A fluorescent cholesterol analog traces cholesterol absorption in hamsters and is esterified in vivo and in vitro

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Abstract **The fluorescent cholesterol analog 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (fluoresterol) was characterized as a tool for exploring the biochemistry and cell biology of intestinal cholesterol absorption. Hamsters absorbed fluoresterol in a concentration- and time-dependent manner, with an efficiency of about 15–30% that of cholesterol. Fluoresterol absorption was blocked by compounds known to inhibit cholesterol absorption, implying that fluoresterol interacts with those elements of the normal pathway for cholesterol absorption on which the inhibitors act. Confocal microscopy of small intestinal tissue demonstrated that fluoresterol was taken up by absorptive epithelial cells and packaged into lipoprotein particles, suggesting a normal route of intracellular trafficking. Uptake of fluoresterol was confirmed by biochemical analysis of intestinal tissue, and a comparison of [^{3}H]cholesterol and fluoresterol content in the mucosa suggested that fluoresterol moved through the enterocytes more rapidly than did cholesterol. This interpretation was supported by measurements of fluoresterol esterification in the mucosa. Four hours after hamsters were given fluoresterol and [^{3}H]cholesterol orally, 44% of the fluoresterol in the intestinal mucosa was esterified, compared to 8% of the [^{3}H]cholesterol. Caco-2 cells took up 2- to 5-fold more [^{3}H]cholesterol than fluoresterol from bile acid micelles, and esterified 21–24% of the fluoresterol but only 1–4% of the [^{3}H]cholesterol. Thus fluoresterol apparently interacts with the proteins required for cholesterol uptake, trafficking, and processing in the small intestine.**—Sparrow, C. P., S. Patel, J. Baffic, Y-S. Chao, M. Hernandez, M-H. Lam, J. Montenegro, S. D. Wright, and P. A. Detmers. **A fluorescent cholesterol analog traces cholesterol absorption in hamsters and is esterified in vivo and in vitro.** ***J. Lipid Res.*** **1999.** 40: **1747–1757.**

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The process by which cholesterol is absorbed in the intestine and transits in lipoprotein particles to plasma has been well studied, and the major steps in this pathway are understood. Whether from a dietary source or from lipoproteins taken up by the liver, cholesterol is solubilized in the lumen of the small intestine by bile acid micelles (1). Cholesterol is taken up from the micelles by the epithelial cells lining the surface of the intestinal villi. This process occurs predominantly in the jejunum (1), with the bile acids themselves being resorbed by specific transporters in the ileum (2). Upon entering the enterocytes, cholesterol is thought to move to the endoplasmic reticulum, where it can be esterified by ACAT and packaged into chylomicrons (1, 3). The newly formed lipoprotein particles exit the absorptive cells along their baso-lateral aspects and become available for uptake by the liver and other tissues.

The initial interaction of cholesterol with the enterocyte brush border is one that is critical to the uptake process, and yet it is not well understood. Arguments have been presented that a specific cholesterol transporter is not required and that uptake is a passive process (4). On the other hand, the existence of very potent and specific compounds that inhibit cholesterol absorption implies that there is a specific target protein critical to the process (5–7). While candidate proteins have been proposed for the role of cholesterol transporter (8), the exact identity of such a protein has remained elusive.

Cholesterol absorption and trafficking has also been studied in vitro, using cell lines that approximate the characteristics of the absorptive epithelium but that are more amenable to experimental manipulation. The most widely used in vitro model is the Caco-2 cell line (3), which was derived from a human intestinal tumor (9). When allowed to reach confluence and differentiate, these cells take up cholesterol from mixed micelles, and the cholesterol uptake is influenced by the composition of the micelles (10). The cholesterol that enters the Caco-2 plasma membrane is transported to the endoplasmic reticulum for packaging into lipoprotein (11) by a process that may involve p-glycoproteins (12, 13). It has also been suggested that sterol carrier protein-2 participates in cholesterol traffick-

Abbreviations: ACAT: acyl-CoA cholesterol acyltransferase; fluoresterol, 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol.

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ing (14). However, many of the biochemical details of cholesterol absorption and trafficking are still not fully characterized.

While the intracellular transit of cholesterol has been traced biochemically and through the use of radioactive probes, no one has directly observed the path of cholesterol absorption in the intestine. A fluorescently labeled molecule would offer the opportunity to make such observations and could prove useful for the identification of additional protein components in the cholesterol transport process. Here we demonstrate that a fluorescent cholesterol analog shares many properties with cholesterol. When given orally to hamsters, it was absorbed into plasma, and absorption was blocked by compounds that block cholesterol absorption. Further, it was esterified by cells both in vivo and in vitro, indicating that it is a substrate for ACAT. We have used this fluorescent probe to directly observe the uptake and movements of cholesterol in the small intestine of hamsters by confocal microscopy.

MATERIALS AND METHODS

Materials

Fluoresterol (22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol) and its oleate ester were purchased from Molecular Probes (Eugene, OR). The structure of fluoresterol is shown in **Fig. 1**. [1,2-^{3}H]cholesterol (43.5 Ci/mmol) was obtained from New England Nuclear (Boston, MA). The two cholesterol absorption inhibitors L-166,143 (Compound #51 in 5) and L-165,313 (CP-148,623 in 6) (**Fig. 2**) were synthesized at Merck Research Laboratories, Rahway, NJ. Solvents used in the quantitation of fluoresterol were all HPLC grade (Aldrich). Caco-2 cells were obtained from ATCC (Bethesda, MD). Cell culture supplies were obtained from Sigma (St. Louis, MO) or Gibco (Grand Island, NY). Phosphate-buffered saline (PBS) was purchased from Gibco. Liquid hamster diet was obtained from Bio-Serv (Frenchtown, NJ) as a powdered concentrate whose composition was 54% carbohydrate, 18% protein, 18% fat, 4% ash, 4% water, and 2% fiber by weight. The powder was dissolved in water at a ratio of 1 gram of diet to 3.96 ml of water.

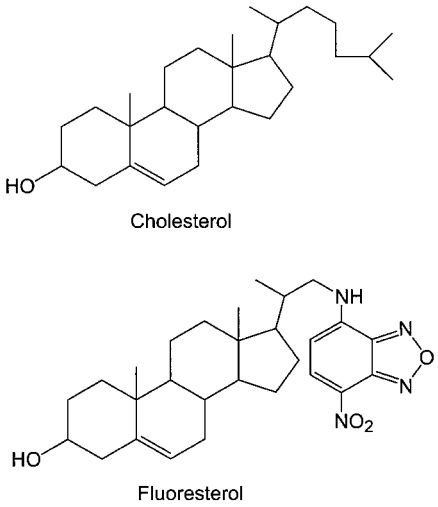

Fig. 1. Chemical structures of cholesterol and fluoresterol.

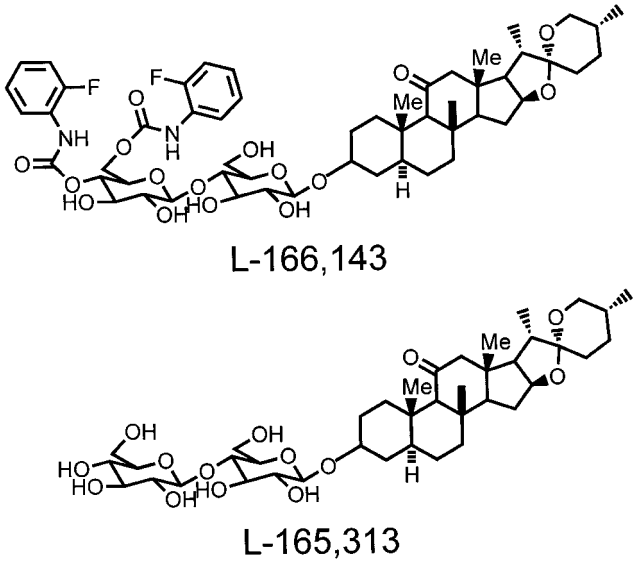

Fig. 2. Chemical structures of the cholesterol absorption inhibitors L-166,143 and L-165,313. L-166,143 ((3β,5α,25*R*)-3-[[4′′,6′′-bis[(2-fluorophenyl)carbamoyl]-β-d-cellobiosyl]oxy]-spirostan-11-one) was described by DeNinno et al. (5) as compound 51. L-165,313 (3β,5α,25*R*-spirostan-11-one cellobioside) was described by Harris et al. (6) as CP-148,623.

Animals

Male golden Syrian hamsters weighing 100–120 g were purchased from Charles River (Kingston, NY). All animals were given free access to water, fed a commercial rodent chow diet, and housed (5 per box) under a regular 12-h light/12-h dark lighting cycle. All animal study protocols were approved by the Merck Institutional Animal Care and Use Committee.

Preparation of fluoresterol solutions in corn oil and liquid diet

Stock solutions of fluoresterol (1–2 mg/ml) were prepared in 100% ethanol with stirring at 50°C. Appropriate amounts of this ethanol solution were mixed with corn oil (Mazola) in a 25-ml screw-cap glass tube. The mixture was purged with argon, while held at 50°C in a water bath, to evaporate the ethanol. The resulting solution of fluoresterol in corn oil was orally administered either directly or after mixing with liquid hamster diet (Bio-Serv, Frenchtown, NJ). To make the mixture, fluoresterol (2.5 mg/ml) in corn oil was added to liquid diet (0.2 ml corn oil per ml liquid diet) containing 0.5% cholic acid and homogenized for approximately 60 sec in a Polytron PT 3000. In some experiments, the corn oil also contained [^{3}H]cholesterol (200 μg and 1 μCi/hamster).

Absorption of fluoresterol and [^{3}H]cholesterol by hamsters

Hamsters were fasted overnight and then orally dosed with fluoresterol and/or [^{3}H]cholesterol. For studies using cholesterol absorption inhibitors, the dose of sterol was preceded by a gavage of 1 ml of 0.25% methylcellulose, with or without compounds. After gavage, the hamsters were returned to their cages and were allowed access to water but were not fed. At the indicated times, the hamsters were killed and weighed. Blood (0.5–1.0 ml) was collected by cardiac puncture and placed in 13 × 100 mm culture tubes containing 10 μl of 0.5 m EDTA. Plasma was collected after centrifugation at 10,000 *g* for 20 min at 4°C. In some experiments, the jejunum was collected and processed for confocal microscopy as described below.

Absorption of [^{3}H]cholesterol in the plasma was quantitated by bleaching 100-μl aliquots of plasma with 25 μl of 30% hydrogen peroxide and then measuring radioactivity in a liquid scintillation counter (15). Fluoresterol was quantitated in hamster plasma after solvent extraction using a modification of a procedure originally described by Dole (16). Briefly, 50 μl of hamster plasma was mixed with 1.2 ml of isopropanol:heptane:125 mm H_2SO_4 80:19:16 in water. The precipitated protein was removed by centrifugation, and the supernatant was transferred to disposable polystyrene cuvettes. Fluoresterol fluorescence was measured in a Spex FluoroMax fluorimeter (excitation 465 nm, emission 535 nm). Plasma samples from hamsters never given fluoresterol were used as blanks, and fluoresterol dissolved in the solvent mixture described above was used to create a standard curve. An experiment in which a known amount of exogenous fluoresterol was added to hamster plasma samples prior to fluoresterol measurement confirmed that the solvents used extracted the fluoresterol quantitatively and that there was no significant quenching of fluoresterol by solvent-soluble plasma components.

The relative absorption of [^{3}H]cholesterol to fluoresterol was calculated as the ratio of plasma concentration of sterol to the amount of sterol given to the hamster, as follows:

$$\frac{(\text{dpm } [^3\text{H}]\text{cholesterol/ml plasma})/(\text{dpm } [^3\text{H}]\text{cholesterol in dose})}{(\text{ng fluoresterol/ml plasma})/(\text{ng fluoresterol in dose})}.$$

Preparation of tissue for confocal microscopy

To prepare intestinal tissue for confocal microscopy, the jejunum was cut into pieces approximately 4–5 cm long. The pieces were cut lengthwise, then flushed and washed with several changes of cold 0.15 m NaCl to remove intestinal debris. After application of OCT embedding medium (Fisher Scientific, Springfield, NJ) to the external surface, each piece was rolled around a toothpick as an inside-out jelly roll, from the proximal to the distal end. The rolls were removed from the toothpicks, stood on end in OCT, and frozen on liquid nitrogen. Sections (30 μm) were cut in a cryostat, fixed on glass slides in 3.7% formaldehyde in PBS at ambient temperature for 5 min, washed twice (5 min each) in PBS, and mounted in ProLong antifade mounting medium (Molecular Probes, Eugene, OR). Sections were immediately observed on a Nikon Optiphot-2 microscope equipped with a Bio-Rad MRC 1024 scanning laser confocal attachment with a krypton–argon laser. Images were collected using either a 10× or 60× objective, with all settings maintained between samples in the same experiment. No autofluorescence was detectable in samples from hamsters that did not receive fluoresterol. The size of randomly selected fluoresterol droplets observed in the intestinal epithelium was quantitated using an ocular micrometer on the collected images.

Measurement of sterol content and esterification in vivo

Mucosa tissue from hamsters dosed with both fluoresterol and [^{3}H]cholesterol was prepared for analysis of sterol content and extent of sterol esterification. The small intestinal mucosa of each hamster was scraped from the underlying muscularis layer and suspended in 5 ml of Dulbecco's PBS. The mucosal suspension was homogenized with a Polytron PT 3000 for 60 sec at setting 14, on ice. Aliquots (100 μl) of the homogenate were counted in a liquid scintillation counter to determine total content of [^{3}H]sterol (expressed as moles). One ml of the homogenate was extracted as described by Bligh and Dyer (17), and the lower phase was analyzed for total fluoresterol content (moles). The same extract was used to determine the amount of [^{3}H]cholesterol or fluoresterol that was esterified. Lipids in the chloroform phase were separated by silica gel TLC using a mobile phase of ether:petroleum ether:acetic acid 50:50:1. Spots corresponding to fluoresterol and its ester were quantitated by scanning the thin-layer plate with a FluorImager 575 (Molecular Dynamics). After this, spots corresponding to cholesterol and cholesteryl ester were identified by iodine staining and scraped for scintillation counting. Sterol esterification was calculated using the values obtained from the TLC plate and is expressed as % of sterol esterified.

Measurement of sterol uptake by Caco-2 cells

Separate micelles containing either [^{3}H]cholesterol or fluoresterol were prepared by mixing the components in chloroform and/or methanol solution, evaporating the solvent under argon gas, and redissolving the micelles in Opti-MEM medium without serum. The micelles contained 10 mm taurocholate, 6 mm egg phosphatidylcholine, and either 200 μm [^{3}H]cholesterol (20 Curie/mole) or 200 μm fluoresterol. Each preparation of micelles was sterile filtered, and the concentration of [^{3}H]cholesterol or fluoresterol in the filtrate was determined. Virtually all of the [^{3}H]cholesterol formed filterable micelles, giving a yield that was usually close to 100%. The yield of fluoresterol in micelles varied from 25–100%. By preparing the two sterols in separate micelles, we could therefore assure that an equimolar ratio could be achieved when they were mixed. The final mixed micelles were used the day of preparation.

Caco-2 cells were maintained in Opti-MEM (Gibco), 5% fetal calf serum, 1% Serum-Max-3 (Sigma), 1% MEM non-essential amino acids, and 1% MEM vitamins. Caco-2 cells grown to confluence in 6-well plates were incubated with the micelles at 37°C for the indicated times, washed, and harvested by scraping. Aliquots of cell homogenate were used to directly measure total ^{3}H content and to measure total fluoresterol fluorescence after dilution with isopropanol. Esterification of each sterol was determined by the TLC method described above.

In vitro ACAT assays

Hamster intestinal microsomes were prepared as described (18), except that microsomes were dispersed in 100 mm potassium phosphate buffer (pH 7.4) containing 1 mm reduced glutathione and a protease inhibitor cocktail obtained from CalBiochem (San Diego, CA) (catalog #539131). These microsomes were used in ACAT assays performed exactly as described by Yang et al. (19), except that both [^{3}H]cholesterol and fluoresterol were present at a final concentration of 50 μg/ml each. After 2.5 min at 37°C, the reactions were terminated by addition of chloroform and methanol, lipids were extracted (17), and sterol esterification was quantitated as described above.

RESULTS

Fluoresterol is absorbed by hamsters

To determine whether fluoresterol was absorbed after oral administraton, we administered fluoresterol to hamsters by gavage and followed its subsequent appearance in plasma. In an initial experiment hamsters received crystalline fluoresterol suspended in water. However, fluoresterol in plasma was hardly detectable (data not shown), suggesting that the crystals were not readily processed to a form that could be absorbed. In order to enhance the bioavailability of the fluoresterol, we dissolved it in corn oil and prepared an emulsion with a standard liquid diet to administer to hamsters by gavage. Absorption of fluoresterol from the emulsion was confirmed in a preliminary experiment to determine an appropriate concentration of fluoresterol to use for subsequent absorption and fluores-

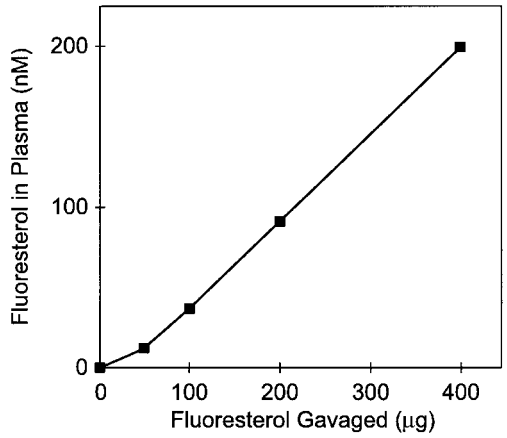

Fig. 3. Absorption of orally administered fluoresterol is concentration-dependent. Single hamsters were gavaged with increasing concentrations of fluoresterol in liquid diet. Blood was collected at 3 h, and fluoresterol in plasma was quantitated.

cent microscopic studies. Increasing concentrations of fluoresterol were given to hamsters, and both blood and small intestines were collected after 3 h. Fluoresterol was readily detected in plasma at this time. Further, absorption was concentration-dependent and linear between 50 and 400 μg/dose (**Fig. 3**). Cryosectioned intestinal material revealed no detectable fluorescence at the lowest dose (50 μg), but stepwise increases in fluorescence present within the villi were apparent at the higher doses (not shown). Therefore 200–400 μg fluoresterol per hamster was chosen as an optimal dose range for further studies.

Absorption of fluoresterol by hamsters was also time-dependent. Hamsters administered 400 μg fluoresterol had detectable fluoresterol in plasma by 30 min, and the concentration increased up to 60 min (**Fig. 4**). Between 1 and 4 h the plasma fluoresterol concentration increased more gradually, appearing to plateau. Four hours, therefore, represented the best choice of time for further studies using plasma measurements to examine absorption of fluoresterol. On the other hand, times less than 4 h appeared to present the best opportunity to monitor trafficking of fluoresterol in the hamster intestine.

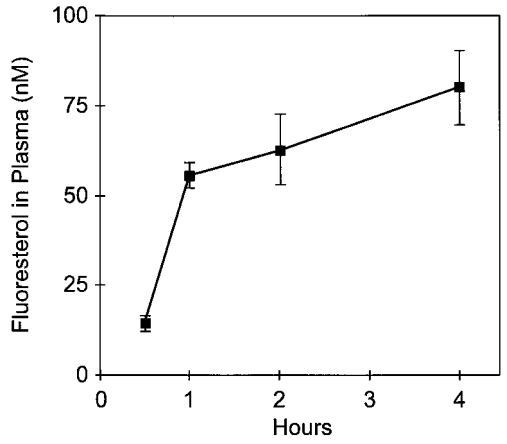

Fig. 4. Absorption of fluoresterol is time-dependent. Hamsters (three per group) were gavaged with 400 μg fluoresterol in liquid diet. At times up to 4 h, blood was collected, and fluoresterol in plasma was quantitated. The data are the mean ± SEM. They are representative of three experiments with similar results.

Absorption of fluoresterol is inhibited by compounds that inhibit cholesterol absorption

Two compounds that inhibit cholesterol absorption were used to verify that the structural changes in the fluorescent analog had little effect on its mechanism of uptake by intestinal epithelial cells. Hamsters were dosed with either L-166,143 or L-165,313 (see Fig. 2 for structures). The hamsters then received both fluoresterol and [^{3}H]cholesterol delivered together in corn oil. Measure-

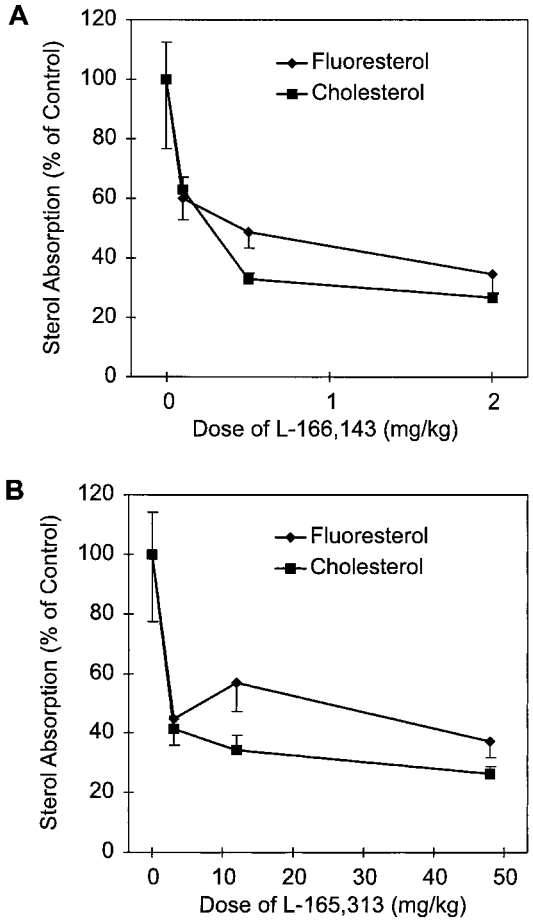

Fig. 5. L-166,143 and L-165,313 inhibit absorption of both cholesterol and fluoresterol in a concentration-dependent manner. A: Hamsters (five per group) were given increasing doses of L-166,143, followed by a mixture of [^{3}H]cholesterol (200 μg and 1 μCi) and fluoresterol (200 μg) in 200 μl corn oil. After 4 h, plasma was collected and both sterols were measured. In control animals that received no L-166,143, the ratio of cholesterol to fluoresterol absorption was 3.6 ± 0.4 (mean ± SEM). B: Hamsters (five per group) were given increasing doses of L-165,313 followed by a mixture of [^{3}H]cholesterol (200 μg and 1 μCi) and fluoresterol (200 μg) in 200 μl corn oil. After 4 h, plasma was collected and both sterols were measured. In control animals receiving no L-165,313, the ratio of cholesterol to fluoresterol absorption was 7.1 ± 2.2 (mean ± SEM).

ment of plasma fluoresterol and [^{3}H]cholesterol content at 4 h showed that the more potent inhibitor, L-166,143, blocked the absorption of both fluoresterol and [^{3}H]cholesterol to a similar extent (**Fig. 5A**). The second, less potent compound, L-165,313, also blocked absorption of both sterols to a similar extent, although 10-fold more than L-166,143 was required to achieve the same effect (Fig. 5B). For both compounds the dose–response curves were very similar for fluoresterol and cholesterol, suggesting that both are absorbed by the intestinal epithelium by a common mechanism. Thus fluoresterol movement into the hamster small intestine is likely to be representative of the early stages of cholesterol movement.

Fluoresterol is absorbed in the hamster jejunum

Fluoresterol trafficking in vivo was followed by confocal microscopy in frozen sections of the hamster jejunum, the primary site of cholesterol absorption in the small intestine (1). Hamsters were gavaged with 400 μg fluoresterol in liquid diet and killed at times up to 4 h. Sections of jejunum from each time point were observed at low and high magnification. Control hamsters received liquid diet and corn oil only, without fluoresterol, and there was no fluorescence present in the jejunums of these hamsters (not shown).

Overall fluorescence from fluoresterol in the jejunum was brightest at 2 h after gavage, offering the most information in low magnification views. Fluoresterol appeared as large, fluorescent droplets, primarily located in the apical cytoplasm of absorptive epithelial cells (**Fig. 6**). The droplet appearance of the fluoresterol was presumably due to the contemporaneous absorption of lipid from the corn oil that was used as a carrier for the fluoresterol. More diffuse fluorescence was also observed in the cytoplasm surrounding the droplets and the nuclei, but nuclei themselves remained unstained. The occasional goblet cell within the epithelial layer did not take up the probe (arrowheads, Fig. 6). Fluoresterol absorption occurred predominantly in the distal portion of the villi. Fluorescence was much reduced in intensity toward the base of the villi, and no fluorescence was visible within the crypts or the submucosa (bottom right of Fig. 6). Bright fluoresterol-containing dots were also visible within the lamina propria, indicating that fluoresterol was able to transit from the apical to the basal aspect of the absorptive cells and become packaged in lipoproteins.

Sections from the time course of fluoresterol absorption viewed at higher magnification revealed that absorption was readily detectable as early as 30 min after gavage (**Fig. 7**).

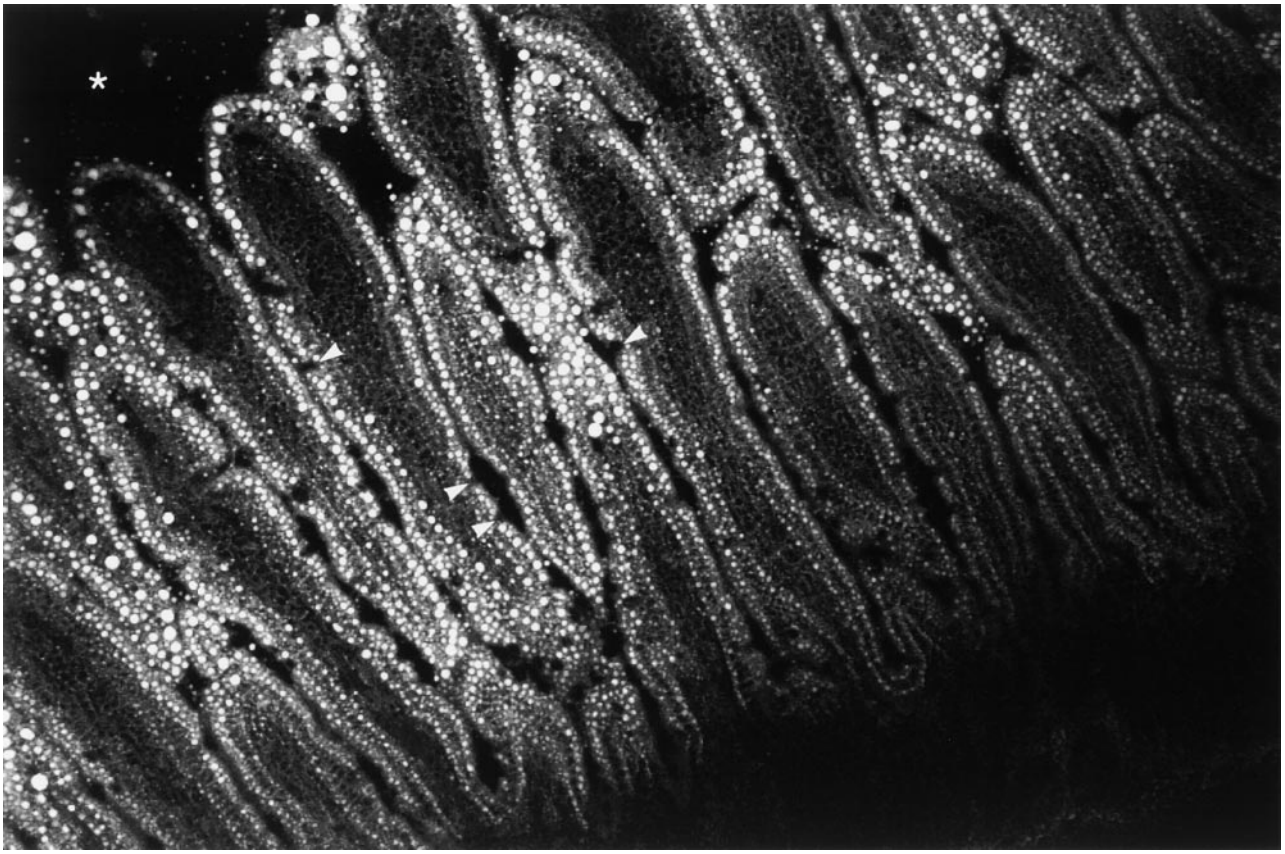

Fig. 6. Fluoresterol trafficks through the intestinal absorptive epithelium of hamsters. Hamsters received an oral dose of 400 μg fluoresterol in liquid diet. Two hours later the jejunum was collected and prepared for confocal microscopy. This is a representative section taken from the proximal half of the jejunum. The villi are oriented with their tips pointing toward the lumen (*) in the upper left of the figure. Crypts are present in the lower right corner, but no fluoresterol was observed in the crypts. Fluoresterol is visible within the absorptive epithelial cells lining the surface of the villi, but not within goblet cells (indicated by arrowheads). 100×.

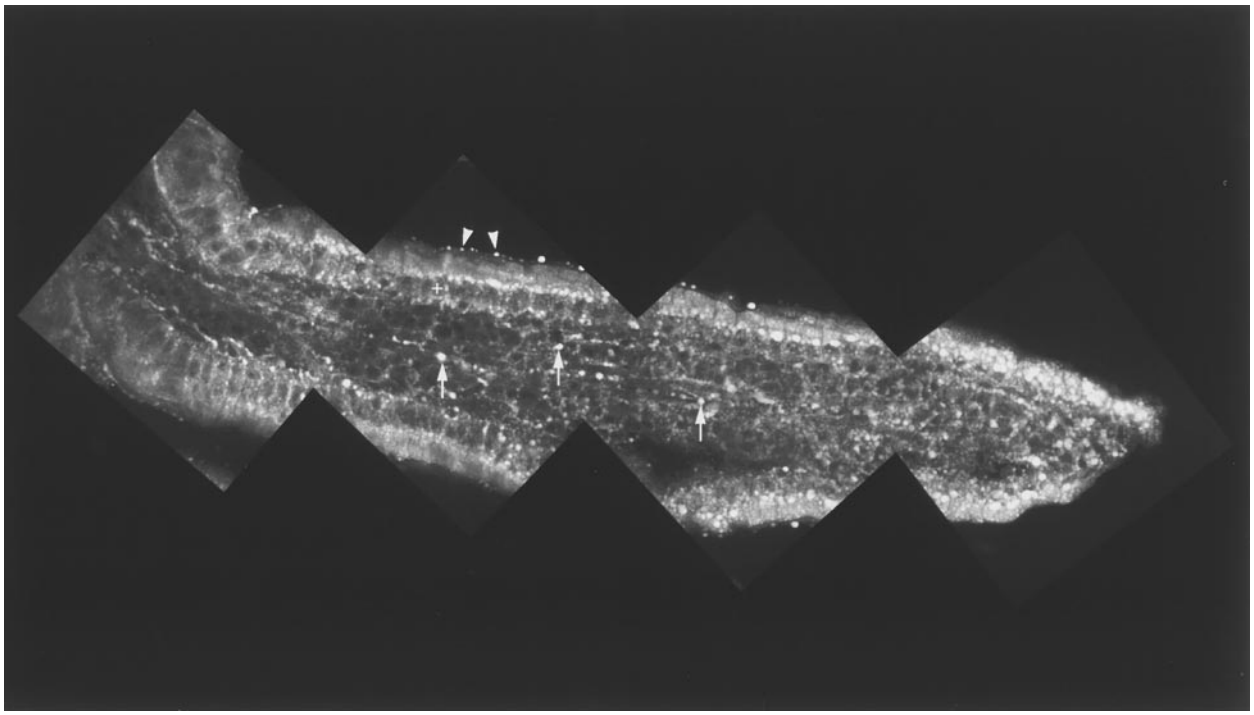

Fig. 7. Localization of fluoresterol within a villus at 30 min. Hamsters were gavaged with 400 μg fluoresterol in liquid diet, and the jejunum was prepared for confocal microscopy after 30 min. This shows one representative villus in the proximal half of the jejunum. At this time, fluoresterol was present primarily in the absorptive epithelium covering the distal half of the villi, with less fluorescence present in the cells of the proximal half. Fluorsterol was associated with the brush border (arrowheads) and also present within the lamina propria (small arrows). 180×.

At this early time point, fluoresterol was visible associated with the brush border of the absorptive epithelial cells (arrowheads, Fig. 7), even in areas of the proximal half of the villi where less absorption was occurring. The fluoresterol within the apical portion of the absorptive epithelial cells was present as multiple, small, fluorescent droplets, with more and larger droplets visible in cells near the tips of the villi. Small fluorescent droplets were present between unlabeled nuclei, suggesting transit of fluoresterol to the baso-lateral membrane. The estimated size range of the droplets was 5–40 μm (n = 50), with the vast majority (80%) of the droplets falling in the 10–20 μm range. Fluoresterol was also present in the lamina propria at 30 min (arrows, Fig. 7), suggesting successful packaging of fluoresterol into lipoprotein particles at this early time. This observation was consistent with the appearance of fluoresterol in plasma by as early as 30 min.

Two hours after gavage the gradient of absorption along the villar length remained, with more uptake occurring near the tips (**Fig. 8A–C**). In addition to the numerous small dots of fluorescence within the cytoplasm of the absorptive cells, there were also more large, fluorescent droplets present than at 30 min. The larger droplets may have resulted from coalescence of small droplets. Many small fluorescent dots were visible toward the lateral and basal aspects of the epithelial layer along the entire length of the villi (Fig. 8A, B, and C), although fluorescence was more concentrated distally than proximally (Fig. 8A, compared to 8C). Fluoresterol was also present in the lamina propria as both small and large punctate structures. This time appears to represent a period of peak flux of fluoresterol into and out of the epithelial layer.

At 4 h, fluoresterol labeling in the villi was reduced to a very faint signal, although lipid droplets could still be detected in the absorptive cells at the very tips of the villi (Fig. 8D). This suggests that fluoresterol may be selectively removed from the droplets prior to removal of lipid. The loss of fluoresterol labeling in the jejunum by 4 h indicates that absorption of fluoresterol was essentially complete by this time. This result is supported by the observation that the fluoresterol concentration in plasma had appeared to reach a plateau by this time.

A cholesterol absorption inhibitor slows uptake of fluoresterol in the jejunum

The effect of a cholesterol absorption inhibitor, L-166, 143, on trafficking of fluoresterol at the cellular level in the small intestine was also observed. Hamsters were treated with L-166,143 1 h prior to administration of fluoresterol in liquid diet. Four h after giving fluoresterol, the jejunum from an untreated animal showed very little residual fluorescence (**Fig. 9A**). In contrast, hamsters that received the inhibitor had fluoresterol present within the absorptive epithelial layer at this time. Droplets were present in the apical cytoplasm (arrowheads, Fig. 9B) and there were many fluorescent particles remaining in the lamina propria (arrows, Fig. 9B). This observation suggests that the compound slowed the entry of fluoresterol

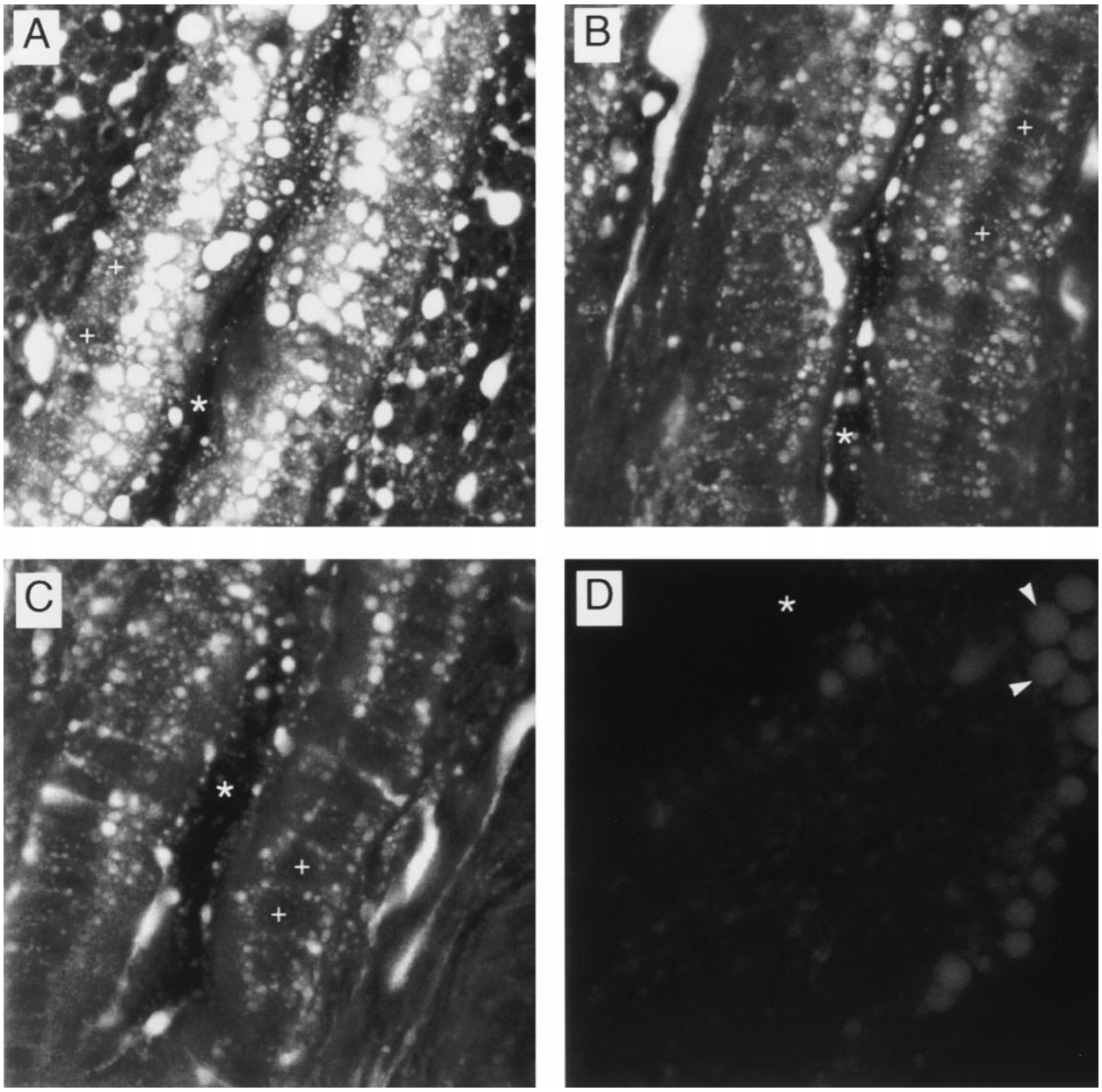

Fig. 8. Localization of fluroesterol in villi at 2 and 4 h. Hamsters were gavaged with 400 μg fluoresterol in liquid diet, and jejunums were prepared for confocal microscopy at 2 h (A, B, and C) or 4 h (D). Panels A–C show views of the epithelial layers from two closely opposed villi with the lumen (*) between them, running roughly down the center of the panels. Panel A is taken from an area on the distal half of the villi; Panel B is from the proximal half of the villi; Panel C is at the base of the villi. A: Strongly fluorescent droplets are visible within the absorptive cells between the apical brush border and the level of the nucleus (+). Fluoresterol is also present within the lamina propria. B and C: Further toward the base of the villi, less fluoresterol is present within the absorptive cells, and the intracellular droplets are smaller. D: At 4 h, near the tip of the villus, there is little fluoresterol remaining, although lipid droplets (arrowheads) are present within the epithelium. 280×.

into the cells but did not prevent the normal trafficking of fluoresterol, once it had entered. The fluorescence in the absorptive epithelium was much less in treated animals at all times than the signal in untreated animals at 30 min to 2 h (Figs. 7, 8, 9, and data not shown), suggesting that, overall, less fluoresterol entered the epithelial cells in the presence of compound. Taken together with the data on plasma fluoresterol levels, these results indicate that fluoresterol absorption by enterocytes is inhibited by a compound known to inhibit cholesterol absorption.

Fluoresterol is esterified in vivo

The images of fluoresterol trafficking through intestinal epithelial cells suggested that fluoresterol was rapidly absorbed and distributed to cytoplasmic compartments. In addition, the appearance of fluoresterol at the baso-lateral aspects of the epithelial cells and within the lamina propria suggested that it was processed normally into lipoprotein particles. To test whether fluoresterol was esterified after its entry into the absorptive cells, we gavaged hamsters with fluoresterol and [^{3}H]cholesterol and followed the uptake

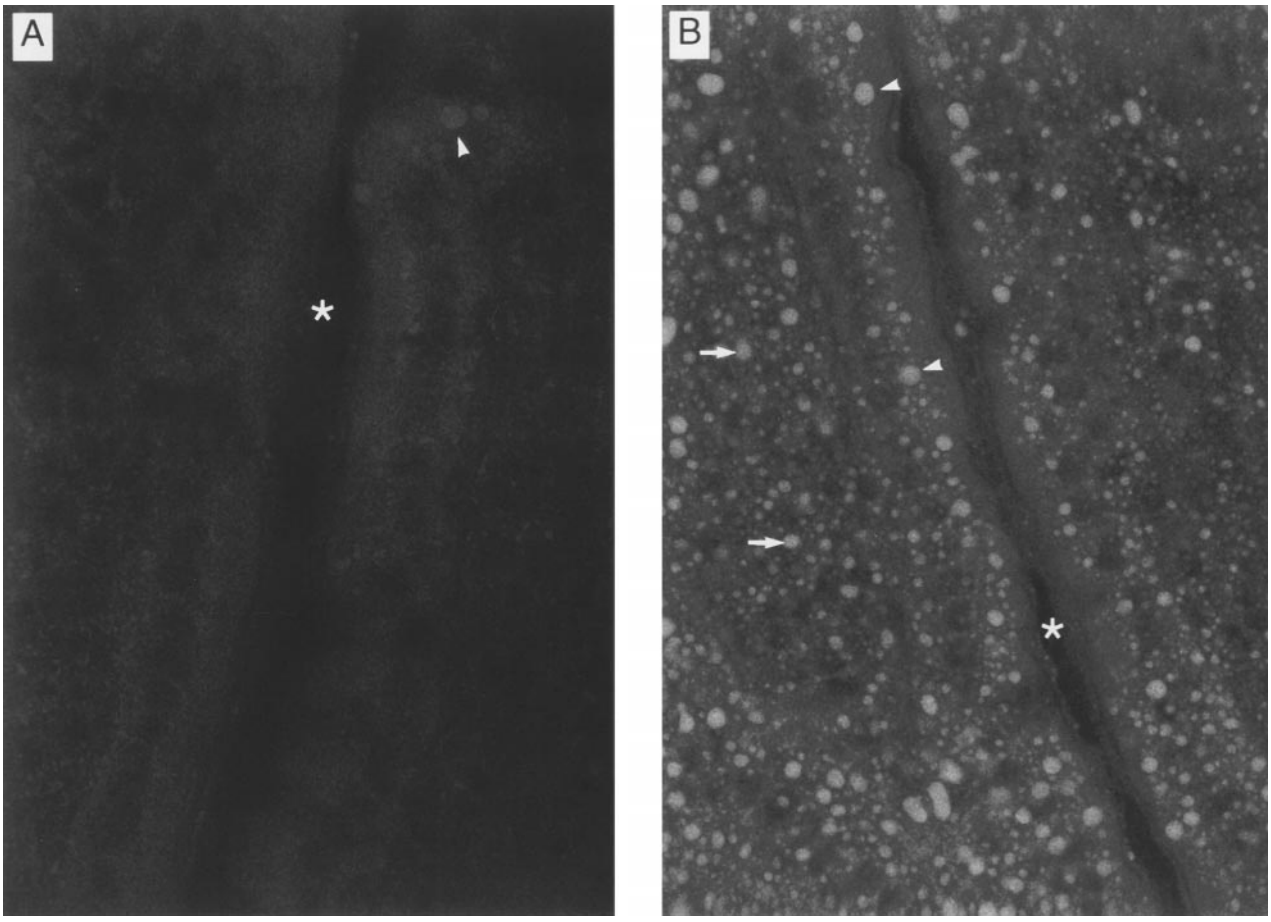

Fig. 9. L-166,143 slows absorption of fluoresterol. Hamsters were gavaged without (A) or with (B) 2 mg/kg L-166,143, followed by 400 μg fluoresterol in liquid diet. After 4 h, jejunums were collected and prepared for confocal microscopy. Both panels show parts of two opposed villi with the lumen (*) between them running from top to bottom. A: Little fluoresterol remains in the villi at this time, although a few slightly fluorescent lipid droplets remain near the tips of the villi (arrows). B: In animals dosed with L-166,143, many more and brighter fluroescent droplets are visible within the absorpive cells (arrowheads) and within the lamina propria (arrows). 280×.

and esterification of the two sterols in the small intestinal mucosa (**Fig. 10**). The mucosa contained more [^{3}H]cholesterol (>25-fold) throughout the time course, which parallels the difference in absolute absorption between the two sterols (see legend to Fig. 5). The mucosal content of [^{3}H]cholesterol increased up to 2 h, then began to decline. In contrast, fluoresterol content was at a maximum by 30 min and declined over the remainder of the time course. The very low level of fluoresterol in the mucosa at 4 h is consistent with the confocal images of this tissue demonstrating extremely little residual fluorescence at this time. Thus fluoresterol appeared to pass through the mucosal cells more rapidly than did cholesterol. The faster transit time of fluoresterol relative to cholesterol suggests that fluoresterol may also move between cellular compartments more rapidly. Indeed at 4 h the percent of fluoresterol that was esterified was about 5-fold greater than the percent esterification of cholesterol (8% of total). This suggests that fluoresterol is a substrate for ACAT.

Cells in vitro take up and esterify fluoresterol

Uptake of fluoresterol and [^{3}H]cholesterol from bile acid micelles and their subsequent esterification was also tested in vitro using Caco-2 cells, a widely used model of small intestinal enterocytes (3, 10, 11, 13) derived from a human intestinal tumor (9). Cultured cells received equimolar fluoresterol and [^{3}H]cholesterol for times up to 22 h, then uptake and esterification of the sterols was quantitated. After 2 h, nearly as much fluoresterol as [^{3}H]cholesterol was taken up by the cells, but by 20–22 h the amount of fluoresterol taken up was only 25–55% of the [^{3}H]cholesterol (**Table 1**). The cells esterified less than 5% of the [^{3}H]cholesterol taken up, in agreement with previous work (13). By 20–22 h more than 20% of the fluoresterol taken up had been esterified. These results in cultured cells are similar to the data from whole animals presented above and verify that fluoresterol is taken up and esterified by enterocytes.

Fluoresterol is esterified by ACAT

To determine whether the esterification of fluoresterol was catalyzed by ACAT, microsomes from hamster intestine were assayed for the ability to esterify fluoresterol and [^{3}H]cholesterol. ACAT enzyme assays were performed as described (19), except that both sterols were present at 50 μg/ml each. After 2.5 min of incubation, 1.5% ± 0.2% of the [^{3}H]cholesterol was esterified, whereas 18% ± 0.3% of

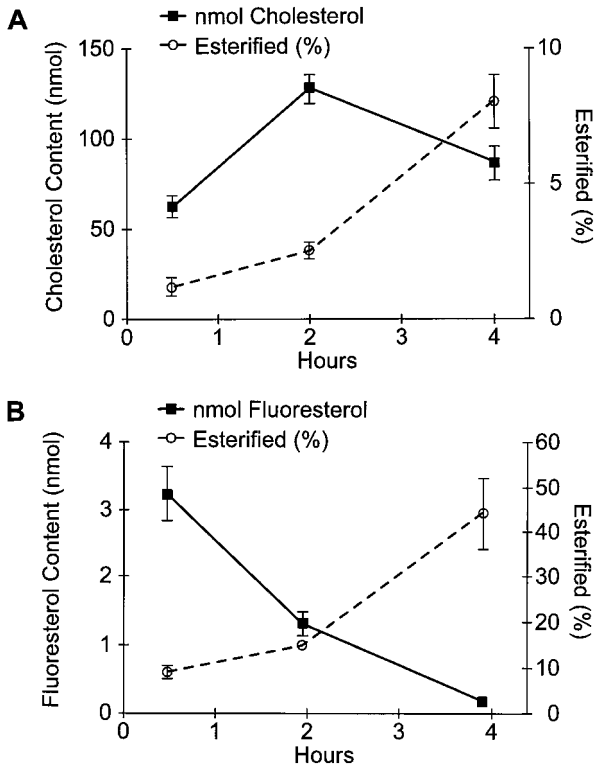

Fig. 10. Fluoresterol is esterified in the hamster mucosa. Hamsters (four per group) were given [^{3}H]cholesterol (400 μg and 1 μCi) and 400 μg fluoresterol in corn oil by gavage. At times up to 4 h, the entire jejunum mucosa was harvested and analyzed for total content of each sterol and for the extent of sterol esterification. Data are mean ± SEM. A: [^{3}H]cholesterol; B: fluoresterol.

the fluoresterol was esterified (mean ± range of duplicates). Parallel reactions containing 5 μm of the ACAT inhibitor DuP 128 (19) showed dramatically decreased esterification of both fluoresterol (85%) and cholesterol (>95%), indicating that the esterification was catalyzed by ACAT.

DISCUSSION

A comparison of a fluorescent analog of cholesterol, dubbed fluoresterol, with [^{3}H]cholesterol demonstrated that fluoresterol, like cholesterol, was absorbed by hamster intestinal epithelial cells in vivo and appeared in the plasma. Fluoresterol absorption was blocked by compounds that inhibited cholesterol absorption, suggesting that it entered the absorptive epithelium of the intestine through the same pathway as cholesterol. The overall absorption of fluoresterol in vivo was, however, less efficient than for cholesterol. This may reflect either a lower affinity of fluoresterol for a cholesterol transporter or decreased solubility in bile acid micelles within the intestinal lumen. Thus, while absorption of fluoresterol resembled cholesterol absorption in many key respects, there were some differences that may be attributed to its altered structure.

As fluoresterol appears to enter the intestinal epithelium via the same route as cholesterol, fluoresterol could be a useful probe for identifying the putative cholesterol transporter. A screen for binding of fluoresterol might lead to the expression cloning of the intestinal cholesterol transporter, much as a fatty acid transporter was identified using a fluorescent fatty acid analog in an analogous approach (20). Further work will be required to determine if such an approach is feasible.

When viewed by confocal microscopy, fluoresterol in the hamster intestine provided a graphic demonstration of the course of sterol absorption. Small fluorescent particles were observed in association with the brush borders of the intestinal epithelial cells, most likely representing the initial interaction of fluoresterol-containing droplets and/or micelles with the plasma membrane. Fluoresterol was rapidly taken up by the epithelial cells, but entered only those cells specialized for absorption, not the secretory goblet cells, demonstrating specificity in the mode of uptake. Within the enterocytes fluoresterol was present most prevalently in large apical droplets. The high concentration of fatty acids present in the intestine from the corn oil used as a carrier probably contributed to the size and distribution of the fluoresterol-containing droplets within the cells, which presumably also contained triglycerides.

Interestingly, there was a distinct gradient of fluoresterol absorption along the length of the intestinal villi, with more absorption observed near the villar tips. This could be explained by a gradient of fluoresterol availability, with less available or accessible nearer the bases of the villi

TABLE 1. Fluoresterol and [^{3}H]cholesterol are taken up and esterified by Caco-2 cells

	Sterol Uptake		% of Sterol Esterified	
	[^{3}H]cholesterol	Fluoresterol	[^{3}H]cholesterol	Fluoresterol
	nmol/mg cell protein		*%*	
Experiment #1 (47 μm each sterol)				
2 h	0.47 ± 0.31	0.38 ± 0.05	ND	ND
20 h	3.0 ± 0.2	0.64 ± 0.06	3.9 ± 0.4	23.9 ± 1.3
Experiment #2 (100 μm each sterol)				
22 h	14.8 ± 0.8	8.2 ± 0.3	0.9 ± 0.04	21.2 ± 1.7

Caco-2 cells were incubated with bile acid micelles containing equimolar [^{3}H]cholesterol and fluoresterol. After various times, cells were harvested and analyzed for cellular content of sterol and for extent of sterol esterification (for details, see Methods). Experiment #1 was performed in duplicate and values given indicate mean and range of duplicates. Experiment #2 was performed in triplicate and values are mean ± SEM; ND, not determined.

compared to the areas projecting further into the intestinal lumen. Alternatively, the differentiation state of the epithelial cells lining the villi may be a factor that contributes to their ability to absorb cholesterol. Less differentiated cells nearer the crypts may express less of the cholesterol transporter or have other deficiencies in components of intracellular cholesterol transport. There is precedent for enterocyte proteins to display a gradient of expression along the crypt-to-villus axis: Chandrasekaran, Coopersmith, and Gordon (21) have shown that the cell–cycle proteins cyclin D1 and cyclin-dependent kinase-2 are more highly expressed in proximal enterocytes that are early in their migration from crypt to villus tip.

Fluoresterol was esterified both in vivo and in cultured cells, indicating that it was able to traffic to the endoplasmic reticulum and act as a substrate for ACAT. Fluoresterol was also shown to be esterified by ACAT in microsomal enzyme assays. Significantly more fluoresterol than cholesterol was esterified in all three settings (hamster mucosa, Caco-2 cells, and enzyme assays). The rapid rate of fluoresterol esterification may indicate that fluoresterol is a better substrate for ACAT than is cholesterol. Cholesterol esterification, however, is believe to be regulated in part by the availability of cholesterol to ACAT in the endoplasmic reticulum (13, 14, 22, 23). Cholesterol esterification in macrophages occurs only after plasma membrane cholesterol content reaches a critical threshold (24), which is set by membrane sphingomyelin content (25). Cholesterol biosynthetic precursors are transported out of the plasma membrane more rapidly than cholesterol itself (11, 12), and this sterol transport process probably requires p-glycoproteins (12, 13). It is possible that fluoresterol is recognized as a cholesterol precursor, and is therefore actively removed from the plasma membrane. Alternatively, the more polar side chain of fluoresterol may change its aqueous solubility or orientation in the membrane, and these properties could contribute to differential processing of fluoresterol versus cholesterol. Another possibility is that the transport of the fluorescent cholesterol analog to the endoplasmic reticulum might be analogous to the rapid transport of a fluorescent ceramide analog to the Golgi apparatus (26). Until more of the processes and components of the intracellular sterol trafficking pathway have been identified, it will be difficult to assess whether fluoresterol interacts with each component in the same way as does cholesterol. Cultured cells might provide a model in which fluoresterol could aid in the identification of additional components involved in the sterol trafficking pathway.

It was evident from the confocal images that fluoresterol was packaged into lipoprotein particles that exited the absorptive epithelial cells along their baso-lateral aspects. The rapid esterification of fluoresterol could also accelerate the packaging of fluoresterol into lipoproteins in enterocytes. Neutral lipids enter the nascent lipoprotein particle from the endoplasmic reticulum and/or the Golgi membranes via the action of the microsomal triglyceride transfer protein (27). The delivery of fluoresterol to the endoplasmic reticulum, followed by esterification, may effectively provide fluoresterol and/or fluoresteryl ester for the transfer protein to incorporate into newly forming particles. Once incorporated into lipoproteins, the fluoresterol would be ensured passage to the plasma.

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