimination of *ent*-cholesterol. Therefore, the enterocyte may confer some specificity in the lumenal enrichment of *ent*-cholesterol, possibly through efflux mediated by ABCG5/8 (22) or other transporters in the brush border.

Rapid intestinal transport and preferential biliary secretion of *ent*-cholesterol strongly suggest that *ent*-cholesterol may have undergone enterohepatic recirculation multiple times. Unexpectedly, a significant amount of this tracer was found in the circulation at 4 h after oral administration. Detailed time-course quantification of biliary tracer excretion using bile fistula hamsters would help clarify the enterohepatic cycling of *ent*-cholesterol, but such studies are currently limited by tracer availability.

ACAT-2 plays a role in cholesterol absorption when intestinal cholesterol levels are high (7). In our studies, only a small fraction of either tracer was esterified by 1 h. Our finding that the mucosal pool of cholesterol was mostly in the unesterified form is consistent with the existence of a metabolic pool of free cholesterol in the mucosa described many years ago (23). In fact, cholesterol was selectively directed into this pool, and its absorption into the systemic circulation was delayed compared with that of *ent*-cholesterol. Little is known of the possible role of this free cholesterol pool in regulating cholesterol absorption, but it seems possible that it may represent a physiological measure of the amount of recently absorbed cholesterol that could regulate the proteins involved in the absorption process.

Noncholesterol sterols can have profound effects on cholesterol metabolism. Active exclusion of phytosterols appears to be critical for the maintenance of normal cholesterol homeostasis (24). The results of this study demonstrate that overall cholesterol absorption is enantioselective. Our data indicate that *ent*-cholesterol is efficiently recognized and resecreted by both the mucosa and the liver. An understanding of the source of this enantioselec-

tivity may provide a rationale for developing better pharmacological agents aimed at reducing the intestinal absorption of both cholesterol and noncholesterol sterols.

This work was supported by National Institutes of Health Grants R01 HL-50420 (to R.E.O.), GM-47969 (to D.F.C.), and T32 HL-07275 (to E.J.W.) as well as by grants to the Washington University Mass Spectrometry Resource (RR-00954), the Washington University Diabetes Center (DK-20579), and the Washington University Clinical Nutrition Research Unit (DK-56341).

REFERENCES

1. Wilson, M. D., and L. L. Rudel. 1994. Review of cholesterol absorption with emphasis on dietary and biliary cholesterol. *J. Lipid Res.* **35:** 943–955.
2. Ostlund, R. E., Jr. 2002. Phytosterols in human nutrition. *Annu. Rev. Nutr.* **22:** 533–549.
3. Sudhop, T., D. Lutjohann, A. Kodal, M. Igel, D. L. Tribble, S. Shah, I. Perevozskaya, and K. von Bergmann. 2002. Inhibition of intestinal cholesterol absorption by ezetimibe in humans. *Circulation.* **106:** 1943–1948.
4. Wang, H. H., N. H. Afdhal, S. J. Gendler, and D. Q. Wang. 2004. Lack of the intestinal Muc1 mucin impairs cholesterol uptake and absorption but not fatty acid uptake in $Muc1^{-/-}$ mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* **287:** G547–G554.
5. Davis, H. R., Jr., L. J. Zhu, L. M. Hoos, G. Tetzloff, M. Maguire, J. Liu, X. Yao, S. P. Iyer, M. H. Lam, E. G. Lund, et al. 2004. Niemann-Pick C1 Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. *J. Biol. Chem.* **279:** 33586–33592.
6. Drobnik, W., B. Lindenthal, B. Lieser, M. Ritter, T. Christiansen Weber, G. Liebisch, U. Giesa, M. Igel, H. Borsukova, C. Buchler, et al. 2001. ATP-binding cassette transporter A1 (ABCA1) affects total body sterol metabolism. *Gastroenterology.* **120:** 1203–1211.
7. Buhman, K. K., M. Accad, S. Novak, R. S. Choi, J. S. Wong, R. L. Hamilton, S. Turley, and R. V. Farese, Jr. 2000. Resistance to diet-induced hypercholesterolemia and gallstone formation in ACAT2-deficient mice. *Nat. Med.* **6:** 1341–1347.
8. McNeish, J., R. J. Aiello, D. Guyot, T. Turi, C. Gabel, C. Aldinger, K. L. Hoppe, M. L. Roach, L. J. Royer, J. de Wet, et al. 2000. High density lipoprotein deficiency and foam cell accumulation in mice with targeted disruption of ATP-binding cassette transporter-1. *Proc. Natl. Acad. Sci. USA.* **97:** 4245–4250.
9. Altmann, S. W., H. R. Davis, Jr., L. J. Zhu, X. Yao, L. M. Hoos, G. Tetzloff, S. P. Iyer, M. Maguire, A. Golovko, M. Zeng, et al. 2004. Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science.* **303:** 1201–1204.
10. Mannock, D. A., T. J. McIntosh, X. Jiang, D. F. Covey, and R. N. McElhaney. 2003. Effects of natural and enantiomeric cholesterol on the thermotropic phase behavior and structure of egg sphingomyelin bilayer membranes. *Biophys. J.* **84:** 1038–1046.
11. Westover, E. J., D. F. Covey, H. L. Brockman, R. E. Brown, and L. J. Pike. 2003. Cholesterol depletion results in site-specific increases in epidermal growth factor receptor phosphorylation due to membrane level effects. Studies with cholesterol enantiomers. *J. Biol. Chem.* **278:** 51125–51133.
12. Westover, E. J., and D. F. Covey. 2003. First synthesis of ent-desmosterol and its conversion to ent-deuterocholesterol. *Steroids.* **68:** 159–166.
13. Kirk, D. N., M. J. Varley, H. L. J. Makin, and D. J. H. Trafford. 1983. Synthesis of [26,27-$^2H_6$]cholesterol and derivatives substituted in the side chain. *J. Chem. Soc. Perkin Trans.* **I:** 2563–2567.
14. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
15. Ostlund, R. E., Jr., F. F. Hsu, M. S. Bosner, W. F. Stenson, and D. L. Hachey. 1996. Quantification of cholesterol tracers by gas chromatography-negative ion chemical ionization mass spectrometry. *J. Mass Spectrom.* **31:** 1291–1296.
16. Bosner, M. S., L. G. Lange, W. F. Stenson, and R. E. Ostlund, Jr. 1999. Percent cholesterol absorption in normal women and men quantified with dual stable isotopic tracers and negative ion mass spectrometry. *J. Lipid Res.* **40:** 302–308.
17. Ostlund, R. E., Jr., J. B. McGill, C. M. Zeng, D. F. Covey, J. Stearns, W. F. Stenson, and C. A. Spilburg. 2002. Gastrointestinal absorption and plasma kinetics of soy Delta(5)-phytosterols and phytostanols in humans. *Am. J. Physiol. Endocrinol. Metab.* **282:** E911–E916.
18. Beaumier-Gallon, G., C. Dubois, M. Senft, M. F. Vergnes, A. M. Pauli, H. Portugal, and D. Lairon. 2001. Dietary cholesterol is secreted in intestinally derived chylomicrons during several subsequent postprandial phases in healthy humans. *Am. J. Clin. Nutr.* **73:** 870–877.
19. Iqbal, J., K. Anwar, and M. M. Hussain. 2003. Multiple, independently regulated pathways of cholesterol transport across the intestinal epithelial cells. *J. Biol. Chem.* **278:** 31610–31620.
20. Igel, M., U. Giesa, D. Lutjohann, and K. von Bergmann. 2003. Comparison of the intestinal uptake of cholesterol, plant sterols, and stanols in mice. *J. Lipid Res.* **44:** 533–538.
21. Repa, J. J., K. K. Buhman, R. V. Farese, Jr., J. M. Dietschy, and S. D. Turley. 2004. ACAT2 deficiency limits cholesterol absorption in the cholesterol-fed mouse: impact on hepatic cholesterol homeostasis. *Hepatology.* **40:** 1088–1097.
22. Yu, L., K. von Bergmann, D. Lutjohann, H. H. Hobbs, and J. C. Cohen. 2004. Selective sterol accumulation in ABCG5/ABCG8-deficient mice. *J. Lipid Res.* **45:** 301–307.
23. Swell, L., E. C. Trout, Jr., J. R. Hopper, H. Field, Jr., and C. R. Treadwell. 1958. Mechanism of cholesterol absorption. II. Changes in free and esterified cholesterol pools of mucosa after feeding cholesterol-4-C14. *J. Biol. Chem.* **233:** 49–53.
24. Yang, C., L. Yu, W. Li, F. Xu, J. C. Cohen, and H. H. Hobbs. 2004. Disruption of cholesterol homeostasis by plant sterols. *J. Clin. Invest.* **114:** 813–822.