Inhibition of lymphatic absorption of cholesterol by cholestane-3β,5α,6β-triol

M. ITO, W. E. CONNOR, E. J. BLANCHETTE, C. R. TREADWELL, and GEORGE V. VAHOUNY

Department of Internal Medicine, University Hospitals, University of Iowa, Iowa City, Iowa 52240, and Departments of Biochemistry and Anatomy, School of Medicine, The George Washington University, Washington, D.C. 20005

ABSTRACT The effect of cholestane-3β,5α,6β-triol (CT) on the intestinal absorption of cholesterol and oleic acid, as well as the absorption of labeled CT, was studied in lymph duct-cannulated rats. Intragastric administration of 50 mg of CT in an emulsion with cholesterol-7α-^{3}H and oleic acid-1-^{14}C resulted in 50% inhibition of sterol transfer into lymph but only 8% depression of fatty acid absorption over an 8 hr period. The absorption of labeled CT into lymph was only 2–3% compared with 50% absorption of cholesterol when each was fed alone. 10% of the fed CT was recovered in the intestinal mucosa, and of this, one-half was associated with the brush border fraction.

In rats fed CT 6 days prior to cholesterol and fatty acid administration, there was no effect on fatty acid absorption, while cholesterol absorption was reduced by almost 30%. When the intestinal mucosa from these animals were investigated by electron microscopy, it appeared that CT feeding resulted in numerous enlarged mitochondria and a marked increase in length of the microvilli. If animals were allowed to recover for 6 days from the CT prefeeding regime, the intestinal mucosa appeared normal, and the absorption of cholesterol approached that in controls.

A possible mechanism for CT inhibition of cholesterol absorption was shown to be competition for the enzyme cholesterol esterase which esterifies cholesterol prior to entrance into the lymphatic system. CT itself is poorly esterified and poorly absorbed, but it is effective in inhibiting esterification of cholesterol in vitro.

SUPPLEMENTARY KEY WORDS intestinal absorption · oleic acid · mucosal esterification · elongated microvilli · enlarged mitochondria · atherosclerosis

Abbreviations: CT, cholestane-3β,5α,6β-triol.

RECENT STUDIES by Aramaki et al. (1) have indicated that cholestane-3β,5α,6β-triol (CT), a structural analogue of cholestanol (Fig. 1), has marked hypocholesteremic and antiatherogenic properties when fed to rabbits on cholesterol diets. Further investigations resulted in the suggestion that the hypocholesteremic effect of the inhibitor was due to increased fecal sterol excretion, resulting from inhibition of cholesterol absorption and (or) increased intestinal excretion (2). Although a direct effect of cholestanetriol on cholesterol absorption was shown, no attempt was made to distinguish between intraluminar and intracellular effects of the inhibitor. It was suggested, however, that cholestanetriol did not form mixed crystals with cholesterol.

Conner, Witiak, Brahmanker, Wartman, and Parker (3) have also found that when rabbits were fed CT at a concentration of 0.5% in a diet containing 0.5% cholesterol and 2.5% peanut oil, the CT completely prevented hypercholesteremia due to cholesterol feeding. When the triol was subsequently omitted from the diet, plasma cholesterol levels were elevated to a maximum after 4 wk. Similarly, triol added to a cholesterol-containing diet for rabbits, which were already hypercholesteremic, caused a marked depression of plasma cholesterol levels to near normal within 3–4 wk.

This study therefore investigated (*a*) the short-term effects of cholestanetriol on cholesterol and fatty acid transfer from intestinal lumen to thoracic duct lymph in rats, (*b*) the absorption and esterification of cholestane-4-^{14}C-triol, (*c*) the effect of the prior feeding of CT on cholesterol and fatty acid absorption, and (*d*) the effect of cholestanetriol on the ultrastructure of the intestinal

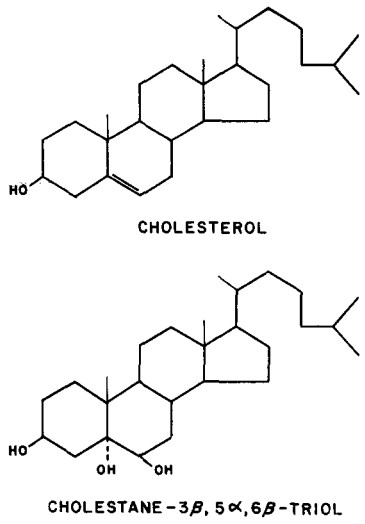

FIG. 1. Structures of cholestane-3β,5α,6β-triol and cholesterol.

mucosa. A preliminary report of this study has appeared (4).

MATERIALS AND METHODS

Cholesterol-7α-^{3}H and oleic acid-1-^{14}C were obtained from the Nuclear-Chicago Corporation (Des Plaines, Ill.) and were diluted with unlabeled cholesterol (purified via the dibromide) and oleic acid (> 99% pure, The Hormel Institute, Austin, Minn.). Cholestane-4-^{14}C-3β,5α,6β-triol was prepared and purified (5) for W. E. Connor. Identification and purity were determined by gas–liquid chromatography as described below.

Animal Procedures

Adult male rats of the Wistar strain (Microbiological Associates, Inc., Bethesda, Md.), each weighing 200–300 g, were maintained on standard laboratory chow and water ad lib. before operation. The thoracic duct was cannulated by a modification of the procedure of Bollman Cain, and Grindlay (6), and the animals were placed in special restraining cages. The rats were then fasted overnight prior to intragastric administration of the test emulsions.

The test emulsions used for intragastric administration were prepared immediately before use, as described earlier (7). The emulsions contained the following components in 3 ml of 0.9% saline: albumin, 50 mg; sodium taurocholate, 288 mg; oleic acid-1-^{14}C, 292 mg; and cholesterol-7α-^{3}H, 50 mg. In certain emulsions, 50 mg of unlabeled cholestanetriol was also included. For the study on the absorption and recovery of cholestane-4-^{14}C-triol, the oleic acid included in the emulsion was unlabeled, and cholesterol was omitted. The amounts of fatty acid and sodium taurocholate were in a molar ratio of 8 and 4, respectively, to 50 mg of cholesterol and were optimal for intestinal absorption of cholesterol (8).

Mucosal homogenates were prepared in 9 volumes of ice-cold 0.278 M mannitol and fractionated into nuclei and cell debris (plus surface mucoprotein), mitochondria, microsomes, and cytoplasm by the procedure of Hogeboom and Schneider (9). Brush border was prepared from the intestinal mucosa according to the method of Miller and Crane (10) as modified by Gallo and Treadwell (11). Invertase activity, used to measure the efficiency of brush border separations, was determined by the method of Börgstrom and Dahlqvist (12).

Everted intestinal sacs were prepared by the procedure of Wilson and Wiseman (13). Incubations were carried out for 120 min at 37°C in 25 ml of phosphate buffer (0.154 M), pH 6.2, containing 0.3% glucose. During the incubation, large amounts of white mucoprotein are released into the medium (14). The sacs were rinsed with a stream of saline, the medium and rinsings were combined, and the solution containing the mucoprotein was centrifuged at 500 *g*. Radioactivity and protein content (15) of the precipitated mucoprotein and remaining supernatant were determined.

Lipid Extraction and Analysis

Aliquots of the individual lymph samples and the mucosal homogenates were extracted in 20 volumes of chloroform–methanol (2:1 v/v), separated into phases, and washed according to the method of Folch, Lees, and Sloane Stanley (16). The chloroform was evaporated under nitrogen, the residue was redissolved in 10 ml of petroleum ether, and 2 ml was placed in a counting vial. The solvent was evaporated, 10 ml of liquid scintillant (100 mg of 1,4-di-2[5-phenyloxazolyl]-benzene + 4 g of 2,5-diphenyloxazole per liter of toluene) was added, and the radioactivity was measured in a liquid scintillation counter (Nuclear-Chicago, Mark I). Measurements in cpm were corrected to dpm by external standardization and the channels ratio method (17).

For the determination of the distribution of radioactivity in the lymph lipids, the lymph samples (four samples were taken from each animal at different times) were pooled and total lymph lipids were extracted as reported above. Lipids were fractionated by thin-layer chromatography on silicic acid in hexane–acetone–acetic acid 88:10:3. After visualization of the lipid bands with iodine vapor, the silicic acid areas corresponding to standards for the major lipid fractions were individually scraped into scintillation vials. 1 ml of methanol and 10 ml of scintillation mixture were added, and the samples were counted as described above.

All lymph and tissue samples from animals fed labeled cholestanetriol were extracted in 25 volumes of ethanol–ether 3:1 by bringing the extract to a rolling boil several

times. After cooling, the volume was adjusted, the samples were filtered, and 5-ml aliquots were placed in scintillation vials for counting. Aliquots were also evaporated and applied to thin-layer silicic acid plates for separation of free and esterified cholestanetriol in hexane–acetone–acetic acid 88:10:3. These two fractions had R_f values different from those of free and esterified cholesterol; they were identified by comparison with known standards and by gas–liquid chromatography.

Cholesterol Esterase Studies

Fresh rat pancreatic juice (free of bile) was used as the enzyme source and was obtained as described previously (18). Preparation and composition of albumin-dispersed substrate mixture for enzymatic synthesis of cholesterol esters has been described in detail previously (19). The mixture for sterol ester synthesis contained 15.5 μmoles of cholesterol-4-^{14}C or cholestane-4-^{14}C-triol (specific activity, 1×10^5 dpm/mg), 46.5 μmoles of oleic acid, 31 μmoles of sodium taurocholate, 100 μmoles of ammonium chloride, and 1.5 mg of albumin (Fraction V) in a final volume of 1.5 ml of 0.154 M phosphate buffer, pH 6.2. For inhibition studies, unlabeled cholestanetriol in increasing concentrations was added to the cholesterol-containing substrate mixture. With this substrate mixture, 0.1–0.5 ml of enzyme was used. Incubations were carried out at 37°C in a Dubnoff metabolic shaker (100 excursions per min). After addition of enzyme, 20-μl samples were withdrawn at 2–5-min intervals and added to 20 μl of acetone–ethanol 1:1. This extract was shaken and immediately heated in a boiling water bath.

The lipid sample in acetone–ethanol was applied as a spot to a silicic acid-coated microscope slide and dried, and the slide was developed in hexane–diethyl ether–acetic acid 83:16:1. The upper and lower halves of the silicic acid on the plate were scraped quantitatively into separate scintillation vials, and 1 ml of spectroanalyzed methanol and 10 ml of scintillant mixture were added. Counting was done in a Nuclear-Chicago liquid scintillation spectrophotometer, and all counts were corrected to dpm by external standardization.

Electron Microscopy

For electron microscopy, small (2-cm) segments of mid-ileum and mid-jejunum were excised, sliced into 1 mm segments, and placed in cold (4°C) phosphate-buffered 3% glutaraldehyde (pH 7.4) for 1 hr. After a brief buffer wash, specimens were postfixed in 2% buffered OsO_4 for 2 hr to improve image contrast. The tissues were dehydrated in graded aqueous acetone mixtures, infiltrated, and embedded in Epon. Sections were prepared on a Reichert ultramicrotome with a diamond knife.

For orientation purposes and light microscopic study, 0.5 μm sections mounted on glass slides were stained with aqueous toluidine blue. Thin sections for electron microscopy were stained with uranyl acetate and lead citrate and examined in an RCA-EMU-3H electron microscope equipped with a heated aperture.

Gas–Liquid Chromatography of Sterols

The sterol mass analyses were performed on a dual-column gas chromatograph (F & M Scientific model 402 high-efficiency gas chromatography; F & H Scientific Corp., Avondale, Pa.) equipped with hydrogen flame ionization detector and an automatic integrator (Infotronic CRT Model 100 digital integrator; Infotronics, Inc., Houston, Texas). The column was a 120 cm glass U-tube, 4 mm I.D., packed with Diatoport S (80–100 mesh) coated with a film (3.8%) of SE-30 (methyl siloxane polymer). Temperatures of column, detector, and flash heater were 230°, 250°, and 300°C, respectively. Helium was the carrier gas at a flow rate of 100 ml/min; the inlet pressure was 40 psi.

The purified cholestane-4-^{14}C-triol had a retention time relative to standard cholestane of 4.18; all radioactivity was associated with the main peak (maximum impurity that could have remained undetected, 5%).

RESULTS

In Vivo Studies

In the initial study, fasting lymph duct-cannulated rats were given, by stomach tube, emulsions containing cholesterol-7α-^{3}H and oleic acid-1-^{14}C with or without 50 mg of cholestane-3β,5α,6β-triol, and lymph was collected in 2-hr periods for 8 hr. The percentage absorption of cholesterol and oleic acid during each collection period is shown in Fig. 2. The inclusion of cholestanetriol in the test emulsion resulted in a delay in absorption of oleic acid, with the peak of absorption then occurring between 4 and 6 hr, and a marked reduction in the appearance of tritium in lymph. The effects on fatty acid and cholesterol absorption appeared to be different, however, in terms of the extent of inhibition by CT. When cumulative absorption of the lipids was plotted, as shown in Fig. 3, it became apparent that by 8 hr there was a significant difference in absorption of cholesterol between the control and experimental groups, while there was little difference in the cumulative appearance of oleic acid-1-^{14}C into lymph. These differences will be discussed more fully below.

The distribution of isotope among the various lymph lipid fractions was obtained by combining aliquots of the individual 2-hr fractions in amounts proportional to the volumes of each fraction. The data, summarized in Table 1, show that in the lymph from control animals, 87% of

TABLE 1 DISTRIBUTION OF ABSORBED CHOLESTEROL-7α-^{3}H AND OLEIC ACID-1-^{14}C IN THORACIC DUCT LYMPH

Additions to Basic Emulsion*	Oleic Acid-1-^{14}C As:						Cholesterol-7α-^{3}H As:	
	TG†	DG	MG	FA	PL	CE	Free	Esterified
None (four rats)	86.7	5.6	0.8	1.7	0.7	4.4	11.8	88.2
CT, 50 mg (three rats)	87.6	5.2	0.7	2.6	1.3	2.7	14.1	85.9

* The basic emulsion consisted of 50 mg of cholesterol-7α-^{3}H, 292 mg of oleic acid-1-^{14}C, 288 mg of sodium taurocholate, and 50 mg of albumin in 3 ml of saline per rat.
† TG, triglyceride; DG, diglyceride; MG, monoglyceride; FA, fatty acid; PL, phospholipid; CE, cholesteryl ester.

the label from oleic acid-1-^{14}C administration was in the form of triglyceride, and 88% of the label from cholesterol-7α-^{3}H feeding was recovered as esterified cholesterol in lymph. The inclusion of cholestanetriol in the diet had no effect on these percentages.

The absorption of cholestane-4-^{14}C-triol was determined by carrying out an isotope recovery experiment in lymph duct-cannulated rats 24 hr after they had been fed an emulsion containing labeled CT, oleic acid, taurocholate, albumin, and saline. Each of the rats in all groups was given 3 ml of the appropriate test emulsion intragastrically under light vinyl ether anesthesia, and lymph was collected for 24 hr in tubes containing 0.05 ml of heparin (1000 units/ml). At the end of the experiment, the animals were killed, the stomach and intestine were removed and flushed with 150 ml of saline, and the washings were combined with the collected feces. The feces plus intestinal contents, and the small intestine to which was added 25 ml of saline, were then separately homogenized. As shown in Table 2, only 2–3% of the administered cholestanetriol was recovered unchanged in lymph compared to 54% recovery of cholesterol during the 24 hr experimental period. Conversely, 80% of the cholestanetriol was recovered in feces and contents during this same period. Of interest, however, was the finding that 10% of the cholestanetriol was recovered unchanged from the intestinal mucosa 24 hr after feeding.

Analysis of the intestinal and lymph extracts by thin-layer chromatography showed that in the *intestine*, 92% of the cholestanetriol and of the cholesterol was unesterified. However, after cholesterol-7α-^{3}H had been fed to the rats 88% of it recovered in *lymph* was esterified, whereas only 23–24% of the labeled cholestanetriol recovered in lymph was in the esterified form.

TABLE 2 ABSORPTION OF CHOLESTEROL-7α-^{3}H AND CHOLESTANE-4-^{14}C-TRIOL IN LYMPH DUCT-CANNULATED RATS

Additions (50 mg) to Intragastric Emulsion*	Percentage Recovery in 24 Hr			
	Lymph	Mucosa	Feces + Intestinal Contents	Total
Cholestane-4-^{14}C-triol				
Rat 1	2.4	10.3	80.6	93.3
Rat 2	3.3	8.4	79.0	90.7
Cholesterol-7α-^{3}H (five rats)	53.6 ± 1.1	8.2 ± 1.3	33.9 ± 3.2	95.6

* The basic emulsion contained, per rat, 3 ml of saline, 292 mg of oleic acid, 288 mg of sodium taurocholate, and 50 mg of albumin. Identification of the sterols by thin-layer and gas–liquid chromatography.

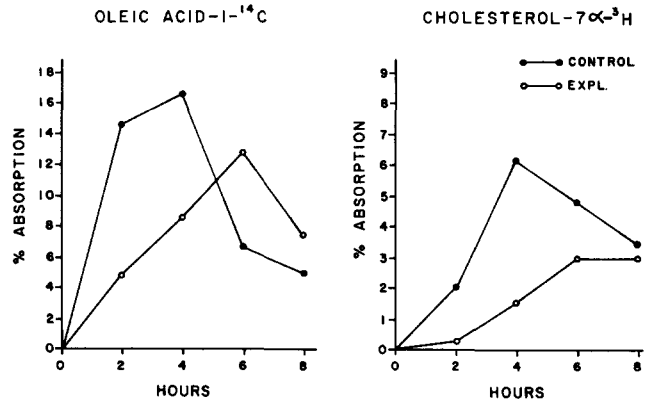

FIG. 2. Time course of absorption of oleic acid-1-^{14}C and cholesterol-7α-^{3}H into thoracic duct lymph in rats. Closed circles represent means from four animals receiving the control intragastric emulsion containing 50 mg of albumin, 50 mg of cholesterol-7α-^{3}H, 288 mg of sodium taurocholate, and 292 mg of oleic acid-1-^{14}C per 3 ml of saline. Open circles represent means from four animals receiving the control emulsion containing also 70 mg of unlabeled cholestane-3β,5α,6β,triol.

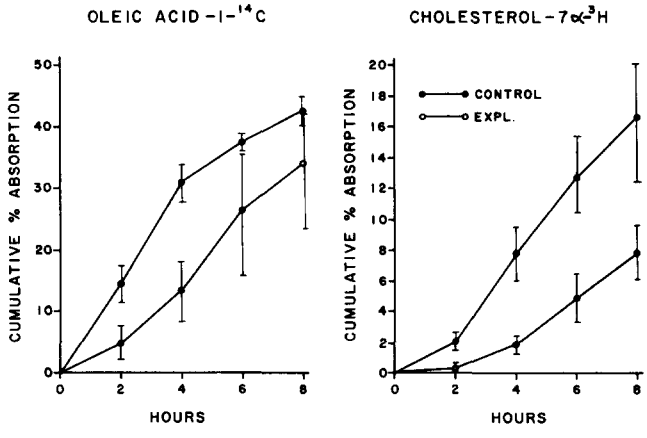

FIG. 3. Cumulative appearance of ^{14}C and ^{3}H in thoracic duct lymph of animals receiving intragastric test emulsions containing oleic acid-1-^{14}C and cholesterol-7α-^{3}H with and without CT. Values are means from four animals ± SEM.

To determine the effect of prior feeding of CT on the absorption of cholesterol and oleic acid, animals were given 50 mg of CT in 1 ml of corn oil intragastrically each day for 6 days, including the day on which the thoracic duct was cannulated. All animals were allowed chow ad lib. in addition to the intragastric feeding. After operation, the animals were fasted for 24 hr before the intragastric administration of the test emulsion containing cholesterol and oleic acid (but no CT). Lymph was then collected from control and experimental animals for 24 hr before they were killed. As shown in Table 3, the prior-feeding of CT reduced lymphatic absorption of cholesterol from 54 to 37% while increasing the percentage of cholesterol in feces and intestinal contents. There was no effect on the amount of isotopic cholesterol recovered in the intestinal mucosa. When animals were prefed CT for 6 days and allowed to recover for 6 days before the operation, the lymphatic absorption of cholesterol was normal. Prior feeding of CT for 6 days had no effect on the lymphatic absorption or recovery of oleic acid-1-^{14}C. Analysis of the distribution of isotope among the various lipid fractions (Table 4) showed no significant effects of prefeeding.

TABLE 3 EFFECT OF PREFEEDING CHOLESTANE-3β,5α,6β-TRIOL (6 DAYS) ON THE LYMPHATIC ABSORPTION AND RECOVERY OF CHOLESTEROL-7α-^{3}H AND OLEIC ACID-1-^{14}C IN 24 HR

	Percentage Recovery			
	Lymph	Mucosa	Feces + Intestinal Contents	Total
Cholesterol-7α-^{3}H				
Control (5)	53.6 ± 1.1	8.2 ± 1.3	33.9 ± 3.2	95.6
Prefed CT for 6 days (5)	36.8 ± 2.8*	8.3 ± 0.5	50.5 ± 3.4	95.6
Recovered 6 days from pre-feeding CT (2)	48.3	11.1	37.0	96.5
Oleic acid-1-^{14}C				
Control (5)	95.6 ± 1.2	1.8 ± 0.4	2.3 ± 1.0	99.7
Prefed CT (5)	93.7 ± 0.7	1.0 ± 0.4	2.0 ± 1.1	98.7
Recovered from pre-feeding (2)	93.4	2.4	1.0	96.8

All animals received the basic emulsion described in Table 1. Figures in parenthesis represent number of animals. Values are means ± SEM.

* Significantly lower ($P < 0.01$).

In Vitro Studies

Since prior feeding of CT was effective in inhibiting cholesterol absorption without interfering with fatty acid transport, it was of interest to determine the intracellular distribution of labeled CT. Fasted animals were given an intragastric feeding of labeled CT (1 μc) in the standard test emulsion. After 24 hr, the small intestine was removed, washed as described above, and cut longitudinally. After removal of the nuclei, cell debris, and mucoprotein by centrifugation at 700 *g* for 10 min, the remaining supernatant fraction was separated into mitochondria, microsomes, and cytoplasm. As a control, labeled CT (2.4 × 10^5 dpm in 0.1 ml of ethanol) was added directly to 10 ml of mucosal homogenate, and the cell fractions were separated as described above. The results, summarized in Table 5, indicate that even 24 hr after CT feeding, about 38% of the radioactivity was associated with the 700 *g* pellet which contains surface mucoprotein. Of the remaining cell constituents, only the CT content of the cytoplasm was significantly different from the control preparations. These data show that the distribution of labeled CT among these cell fractions was not artifactual. However, the remaining isotope distribution (i.e. between microsomes and mitochondria) was

TABLE 5 DISTRIBUTION OF RADIOACTIVITY IN INTESTINAL MUCOSAL SUBCELLULAR FRACTIONS AFTER THE FEEDING OF CHOLESTANE-4-^{14}C-TRIOL

	Distribution of ^{14}C	
Cell Fraction	After Feeding	Direct Addition to Homogenized Mucosa*
700 *g* pellet (nuclei, cell debris, mucoprotein)	*37.7*	*2.5*
700 *g* supernatant	63.3	97.5
Mitochondria % of supernatant ^{14}C	23.4	24.2
Microsomes % of supernatant ^{14}C	52.6	66.1
Cytoplasm % of supernatant ^{14}C	*23.9*	*9.7*

* 2.4 × 10_5 dpm of cholestane-4-^{14}C-triol in 0.1 ml of ethanol was added to 10 ml of mucosal homogenate prior to centrifugation.

TABLE 4 DISTRIBUTION OF ABSORBED CHOLESTEROL-7α-^{3}H AND OLEIC ACID-1-^{14}C IN THORACIC DUCT LYMPH

	Oleic Acid-1-^{14}C As:						Cholesterol-7α^{3}-H As:	
	TG	DG	MG	FA	PL	CE	Free	Esterified
Control	63.5	13.7	7.9	3.6	2.1	9.3	13.1	86.9
Prefed CT for 6 days	75.7	11.0	2.6	3.2	3.2	5.7	15.3	84.7

All animals received the basic emulsion described in Table 1. Abbreviations as in Table 1.

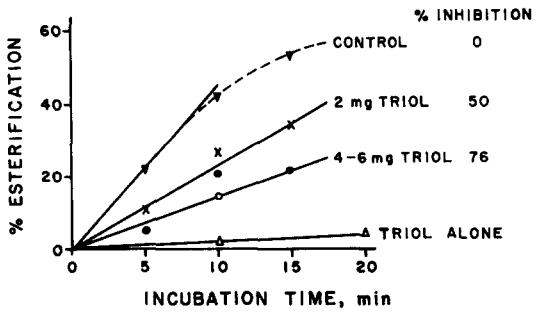

FIG. 4. Esterification of cholestane-3β,5α,6β-triol by pancreatic juice cholesterol esterase in vitro and the effect of CT on esterification of cholesterol. Δ, 6 mg of CT; ▲, 6 mg of cholesterol; remainder, all with 6 mg of cholesterol and, X, 2 mg of CT; ○, 4 mg of CT; and ●, 6 mg of CT.

similar whether the triol was fed or merely added to a mucosal homogenate.

When the mucosa was separated into brush border and nonbrush border fractions (Table 6), 50% of the original mucosal-labeled CT was associated with the microvillar surface. This fraction also had a higher value for dpm/mg protein than did the total mucosal homogenate. Incubation of everted intestinal sacs from animals fed labeled CT resulted in substantial release (32%) of original mucosal isotope into the incubation medium (Table 6); of this, almost 85% was sedimented with mucoprotein by centrifugation at 500 *g* for 20 min, and this material had twice the dpm/mg protein of the original mucosal homogenate.

The enzymatic esterification of cholesterol and CT in vitro is shown in Figure 4. The esterification of 6 mg of CT by pancreatic juice cholesterol esterase was only 2% in 10 min compared to 42% for cholesterol during the same period. The addition of 2 mg of CT to the incubation medium containing cholesterol resulted in 50% inhibition of cholesterol esterification; 4 or 6 mg of CT caused a 76% inhibition of esterification.

For electron microscopy, control animals were fasted overnight or fed a cholesterol–oleic acid emulsion 24 hr before sacrifice. Experimental rats were given 50 mg of CT for 6 days prior to sectioning; some were allowed to recover from the CT feeding for 6 days. Typical sections of the jejunal brush border for control and experimental rats are shown in Figs. 5–8. In jejunal specimens from rats, 24 hr after ingestion of the cholesterol–oleic acid emulsion, the microvillous brush border exhibits a surface coat. The fibrous core for the microvillus extends into the terminal web area *TW* (Fig. 5). The adjacent cells are closely aligned and exhibit cell border specializations: zonula occludens, desmosomes, and lateral interdigitations. Profiles of agranular endoplasmic reticulum (Fig. 6) are present along with the more predominant form of granular endoplasmic reticulum. There was little evidence of unusual mitochondria in any of the sections.

TABLE 6 DISTRIBUTION OF RADIOACTIVITY IN INTESTINAL MUCOSAL FRACTIONS AFTER CHOLESTANE-4-^{14}C-TRIOL FEEDING

Cell Fraction	% of Invertase Activity	% of Total ^{14}C	dpm/mg Protein
Mucosal homogenate	100	100	75
Brush border	85.1	49.7	104
Mucosal homogenate (after 2 hr incubation of everted sac)	—	67.9	55
Incubation medium		32.2	45
Insoluble mucoprotein			
500 *g* pellet	—	27.6	126
500 *g* supernatant	—	4.6	14

In rats fed CT for 6 days, two morphological changes were apparent in the jejunal epithelial cells (Figs. 7, 8). There was invariably a marked increase in length of the microvilli (compare Fig. 7 with Fig. 5). It was also possible to demonstrate intermediate lengths at 3 days of feeding CT or 6 days following the feeding period. Secondly, several mitochondria were enlarged and were aligned closely with the cisternae of the granular endoplasmic reticulum (*GER*, Fig. 8). This finding was consistent and is a subject for further study.

DISCUSSION

The present report confirms the original observations (2) that cholestane-3β,5α,6β-triol, administered in an aqueous emulsion together with cholesterol to rats, results in a depression of lymphatic absorption of the cholesterol and in increased fecal excretion of sterols. With thoracic duct-cannulated rats, 50 mg of CT administered with 50 mg of cholesterol-7α-^{3}H and 288 mg of oleic acid-1-^{14}C resulted in approximately 50% inhibition of cholesterol transfer into lymph but only 8% inhibition of oleic acid transport during the 8 hr experimental period. Some interference with fatty acid absorption was evidenced by a delay in the appearance of isotope in lymph. However, by the end of the experiment, the cumulative absorption of oleic acid in the animals receiving CT was not significantly different from that in the controls. Despite these effects, CT had no influence on the distribution of labeled oleic acid among the intestinal or lymph lipid fractions nor on the percentages of free and esterified cholesterol-7α-^{3}H in these tissues.

To determine whether the effect of CT on cholesterol absorption was due to competition for sterol transport pathways, we studied the absorption of cholestane-4-^{14}C-triol in a 24 hr recovery experiment (Table 2), in which only about 3% of the administered CT was recovered in thoracic duct lymph, 75% of it in the unesterified form. By contrast, 50% of the fed cholesterol was recovered, 85% of it esterified. Since it has been suggested that

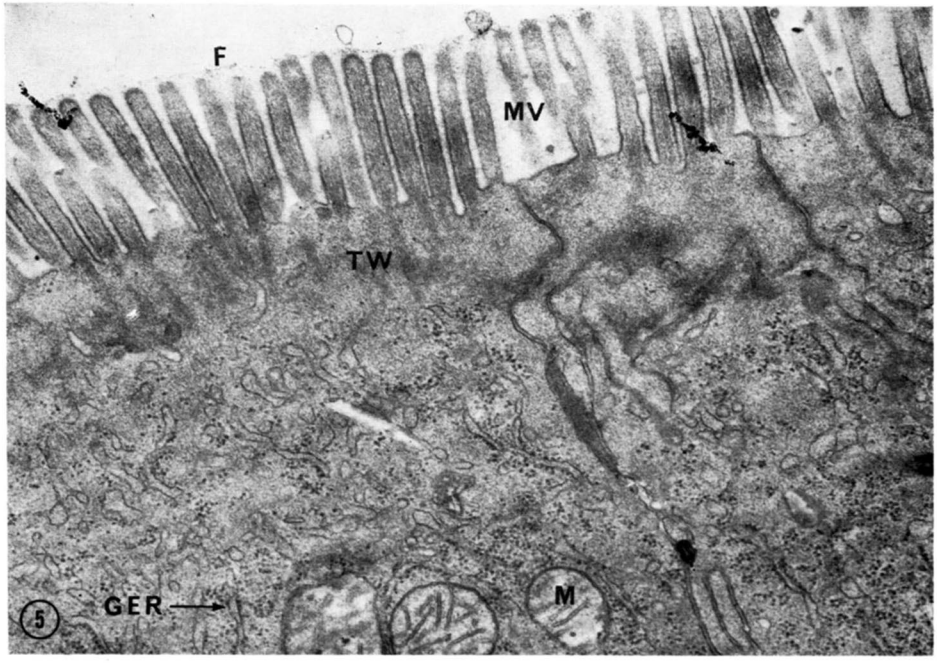

FIG. 5. Electron micrograph of the apical portion of cells from the rat jejunum 24 hr after the administration of a cholesterol–oleic acid emulsion. The microvilli (*MV*) exhibits the typical "fuzzy coat" (*F*). Below the microvillus surface the terminal web (*TW*) is apparent, extending laterally to the junctional complex of adjacent cells, which corresponds to the terminal bar seen with the light microscope. Mitochondria (*M*) and granular endoplasmic reticulum (*GER*) are present in the cytoplasm. × 21,800.

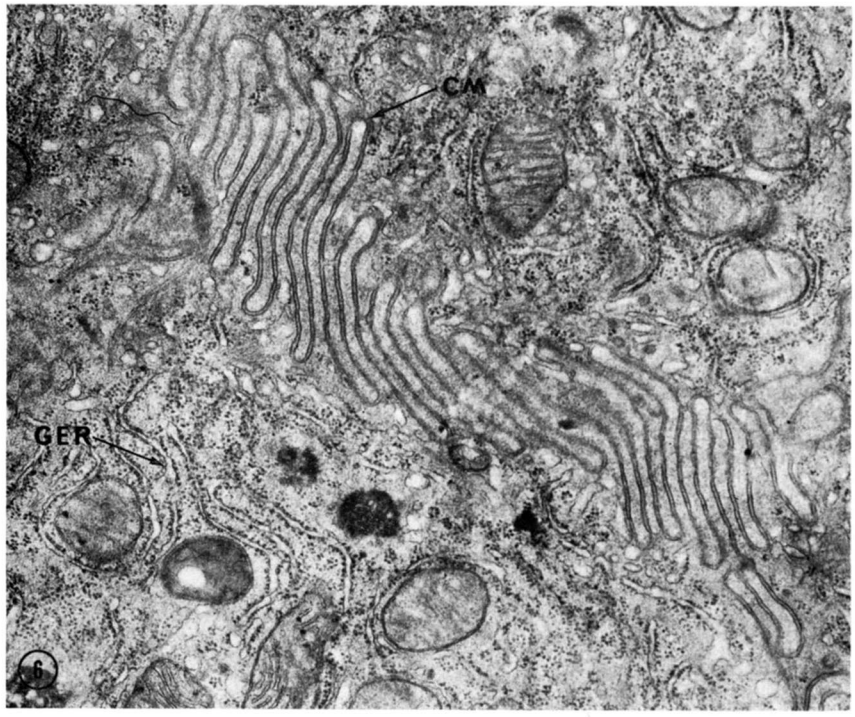

FIG. 6. Electron micrograph of portions of two intestinal epithelial cells of a control rat fasted overnight, showing the complex lateral interdigitations between adjacent jejunal cell membranes (*CM*). Compact cisternae of the granular endoplasmic reticulum (*GER*) are in close association with the mitochondria. × 22,000.

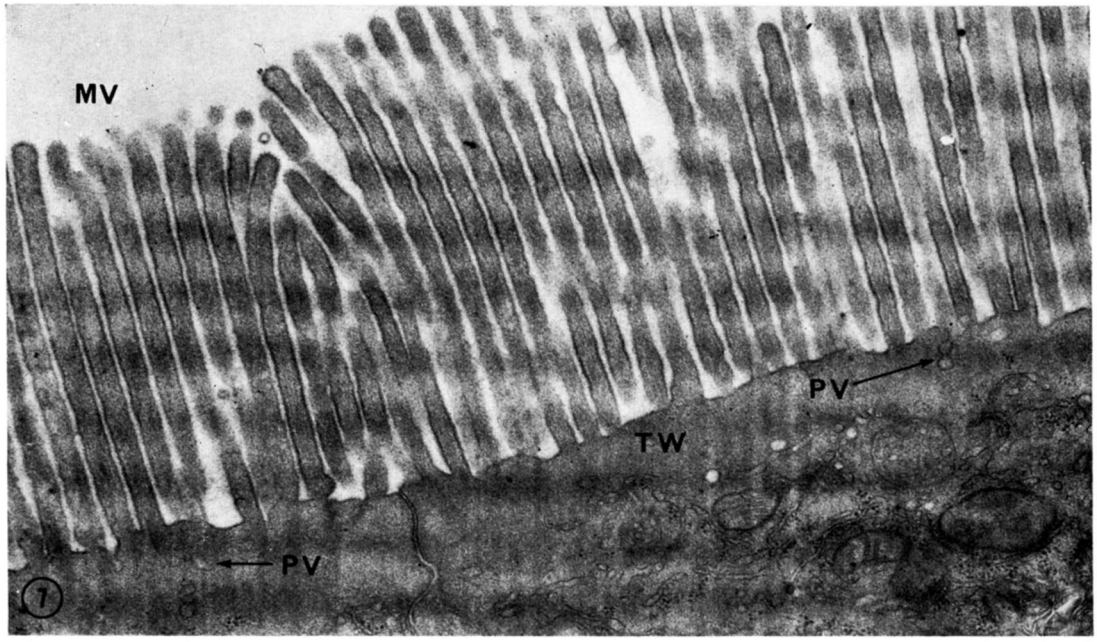

FIG. 7. Electron micrograph of the apical portion of cells from the jejunum of rats fed CT for 6 days. The well-developed brush border of two adjacent epithelial cells exhibits an increased microvillar (*MV*) length (compare with Fig. 5). Small vesicular invaginations (*PV*) of the cell surface are prevalent among the microvilli. Small vesicles are also found deep in the terminal web (*TW*). No enlarged mitochondria are evident in this picture (the reason for this difference from Fig. 8 is not known). × 21,000.

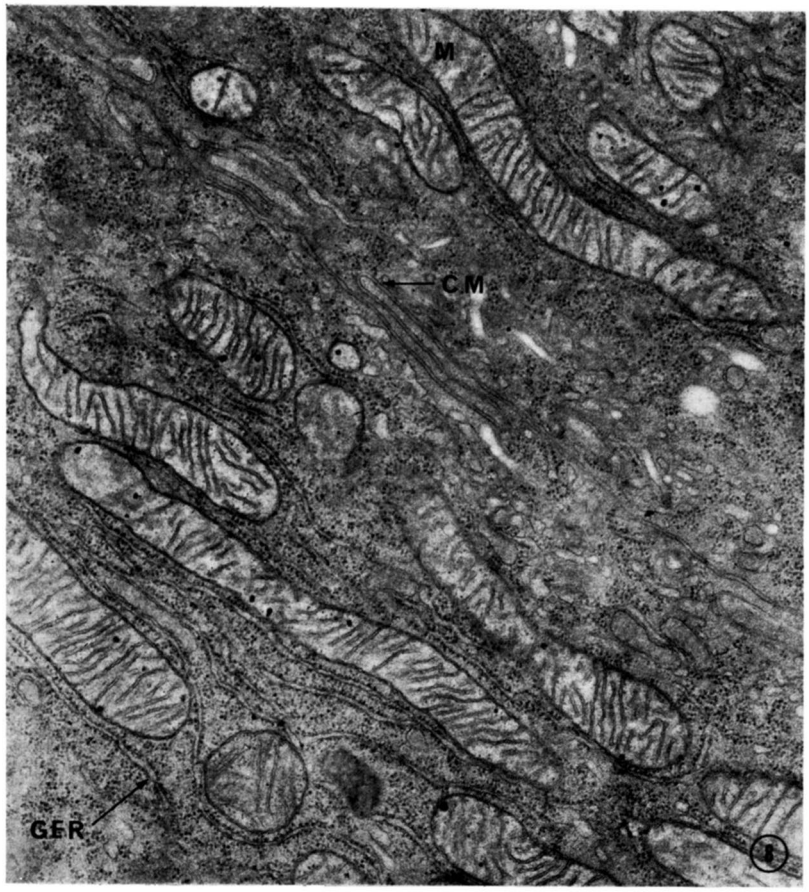

FIG. 8. Electron micrograph of the apical portion of cells from the jejunum of rats fed CT for 6 days. Elongated mitochondria (*M*) exhibit well-developed cristae and are aligned parallel with the lateral cell membrane (*CM*) of adjacent cells. Prominent cisternae of granular endoplasmic reticulum (*GER*) are closely aligned with and encircle mitochondria. × 23,000.

esterification of cholesterol by mucosal cholesterol esterase is an important mechanism for the final transport of cholesterol into lymph (18, 19), the inefficient absorption of cholestanetriol may well be the result of ineffective esterification of this sterol by the esterifying systems. Since CT was transported into the mucosa to the extent of 10% of the fed dose but did not appear in lymph to the same extent, the defect in transfer of CT into lymph must have occurred at some terminal intracellular stage of absorption. Of the 10% CT recovered in the mucosa, even 24 hr after feeding, 38% sedimented at 700 *g* with the cell debris, nuclei, and mucoprotein fraction. Of the total CT associated with the mucosal cell, 50% was recovered with the brush border or microvilli, suggesting that one site of CT action might be on the mucosal cell surface. This may also be related to the fact that feeding CT for 6 days resulted in an elongation of the microvilli (Fig. 7). In the electron micrographs, small invaginations of the cell membrane at the bottom of the clefts between microvilli could be seen in CT-treated animals (Fig. 7). The presence of these invaginations vesicles, and elongated microvilli suggests that, among various possibilities, there may be structural adaptations to decreased sterol absorption or to increased CT content of the mucosal membranes. However, the increased membrane surface, due to the increase in length of the brush border, also suggests the possibility that there are increases in the other absorptive functions of the epithelial cells. This possibility is currently under study.

In an in vitro esterifying system, CT was esterified with oleic acid very poorly (2%) while cholesterol was effectively esterified (42%) during the 10 min incubation period. These data show CT to be a poor substrate for cholesterol-esterifying systems and may be the explanation for the limited transport of CT from mucosal cell to lymph.

The accumulation of CT in the intestinal mucosa led to the possibility that CT might saturate intracellular transport systems for sterols and thus prevent effective cholesterol absorption. An example of this type of effect was shown in the studies on cholesterol esterase activity in vitro (Fig. 4), where the addition of 2 mg of CT to a substrate system containing 6 mg of cholesterol resulted in 50% inhibition of cholesterol esterification. A similar effect in vivo might result in depressed esterification of cholesterol in the mucosa and transport into lymph.

The findings in the present report are of interest in relation to development of atherosclerosis since the accumulation of CT in the mucosal cell following feeding specifically interferes with cholesterol absorption, with no significant effect on absorption of fatty acid. Of additional interest is the finding that further transport of CT from mucosal cell into lymph is limited.

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