Absolute Requirement for Free Sterol for Absorption by Rat Intestinal Mucosa.* (29289)

GEORGE V. VAHOUNY AND C. R. TREADWELL
Department of Biochemistry, George Washington University School of Medicine, Washington, D.C.

Several types of evidence indicate that the mechanism of transfer of cholesterol from the intestinal lumen into mucosal cells is specific for the free sterol. It has been shown that there is extensive hydrolysis of fed cholesterol esters in the intestinal lumen, whereas essentially no esterification of dietary free cholesterol occurs intraluminarly(1). Also, irrespective of whether free or esterified cholesterol-4-C^{14} is administered, only 25% of the labeled sterol in the intestinal mucosa is present in the esterified form, while in lymph, 80-90% exists as esterified cholesterol-4-C^{14} (2). Thus, the apparent mechanism of absorption of either form is the same. Finally, the rate and extent of transfer of various cholesterol esters from lumen to thoracic duct lymph is directly related to the susceptibility of these esters to cholesterol esterase hydrolysis *in vitro*(3,4). Cholesterol trimethylacetate is not split enzymatically *in vitro*, and its administration *in vivo* does not produce a chemical increase in lymph cholesterol over a 24-hour period(3).

These studies, however, have not provided direct evidence on the absorbability of intact, or unhydrolyzed, cholesterol esters into mucosal cells and lymph. The present study was undertaken to obtain such direct evidence. A sterically-hindered, disubstituted fatty acid ester of cholesterol-4-C^{14} has been synthesized and studied for susceptibility to cholesterol esterase splitting *in vitro*, and absorbability *in vivo* using lymph fistula rats. Further, the ester was solubilized in phospholipid-bile salt micelles to provide an optimum medium for enzymatic hydrolysis *in vivo*(5).

Materials and methods. Cholesterol-4-C^{14} α,α-methyl ethyl caproate† (C-MEC) was prepared according to Swell and Treadwell (6), and the reaction products were extracted in 20 volumes of 2:1 chloroform-methanol. After addition of water and separation of the phases, the chloroform extract was evaporated to dryness under nitrogen on a steam bath. The residue was reextracted into petroleum ether, the extract was dried over anhydrous sodium sulfate, and the ester was repeatedly purified by silicic acid column chromatography(7). Purity was determined by methods described by Stern and Treadwell (8), and by quantitative micro-thin layer chromatography for free and esterified cholesterol-4-C^{14}(9). The final product had a melting point of 58°C, and contained 0.35% contamination of free cholesterol-4-C^{14}.

Phospholipid-bile salt micellar medium containing the labeled ester was prepared by homogenizing 6.8 mg C-MEC, 14 mg sodium taurocholate and 20 mg phosphatidyl choline per 5 ml isotonic saline. The mixture was sonicated‡ for 30 minutes prior to use and had very slight opalescence(5).

Adult male rats (225-250 g) of the Carworth strain were used and cannulation of the thoracic duct was performed as described earlier(10). To avoid gastric alteration of the micellar dispersion, an infusion catheter was placed in the duodenum through which the preparation was administered. The animals were placed in restraining cages and supplied with 0.9% saline but no food. After 24 hours, 5 ml of the micellar medium was introduced into the small intestine, and lymph was collected in a heparinized tube for 12 hours. Feces were quantitatively collected, and after sacrifice of animals, the entire small intestine was removed and washed 3 times with 0.9% saline. These saline washings were pooled with the feces of the respective animal, and the mixture was homogenized. The small intestine was homogenized in 5 ml saline.

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† Cholesterol-4-C^{14} was from Nuclear-Chicago Corp.; α,α-methyl ethylcaproic acid, from Henley and Co., Inc., New York.

‡ MSE ultrasonic disintegrator, Instrumentation Associates, Inc., New York.

TABLE I. Hydrolysis of Micellar Cholesterol Caproate, Oleate and α,α-Methyl Ethyl Caproate (MEC) by Pancreatic Juice Cholesterol Esterase *in vitro*.

Cholesterol ester*	% Hydrolysis, 8 min
Caproate	14.5
Oleate	16
MEC	0 (3 hr)

* Preparation of substrates, assay and determinations have been described(5).

Aliquots of lymph and of the intestinal homogenates were extracted in 25 volumes of boiling acetone-ethanol (1:1), while intestinal contents and feces were extracted 3 times in the same manner to insure complete recovery of the ester.§ The acetone-ethanol extracts were taken to dryness under nitrogen on a steam bath, and the residues were re-extracted into petroleum ether. Free and easily-hydrolyzable cholesterol esters(11) were precipitated with digitonin overnight, the digitonides were washed successively with 2 ml acetone-ether (1:1) and ether(11), and were recrystallized from methanol overnight (12). The digitonides were dissolved in methanol and counted in a proportional liquid scintillation counter using 10 ml of a scintillation mixture containing diphenyloxazole (4 g/l), POPOP‖ (100 mg/l) and toluene. In addition, difficultly-hydrolyzable C-MEC(8) was determined directly chemically with Liebermann-Burchardt reagent (10), after removal of digitonin-precipitable free and esterified cholesterol, and for radioactivity by the method of Vahouny *et al*(9).

Results and discussion. Table I shows the results of *in vitro* studies on the hydrolysis of representative micellar "solubilized" cholesterol esters by pancreatic juice cholesterol esterase. The collection of bile-free pancreatic juice, substrate preparation and assay procedures were as described previously(5). It is apparent that micellar-dispersed cholesterol caproate or oleate is enzymatically hydrolyzed very rapidly (approximately 15% hydrolysis in 8 min). However, micellar C-MEC was completely resistant to enzymatic cleavage for up to 3 hours under the same conditions.

The data in Table II summarize recoveries of infused micellar C-MEC in the 12-hour lymph samples, intestinal tissues, and feces plus intestinal contents. The values represent averages for 4 animals ± standard error. Of the labeled sterol ester administered (5.99×10^5 dpm/rat), 98.1% was recovered unchanged in the feces plus intestinal contents, with no evidence of labeling in the free or easily-hydrolyzable sterol ester fractions. Thus, the ester was as resistant to *in vivo* as to *in vitro* enzymatic splitting. The free and esterified cholesterol fractions of lymph and intestinal extracts contained very small quantities of C^{14} radioactivity (13-36 cpm above background) and this was accounted for by the small amount of free sterol in the administered preparation (*i.e.*, 0.35% free cholesterol-4-C^{14}, 99.65% C-MEC). No C-MEC was detected in either the lymph or intestines of the 4 animals.

To be certain of complete extraction of all C^{14} from tissues, the residues after solvent extraction were placed in counting vials with 1 ml hyamine hydroxide and 10 ml scintillation mixture. These residues contained no radioisotope, indicating quantitative recoveries.

The present study provides convincing evidence for the non-absorbability of intact cholesterol esters into intestinal mucosa and lymph, even when provided in micellar medium. These data further support the concept that the initial transfer of sterol from lumen to mucosa cell is specific for *free* sterol, and that esterification of absorbed sterol prior to appearance in lymph occurs at a later step (4,13).

Summary. An enzymatically unhydrolyzable ester of cholesterol, cholesterol-4-C^{14} α,α-methyl ethyl caproate, has been synthesized. This ester was infused in micellar dispersion into the duodenum of lymph fistula rats. After 12 hours, 98.1% of the administered ester was recovered unaltered from the feces and intestinal contents. There was none of this ester in intestinal tissue or lymph. The data provide convincing evidence that the

§ It was found that single extractions of feces plus intestinal contents do not give quantitative recoveries of branched-chain fatty acid esters of cholesterol-4-C^{14}.

‖ 1,4-di·2(5-phenyloxazolyl)-benzene.

TABLE II. Recovery of Fed Micellar Cholesterol-4-C^{14}-α,α-Methyl Ethyl Caproate (C^{14}-MEC).

Tissue*	% of C^{14} recovered as Free sterol-C^{14}	Ester-C^{14}	C^{14}-MEC	Total
Lymph (0-12 hr)†	Trace	Trace	0	Trace
Intestinal tissue†	"	"	0	"
Feces plus contents	0	0	98.1	98.1 ± 1.0

* Represents average of 4 animals ± S.E. Each lymph fistula rat received 6.8 mg cholesterol-4-C^{14} MEC (5.99×10^6 dpm) solubilized in phospholipid-bile salt micelles(5).

† The counts of free and esterified sterol-C^{14} from 2 ml lymph and approximately 1 g small intestine represented 13-36 cpm above background, and can be accounted for by 0.35% contamination of C^{14}-MEC.

initial transfer of cholesterol into mucosal cells is specific for free cholesterol, and that cholesterol esters are not absorbed from the intestinal lumen prior to hydrolysis.

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In vitro Uptake of Tritiated Thymidine by Erythrocytic Percursors Following Hemorrhage in the Dog.* (29290)

N. O. NIELSEN,† L. E. FEINENDEGEN,‡ V. P. BOND, N. ODARTCHENKO§ AND H. COTTIER‖

Medical Research Center, Brookhaven National Laboratory, Upton, L. I., N. Y.

Following hemorrhage, the bone marrow responds with an increased output of red cells which can reach 7 times normal values(1). The nature of the cellular response by which this is achieved has not been firmly established. An expansion of the mass of erythrocytic precursor cells has been reported, as well as an increase in reticulocyte count(2-4, 12). It has remained questionable whether the cellular response involves also an increase in rate of maturation, and change in rate of DNA synthesis.

The value of tritiated thymidine and autoradiography in the study of *in vivo* cell kinetics has been well established(5-7). *In vitro* studies have also been used(8-10). The incubation of freshly drawn bone marrow with tritiated thymidine *in vitro* results in incorporation of the isotope into the nucleus of those cells that are synthesizing DNA. They are recognized autoradiographically. The mean number of grains over nuclei of mor-

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† College of Vet. Med., University of Minnesota, St. Paul.

‡ Euratom, Brussels, Belgium.

§ Centre Anti-Cancereux Romand, Hopital Cantonal, Lausanne, Switz.

‖ Inst. of Pathology, Univ. of Bern, Switz.