Role of acyl CoA:cholesterol acyltransferase in cholesterol absorption and its inhibition by 57-118 in the rabbit.

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Abstract Esterification of cholesterol in rabbit small intestine mucosal microsomes by acyl CoA:cholesterol acyltransferase (ACAT, Ec 2.3.1.26) and mucosal cytosol by cholesterol esterase (EC 3.1.1.13) was studied. Compound 57-118, N-(1-oxo-9-octadecenyl)-DL-tryptophan(Z)ethyl ester, an inhibitor of cholesterol absorption, was found to inhibit in vitro ACAT in mucosal microsomes at concentrations of 2–20 nmol/0.5 ml incubation mixture, but had no effect on cholesterol esterase in the cytosol at similar concentrations. A kinetic analysis using a Lineweaver-Burk plot indicates that 57-118 acts as a competitive inhibitor of ACAT. An ex vivo study in the rabbit where 57-118 was given by gavage at a dose of 200 mg/kg also showed inhibition of ACAT but not of cholesterol esterase. High performance liquid chromatography determination of 57-118 in various subcellular fractions demonstrated the presence of this substance after oral administration in concentrations in mucosal microsomes equivalent to those required to show inhibition of ACAT in vitro. These data support the work of Norum et al. (1979. *Eur. J. Clin. Invest.* **9:** 55–62) indicating mucosal ACAT plays a significant role in cholesterol absorption.—**Heider, J. G., C. E. Pickens, and L. A. Kelly.** Role of acyl CoA:cholesterol acyltransferase in cholesterol absorption and its inhibition by 57-118 in the rabbit. *J. Lipid Res.* 1983. **24:** 1127–1134.

Supplementary key words cholesterol esterification • intestinal microsomes

It is generally accepted that cholesterol is absorbed as free cholesterol (1) and is predominantly esterified by the time it reaches the lymph (2). The mechanism by which this esterification takes place and its importance in cholesterol absorption has been subject to much controversy. Early studies suggested that the pancreas was the major source of the intestinal esterifying enzyme. For example, Swell, Byron, and Treadwell (3) showed that rat intestine homogenates lost most of their cholesterol esterifying activity after 95% pancreatectomy. Hernandez, Chaikoff, and Kiyasu (4) showed that duodenal homogenates from rats in which pancreatic juice was diverted from the intestine almost completely lacked the capacity to promote esterification. Borja, Vahouny, and Treadwell (5) demonstrated a link between bile and pancreatic juice in cholesterol absorption and esterification. More recently Gallo et al. (6) have presented evidence that cholesterol is esterified by pancreatic cholesterol esterase (EC 3.1.1.13). They demonstrated, by immunocytochemical techniques, the presence of this enzyme within the mucosal cells as opposed to the absorptive surface (7). The data from these studies suggested to Gallo et al. (6) that pancreatic cholesterol esterase plays an essential role in the mucosal esterification of absorbed cholesterol prior to lymphatic transport. This enzyme, found in the cytosol (8), is not CoA-dependent but does require, at least in vitro, trihydroxy cholanic acids as a cofactor (7, 9).

On the other hand, Murthy and Ganguly (10) found that increasing the cholesterol content in the diet increased intestinal esterifying activity several fold whereas pancreatic activity remained unchanged, suggesting that the mucosal enzyme was different from the pancreatic enzyme. Watt and Simmonds' experiments (11) in the rat did not support an effect of pancreatic juice on the efficiency of absorption and esterification of luminal exogenous cholesterol and suggested an alternative enzyme dependent directly or indirectly on the presence of micellar bile salts in the lumen to explain intestinal cholesterol-esterifying activity during absorption. Norum and coworkers have demonstrated the presence of acyl CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26) in the mucosal cells from rat (12), guinea pig (13), and human (14) small intestines. This enzyme is found in the microsomes (12, 13). However the significance of the role played by either of these enzymes in cholesterol absorption has not been established.

In support of what Norum et al. (12–14) have found in other species, we have determined the presence of ACAT in the microsomal fraction of mucosal cells from the small intestines of rabbits. Furthermore, we have

Abbreviations: HPLC, high performance liquid chromatography; CMC, carboxymethylcellulose; TLC, thin-layer chromatography; 57-118, N-(1-oxo-9-octadecenyl)-DL-tryptophan(Z)ethyl ester.

developed a series of compounds (e.g., 57-118, N-(1-oxo-9-octadecenyl)-DL-tryptophan(Z)ethyl ester reported here) that inhibit the accumulation of cholesteryl esters in cells in culture (e.g., Fu5AH, smooth muscle cells and human fibroblasts) by the inhibition of ACAT activity.[1] The data presented here show that 57-118 inhibits cholesterol absorption and this correlates with its in vitro and ex vivo inhibition of ACAT in mucosal cells. Kinetic studies indicate that this substance is an apparent competitive inhibitor of ACAT.

MATERIALS AND METHODS

Chemicals

[1-2-^{3}H]Cholesterol (53 mCi/mmol), [4-^{14}C]cholesterol (0.5 mCi/mmol), [1-^{14}C]oleoyl-CoA (60 mCi/mmol) were obtained from New England Nuclear, Boston, MA. [^{3}H]- and [^{14}C]cholesterol were purified by HPLC before use. Oleoyl-CoA, oleic acid, sodium taurocholate, and bovine serum albumin (essentially fatty acid-free) were obtained from Sigma Chemical Co., St. Louis, MO. All solvents were from J. T. Baker, Phillipsburg, NJ. HPLC solvents as well as chloroform and methanol were HPLC grade. 57-118, N-(1-oxo-9-octadecenyl)-DL-tryptophan(Z)ethyl ester was synthesized by Dr. F. Kathawala, Medicinal Chemistry Department, Sandoz, Inc., NJ. All other chemicals used were standard commercial high purity materials.

Measurement of cholesterol absorption via the Zilversmit dual isotope method (15)

Animals and diet

Male, New Zealand rabbits were divided into approximately equal weight groups (1.9 ± 0.5 kg) for each experiment and were caged individually. Rabbit chow with 1% cholesterol (BioServ Inc., NJ) and water were given ad libitum for 2 days before and throughout the study except for an overnight fast before isotope administration and until 4 hr after the isotope dosing. Rabbits in another group were fed normal rabbit chow (BioServ Inc., NJ) and were fasted in a similar manner.

Preparation of radioactive isotope cholesterol doses

Intravenous. Fifty μl of [1-2-^{3}H]cholesterol in 95% ethanol was added to 1 ml of 0.9% saline and mixed on a vortex mixer. Immediately thereafter two 0.1-ml aliquots were taken for assay and 0.5 ml was used for intravenous injection into the marginal ear vein of each animal within 4 min of preparing the dose. Each animal received ≃5 μCi/0.11 nmol of [1-2-^{3}H]cholesterol.

Oral. [4-^{14}C]Cholesterol was dissolved in corn oil. The corn oil solution was added to 6.8% powdered skim milk suspension in a ratio of 1:4. The resulting mixture was homogenized by sonicating with a Bronwill Biosonik IV Sonicator using a microprobe at a "LO" setting of 50 until a homogeneous suspension was formed. Each animal received by gavage 1 ml of this preparation containing ≃10 μCi/187 nmol of [4-^{14}C]cholesterol. Aliquots were taken for assay at the start, midpoint, and end of the oral isotope administration to the animals.

Drug dose

Doses of 15, 50, and 150 mg/kg of 57-118 were administered by gavage as a suspension in a 1.5% aqueous solution of carboxymethylcellulose (CMC). The control group received an equal volume of CMC solution. The drug was administered for 7 days. On the third day the drug was given 15 min before oral isotope and 17 min before intravenous isotope administration. The drug was given in the morning of each day of the study and the time interval between doses was the same for each animal and all doses. The rabbits fed normal rabbit chow were divided into two groups; one group received a daily dose of 200 mg/kg of 57-118 in CMC and the other group received an equal volume of CMC under the same conditions as the cholesterol-fed rabbits.

Sample collection

Samples of whole blood were taken at 72 hr from the ear vein (opposite ear to the one receiving the isotope injection) from each animal using a heparinized needle, and by cardiac puncture at 120 hr after isotope dosing. Duplicate 0.2-ml aliquots of whole blood were placed on combustion papers and dried before combustion in the Packard 306 Tri-Carb Sample Oxidizer for the separation of the two isotopes.

Radioactive analysis

The radioactivity was determined by liquid scintillation counting in a Beckman model LS 7500 liquid scintillation spectrometer. The scintillation fluids for ^{14}C were Carbsorb II and Permafluor V. Monophase was used for ^{3}H. Permafluor V was used for direct counting of the dose assays. Samples were counted at a two-sigma confidence level of less than 3.65.

Calculations

The combined efficiency of the oxidizer and liquid scintillation counter was determined for each isotope from standards of known specific activity. After blank subtraction the dpm of the samples were determined. Absorption was calculated from the following equation:

[1] Heider, J. G., et al. Unpublished data.

$$\text{Absorption (\%)} = \frac{\frac{^{14}\text{C sample (dpm)}}{^{14}\text{C oral dose (dpm)}}}{\frac{^{3}\text{H sample (dpm)}}{^{3}\text{H i.v. dose (dpm)}}} \times 100.$$

Inhibition of ACAT activity

Animals and diet

Male New Zealand rabbits, 1.9 ± 0.5 kg, were placed on 1% cholesterol-supplemented rabbit chow and water ad libitum for 2 weeks prior to the study. The animals, individually caged, were divided into two groups. Group I (controls) received 2 ml/kg of 1.5% CMC by gavage and Group II received 57-118, 200 mg/kg, as a CMC suspension (2 ml/kg) by gavage at 2 hr and again at 0.5 hr before being killed with Sleepaway, Fort Dodge Labs, IA.

Preparation of subcellular fractions

The intestines were removed immediately and placed on ice. The first 12 cm from the stomach was removed and discarded. The next 75 cm was taken for study. This portion of the intestines was divided into thirds and the lumen was washed with ice-cold saline solution. After being opened longitudinally, the intestinal wall was washed with a fine jet of cold saline and the wash was absorbed on a strip of paper towel. The process was repeated four times. The mucosa was scraped with a microscope slide and placed in 10 ml of 0.25 M sucrose solution. After gentle mixing by inverting the stoppered centrifuge tubes several times, the mucosal cell suspension was centrifuged at 900 *g* for 10 min. The supernatant was retained for HPLC analysis of 57-118 (cell wash 1) and the cells were resuspended in a final volume of 10 ml of 0.154 M phosphate buffer (pH 6.2); after gentle mixing as before, they were centrifuged at 900 *g* for 10 min. The supernatant was retained for HPLC analysis of 57-118 (cell wash 2). The mucosal cells were again resuspended in a final volume of 5 ml of phosphate buffer and homogenized (six 5-second strokes with a motor-driven Teflon pestle at 0–4°C). The subcellular fractions were prepared by the method used by Hashimoto et al. (16) for rabbit aortas with some minor modifications. The mucosal homogenate was centrifuged at 1000 *g* for 15 min to isolate nuclei and cell debris. The resulting supernatant was centrifuged at 12000 *g* for 15 min to isolate the mitochondria. The resulting supernatant was centrifuged at 107,000 *g* for 30 min to isolate the microsomes. This supernatant was taken for cholesterol esterase assay and the microsomes were resuspended in 0.154 M phosphate buffer (pH 7.4) for use in the ACAT assay. A portion of each fraction was retained for analysis of 57-118, protein, and electron microscopy.

Assay of ACAT

ACAT activity was determined essentially by the method of Helgerud, Saarem, and Norum (17). Endogenous cholesterol of the microsomal fraction and exogenous [1-^{14}C]oleoyl CoA were used as the substrates. The incubation mixture of 0.5 ml of 0.154 M potassium phosphate buffer (pH 7.4), containing 18 nmol of bovine serum albumin and 18 nmol of [1-^{14}C]oleoyl CoA (0.006 μCi/nmol) was preincubated at 37°C for 5 min before the addition of 20 μl of the microsomal fraction (0.2–0.3 mg of protein). The incubation times are described in Results. The reaction was stopped by the addition of 12.5 ml of chloroform–methanol 2:1. To this, 2.5 ml of water was added. After shaking, the aqueous phase was removed and discarded and the chloroform phase was washed by the method of Folch, Lees, and Sloane Stanley (18) before a 6-ml aliquot was taken to dryness. The residue was taken up in 20 μl of n-hexane containing 2 mg/ml of cholesteryl oleate as carrier and quantitatively transferred to silica gel G plates (Merck, Darmstadt) for TLC using diethyl-ether–petroleum ether (bp 39–55°C) 10:190 as a solvent system. The efficiency of the extraction method was determined to be 93.2 ± 2.4%, and for the combined extraction and TLC 85.5 ± 3.4%. These values were confirmed in some studies where [1,2,6,7(N)^{3}H]-cholesteryl oleate was used as internal standard. The plates were developed using the multiple development technique, i.e., developed to 5 cm above origin, removed and dried under nitrogen; developed to 10 cm and dried in a similar manner; and finally developed to 15 cm and dried as above. Cholesteryl oleate was visualized with iodine vapor and the area was scraped into counting vials and the radioactivity was assayed by liquid scintillation counting with ACS scintillation fluid, Amersham, Arlington Heights, IL, in a Beckman model LS 7500 liquid scintillation spectrometer. Samples were counted at a two-sigma confidence level of less than 3.65. Efficiency was determined by internal standardization using ^{14}C-labeled Spec-Chec, Packard, Downers Grove, IL. Esterification rates were calculated as pmol of cholesteryl [1-^{14}C]oleate formed · mg microsomal protein^{-1} · min^{-1}.

Assay of cholesterol esterase

The supernatant from the microsomal preparation was taken for cholesterol esterase activity essentially by the method of Gallo and Treadwell (8) with some modifications. An aliquot (0.75 ml) of cytosol in 0.154 M phosphate buffer, pH 6.2, was preincubated for 10 min at 37°C. After the addition of 0.25 ml of substrate mixture, the samples were incubated for 3 hr. The final

incubation mixture contained, besides the cytosol, 11.6 μmol of oleic acid, 0.54 μCi/3.9 μmol of cholesterol, 8.2 μmol of sodium taurocholate, and 25 μmol of albumin in a final volume of 1 ml. All assays were run in triplicate. Substrate blanks were run with 0.154 M phosphate buffer instead of cytosol, and enzyme blanks were run with cytosol inactivated for cholesterol esterase activity by heating at 70°C for 10 min. Various concentrations of 57-118 (0.2–100 nmol/incubation mixture) were added in 5 μl of DMSO solution. Control samples received 5 μl of DMSO alone. The reaction was stopped with 10 ml of chloroform–methanol 2:1. To this was added 2 ml of 0.29 M NaCl. After shaking, the aqueous phase was removed and discarded and the chloroform was washed with another 2 ml of the salt solution; a 6-ml aliquot of the chloroform was then taken to dryness. The residue was taken up in 20 μl of n-hexane containing 2 mg/ml of cholesteryl oleate as carrier and quantitatively transferred to a TLC plate and treated as described above (Assay of ACAT).

Protein assay

Protein was determined by the method of Lowry et al. (19).

High performance liquid chromatography

HPLC analysis was performed on a Beckman model 332 gradient liquid HPLC instrument attached with a Beckman model 155-40 variable wavelength spectrophotometer and a Perkin-Elmer model 650S fluorospectrophotometer in series. The solvents were degassed under vacuum in a Mettler Ultrasonic Cleaner for 5 min. The mobile phase was made up of 50% each of two solvent systems. Solvent A was composed of acetonitrile–isopropyl alcohol 60:40 and solvent B was composed of acetonitrile–isopropyl alcohol–heptane 40:40:20. The flow rate was 1 ml/min through a 25 cm × 4.6 mm Ultrasphere ODS C-18 reverse phase column (Beckman). Under these conditions lipids of biological origin, e.g., cholesterol, free and individual esters, phospholipids, and triglycerides, were separated and monitored with the UV detector at 206 nm. Compound 57-118 was detected via fluorescence with an excitation wavelength of 288 nm (slit 4 mm) and an emission wavelength of 344 nm (slit 4 mm). The lower limit of detection for 57-118 was 0.5 ng (1 pmol).

RESULTS

Inhibition of cholesterol absorption

The effects of various doses of 57-118 on the inhibition of cholesterol absorption are shown in **Table 1.** In an earlier study 57-118 was found to inhibit absorption by 65% at 200 mg/kg. The inhibition of cholesterol absorption decreased in a dose-related manner from 46% at 150 mg/kg, 26% at 50 mg/kg, to no effect at 15 mg/kg. The differences observed in the data obtained at 72 hr vs. 120 hr are addressed in the Discus-

TABLE 1. Dose effect of 57-118 on cholesterol absorption in the rabbit

Treatment	N	% Cholesterol Absorption (% difference)[a] 72 Hr[b]	120 Hr[b]
Normal control		63.4 ± 15.9	55.8 ± 14.9
Normal 200 mg/kg		66.7 ± 6.7 (+5.2%)[c]	58.6 ± 15.7 (+6.5%)[c]
Control	8	56.7 ± 5.2	45.7 ± 4.2
150 mg/kg	4	30.6 ± 8.3 (−46%)[d]	26.4 ± 8.0 (−42%)[d]
Control	8	56.7 ± 5.2	45.7 ± 4.2
50 mg/kg	5	41.7 ± 1.8 (−26%)[d]	35.0 ± 1.7 (−23%)[d]
Control	4	40.4 ± 8.0	34.0 ± 6.8
15 mg/kg	5	39.6 ± 8.2 (−1.9%)[c]	31.6 ± 6.5 (−7.1%)[c]

[a] Percent difference = $\frac{\text{drug} - \text{control}}{\text{control}} \times 100$.

[b] Time of blood sample for dual label analysis.

[c] Not significant.

[d] Significant at $P < 0.05$.

Normal control rabbits and normal rabbits given 200 mg/kg 57-118 were fed normal rabbit chow. The remaining control and drug-treated groups received a 1% cholesterol-supplemented diet for 2 days prior to the study.

sion. No effect was observed with 57-118 in the normal fed rabbits.

Inhibition of ACAT

In vitro enzyme studies

The formation of cholesteryl [1-^{14}C]oleate from mucosal microsomes from rabbits on a 1% cholesterol-fed diet was linear up to 4 min (**Fig. 1A**). Therefore, a 2-min incubation time was used in subsequent experiments. There was a linear relationship between ACAT activity and microsomal protein content up to 800 μg of protein (Fig. 1B). Increasing the concentration of [1-^{14}C]oleoyl CoA affected ACAT activity as shown in **Fig. 2.** When a fixed amount of bovine serum albumin (36 nmol, assuming mol wt of 60,000) was used, optimum activity was reached at 36 nmol of [1-^{14}C]oleoyl CoA, and when bovine serum albumin was maintained at equimolar concentrations with oleoyl CoA, optimum activity reached a peak at 18 nmol of [1-^{14}C]oleoyl CoA and remained on a broad plateau with a slight decline

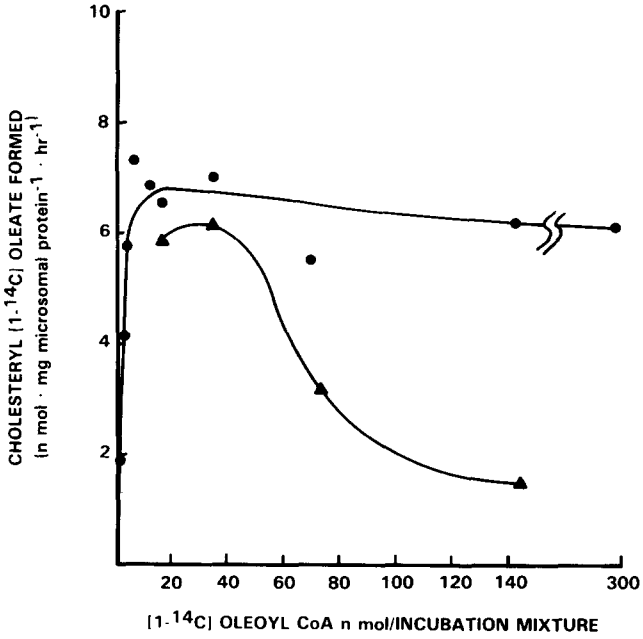

Fig. 2. Effect of [1-^{14}C]oleoyl-CoA concentration on ACAT activity; (● —— ●), equal molar concentration of albumin; (▲ —— ▲), fixed amount of albumin, 36 nmol/incubation mixture.

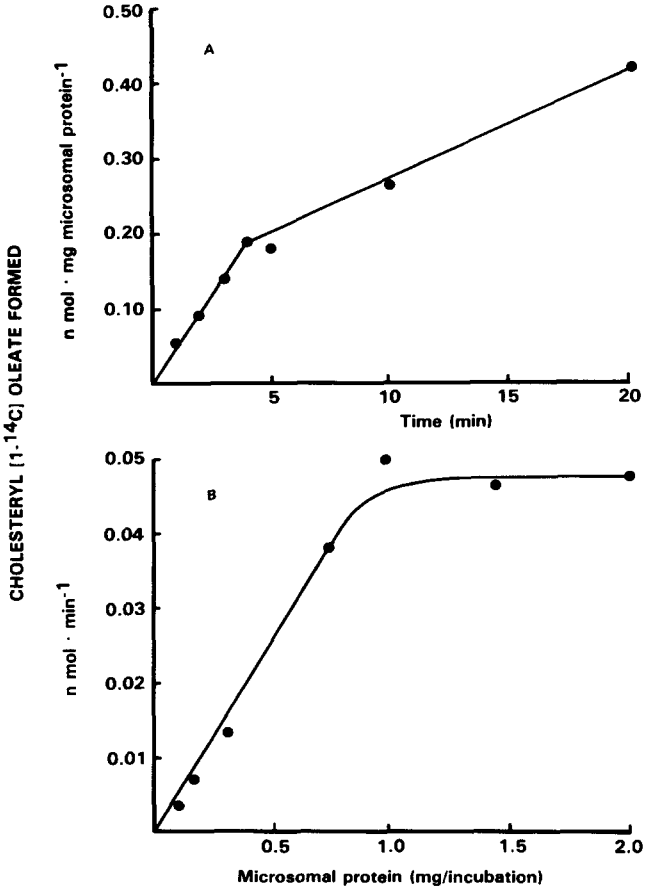

Fig. 1. The effect of incubation time (A) and microsomal protein (B) on ACAT with [1-^{14}C]oleoyl-CoA as the labeled substrate. The incubation conditions were as described under Methods except for the variations shown. Each value is the mean of duplicate assays.

up to 288 nmol. This in vitro ACAT activity was inhibited by 57-118 in a dose-related manner, whereas no effect was found for 57-118 on cytosolic cholesterol esterase activity (**Table 2**). Since the cholesterol esterase incubation mixture had a greater volume than that for the ACAT assay, the concentration of 57-118 was in-

TABLE 2. The in vitro effect of 57-118 on cholesterol esterification in mucosal microsomes and cytosol

Concentration[a] of 57-118	Cholesterol Esterification[b]	
	Microsomes	Cytosol
0	100.0 ± 7.4	100.0 ± 2.8
0.2	66.7 ± 13.8	93.8 ± 6.3
0.6	44.4 ± 9.5	100.5 ± 10.0
2.0	25.4 ± 5.6	100.5 ± 8.8
6.0	22.6 ± 2.1	102.5 ± 4.4
10.0	14.5 ± 3.5	93.4 ± 0.2
20.0	9.4 ± 3.2	99.9 ± 3.2
40.0		97.2 ± 7.5
100.0		100.6 ± 6.9

The animals from which the mucosal subcellular fractions were obtained were on a 1% cholesterol diet for 2 weeks prior to the study. The enzyme assay conditions and chemical determinations were performed as described under Methods.

[a] The concentration of 57-118 is nmol/incubation mixture (1 ml) and was added as a DMSO solution. The control samples (0 conc. 57-118) contained an equal volume of DMSO (5 μl).

[b] Enzyme activity is given as percent of controls. ACAT controls 44.3 ± 3.7 pmol cholesteryl [1-^{14}C]oleate · mg microsomal protein^{-1} · min^{-1} and cholesterol esterase controls 38.8 ± 4.8 pmol [4-^{14}C]-cholesteryl oleate · mg cytosol protein^{-1} · min^{-1}. The values are the mean of triplicate samples ± standard deviation.

creased to 100 nmol/incubation mixture (Table 2). This exceeds the concentration of 57-118 in the microsomal preparation (83.3 nmol vs. 73.5 nmol) when calculated on a mg of protein basis. A double reciprocal plot shown in **Fig. 3** suggests that 57-118 is acting as an apparent competitive inhibitor of ACAT activity.

Ex vivo

The results of the effect of 57-118 given orally on ACAT activity from mucosal microsomes are shown in **Table 3.** Under the conditions of this experiment, 57-118 was found to inhibit cholesterol esterification using a system optimized for ACAT activity. No effect was observed in a system optimized for the analysis of cholesterol esterase activity (Table 3).

The results of the HPLC determination of 57-118 are shown in **Table 4.** From these data it can be seen that the procedure removed any 57-118 that may have been present in the lumen of the gut at the time of killing and eliminated the possibility of contamination of the mucosal cell preparation.

Analysis of the various subcellular fractions indicates that 57-118 was present in all fractions. The concentrations found in both the microsomes and cytosol were of the same order of magnitude as those used in the in vitro studies.

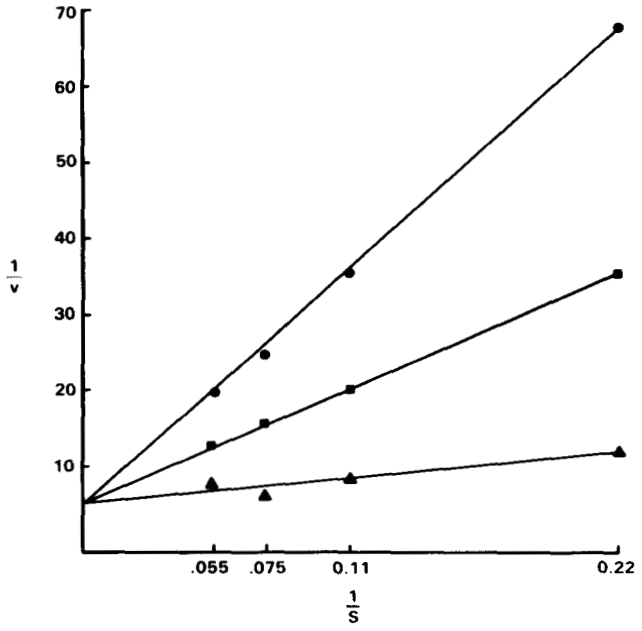

Fig. 3. Lineweaver-Burk plot of the effect of 57-118 on ACAT activity. (▲——▲), no 57-118; (■——■), 0.2 nmol 57-118; (●——●), 0.6 nmol 57-118; v, nmol cholesterol [1-^{14}C]oleate formed · mg microsomal protein^{-1} · hr^{-1}; S, [1-^{14}C]oleoyl-CoA nmol/incubation mixture.

TABLE 3. The ex vivo effect of 57-118 on cholesterol esterification in mucosal microsomes and cytosol

Treatment	Cholesterol Esterification	
	Microsomes	Cytosol
Control (n = 9)	133.3 ± 65.9	38.7 ± 14.3
57-118 (n = 10)	54.8 ± 19.2	38.0 ± 17.7

The animals from which the mucosal subcellular fractions were obtained were on a 1% cholesterol diet for 2 weeks prior to the study. The animals were fasted 12.5–15 hr before being killed. The control animals received 2 ml/kg 1.5% CMC at 2 and 0.5 hr before being killed. Compound 57-118 was given by gavage at 200 mg/kg in 1.5% CMC (2 ml/kg) at 2 and 0.5 hr before the rabbits were killed. Conditions and values are the same as in Table 2.

DISCUSSION

The development of 57-118 arose from a program initially directed toward finding agents that inhibit cholesteryl ester accumulation in cells of the arterial wall. Using a cell culture system with various cell types, several substances were found to be active. A structure-activity program was initiated to determine the active sites of these leads. In an attempt to develop an active drug that would have the greatest potential for being nontoxic, compounds were prepared incorporating these active sites from beneficial or innocuous naturally occurring substances. The amide of oleic acid and the ethyl ester of tryptophan (57-118) was one of the results of these efforts. Subsequent studies showed that it prevented cholesteryl ester accumulation in cells in culture by the inhibition of the ACAT enzyme. Studies on cholesterol-fed rabbits showed marked reduction in the development of atherosclerosis in the drug-treated animals. However, the markedly lower serum cholesterol levels in the drug-treated group compared to controls

TABLE 4. Concentration of 57-118 in various subcellular fractions of mucosal cells after oral administration[a]

Fraction	57-118[b]
Mucosal homogenate	2.12 ± 0.19
Nuclei and cell debris	3.12 ± 0.18
Mitochondria	1.96 ± 0.42
Microsomes	2.24 ± 0.11
Cytosol	2.80 ± 0.03
Wash 1	0.09 ± 0.01
Wash 2	0.04 ± 0.02

[a] Compound 57-118 was given by gavage at 200 mg/kg in CMC (2 ml) at 2 and 0.5 hr before the rabbits were killed. Homogenate subcellular fractions and washes were prepared as described in Methods.

[b] Concentration of 57-118 in subcellular fractions is given as nmol · mg protein^{-1} and in washes as nmol · total wash^{-1}. Values are the mean ± SD (n = 3).

throughout the study suggested the inhibition of cholesterol absorption by 57-118. The use of radiolabeled 57-118 showed that the drug was very poorly absorbed, indicating it was acting primarily at the intestinal level. The studies described here were made to establish the effect of 57-118 on cholesterol absorption and to determine if this correlated with its inhibition of ACAT in intestinal mucosal cells. Clinical studies are in progress to determine the effect of this agent in man.

The Zilversmit dual isotope method (15) for the determination of cholesterol absorption has been validated in several species (15, 20, 21, 22). The basic assumption of this method is that after approximately 24 hr the two isotopes of cholesterol administered by oral and intravenous routes equilibrate with the body pools and their blood decay curves are parallel (15). If this assumption is correct, then the percent absorption calculated from the isotope ratios obtained from blood samples will be the same at any time period after 24 hr. From the data given in Table 1 it can be seen that this does not hold in the rabbit, confirming the prediction of Zilversmit.[2] Therefore, the absolute determination of absorption by this method cannot be made. However, the primary purpose of this study was to determine if 57-118 inhibited cholesterol absorption and, although the calculated percent absorption varied with time (e.g., controls 56.7% at 72 hr vs. 45.7% at 120 hr), it has been demonstrated that 57-118 does inhibit absorption in a dose-related manner and the percent inhibitions determined from the two time periods are in reasonably good agreement.

The problem of different decay curves of the intravenously vs. orally administered isotopic cholesterol has been resolved in subsequent studies by administering [^{3}H]cholesterol orally to a rabbit, exsanguinating after 24 hr, and using the serum from this animal for the intravenous injections, while [^{14}C]cholesterol is given orally as described in Methods. Animals treated in this manner show parallel decay curves for the two isotopes and the calculated percent absorption is similar for all blood sampling times after 24 hr.

The conditions for optimizing ACAT activity in rabbit mucosal microsomes obtained in this study are, in general, in agreement with those obtained in humans by Helgerud et al. (17). The in vitro inhibition by 57-118 of this enzyme from mucosal microsomes is similar to that found in microsomes from Fu5AH rat hepatoma cells,[3] monkey smooth muscle cells, and human fibroblasts, as well as from rabbit liver and adrenals.[1] A double reciprocal plot shown in Fig. 3 suggests that 57-118 is acting as an apparent competitive inhibitor.

Our studies in cells in culture as well as in the mucosa from normal and cholesterol-fed rabbits confirms the work of Rothblat, Naftulin, and Arbogast (23) and Drevon and Hovig (24) that ACAT activity is highly dependent on cholesterol concentration. This presented a problem in demonstrating the ex vivo inhibition of ACAT activity with 57-118. If it inhibited cholesterol absorption by other mechanisms (e.g., inhibition of cholesterol solubilization in the micelles in the gut), the decrease in the cholesterol substrate would lead to less ACAT activity. Therefore, the animals were primed by cholesterol feeding for 2 weeks. Then the rabbits were fasted for 12–15 hr before oral administration of 57-118. Gastrointestinal transit times determined by Oil Red O and cholesterol indicated that by this time all exogenous cholesterol was gone from the lumen of the small intestine. ACAT activity in the mucosal cells from cholesterol-fed control animals was found to be still elevated at this time period. This assured that the drug was not acting in the lumen on exogenous cholesterol. The careful washings of the mucosa lining were made to eliminate the contamination of the mucosal homogenate with extracellular 57-118 (excluding tightly surface-bound drug if any). The quantitative determination of 57-118 in the various cell fractions must, however, be taken with caution since it is possible that during the ultracentrigation separation of the subcellular fractions the compound was redistributed.

The fact that 57-118 inhibits cholesterol absorption and that it has been shown to inhibit ACAT activity in vitro as well as ex vivo strongly suggests that ACAT plays an important role in cholesterol absorption. However, since absorption cannot be completely inhibited, it is possible that 57-118 does not reach the endoplasmic reticulum of the mucosal cells in sufficient concentrations to inhibit the enzyme completely or that more than one mechanism for cholesterol absorption exists. The studies of Gallo et al. (6, 7) suggest that cholesterol esterase may play a role in an alternative pathway. Since 57-118 requires a cholesterol-supplemented diet for activity, one might speculate that the ACAT pathway plays the predominant role under conditions of a cholesterol load and that another mechanism (possibly cholesterol esterase) is predominant under normal conditions.

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[2] Zilversmit, D. B. Personal communication.
[3] Gift of Dr. George Rothblat.

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