Acyl-CoA:cholesterol acyltransferase in human small intestine: its activity and some properties of the enzymic reaction

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Abstract Esterification of endogenous cholesterol in human small intestinal mucosa by acyl-CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26) was studied using [1-^{14}C]oleoyl-CoA as substrate. The reaction was linear for 2 min only. The esterification of cholesterol was stimulated by albumin, but this effect was dependent on the oleoyl-CoA concentration. When the albumin concentration was 5 g/liter, maximal esterification was obtained with 35 μM oleoyl-CoA. The pH optimum was 7.2–7.8. The ACAT specific activity was highest in microsomal preparations from jejunum (0.21 ± 0.19 (n = 8) nmol cholesteryl oleate·mg microsomal protein^{-1}·min^{-1}), and lower in proximal duodenum and distal ileum. Whole homogenates of biopsies had about ¼ of the activity of the corresponding microsomal preparation. Microsomal preparations from jejunum contained acyl-CoA hydrolase (EC 3.1.2.2) which under the prevailing conditions had a maximal activity of 4.4 nmol oleate formed·microsomal protein^{-1}·min^{-1} The high activity of intestinal ACAT in man renders it possible that this enzyme plays a role in cholesterol absorption.—**Helgerud, P., K. Saarem, and K. R. Norum.** Acyl-CoA:cholesterol acyltransferase in human small intestine: its activity and some properties of the enzymic reaction. *J. Lipid Res.* 1981. **22:** 271–277.

Supplementary key words acyl-CoA hydrolase · cholesterol esterification · cholesterol absorption · chylomicrons

Recent work in this laboratory has shown that CoA-dependent cholesterol esterifying activity is present in the "microsomal fraction" of small intestinal mucosal cells from rats (1), guinea pigs (2), and humans (3). Our data strongly indicate that cholesterol esterification is catalyzed by an acyl-CoA: cholesterol acyltransferase (ACAT) (EC 2.3.1.26). In these studies, the enzyme activity was determined using as substrate endogenous cholesterol labeled with trace amounts of [7α-^{3}H]cholesterol (4), and complete equilibration of isotope with endogenous cholesterol pools was assumed in the calculation of total and specific ACAT activities. A major disadvantage of this assay system is the uncertainty concerning the degree of isotopic equilibration (5, 6). Data from rat liver (7–9) and rabbit aortic (10, 11) microsomes suggest that there are different compartments of cholesterol and that not all of these are substrates for the ACAT reaction. This may also be true for intestinal microsomes, giving overestimation of the enzyme activity as indicated for ACAT activity in Ehrlich cell microsomes (12).

The most direct method for examination of ACAT activity is based on labeled activated fatty acid as introduced for rat liver ACAT by Goodman, Deykin, and Shiratori (13), later modified by others and used in other tissues (12, 14–17). In this report we have examined the ACAT activity in human small intestine using [1-^{14}C]oleoyl-CoA as substrate and thus confirmed the existence of the enzyme. In order to evaluate its activity, we have tested first the optimal conditions of the assay using microsomal preparations of jejunum and then the specific activity in different segments of the small intestine.

Material from human intestinal mucosa is obtainable as a routine method only through small biopsies not suitable for the preparation of microsomes. We have therefore also tested the ACAT activity in whole homogenates of biopsies from distal duodenum-proximal jejunum and compared this to the microsomal activity.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]Oleoyl-CoA (ca. 45 mCi/mmol) was from New England Nuclear, Boston, MA. Unlabeled oleoyl-CoA and CoA were purchased from Sigma Chemical Co., St. Louis, MO. The oleoyl-CoA was dissolved in

Abbreviation: acyl-CoA:cholesterol acyltransferase (EC 2.3.1.26), ACAT.

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sodium acetate buffer (0.01 M, pH 6.0). The concentration was controlled by measuring the absorbance at 260 nm using an extinction coefficient of 16×10^3 $M^{-1} \cdot cm^{-1}$ (18). Both labeled and unlabeled oleoyl-CoA were stored at −70°C. [1-^{14}C]Oleic acid (59 mCi/mmol) was from The Radiochemical Centre, Amersham, England. Bovine serum albumin (fraction V; Sigma Chemical Co.) was essentially fatty acid-free. It was dissolved in potassium phosphate buffer (0.2 M, pH 7.4), heat-inactivated at 60°C for 30 min, and stored at −20°C. All other chemicals and solvents were standard commercial high purity materials.

Human material

Mucosal material from proximal duodenum and jejunum or distal ileum was obtained from resections made because of cancer or morbus Crohn, or resections were made at the time of kidney removal because of brain death. Finally, after informed consent, minor resections of jejunum were obtained during jejuno-ileal bypass for obesity.

From the specimens of proximal jejunum, small "biopsies" were taken and stored at −70°C in potassium phosphate buffer (0.2 M, pH 7.4) along with biopsies previously taken in normal men by a modified Crosby capsule (3). From these, fresh whole homogenates were prepared on the day of enzyme assay as previously described (3).

Except for homogenization and centrifugation in ice-cold 0.25 M sucrose, the preparation of a "microsomal fraction" was as previously described (3). The pellet was resuspended in the same buffer and stored in the same manner as the biopsies.

Chemical analyses

The protein determination was according to Lowry et al. (19) using bovine serum albumin as standard.

The content of cholesterol in whole homogenates and microsomal preparations was determined as previously described (20).

ACAT assay

The activity of ACAT was determined by the formation of labeled cholesteryl ester from [1-^{14}C]oleoyl-CoA and endogenous cholesterol. Incubations were carried out in plastic vials (2 ml) shaken continuously at 120 strokes per min in a water bath held at 37°C. To ensure this temperature at start of the short standard incubation period of 2 min, all vials were preincubated for 5 min before addition of the labeled substrate. In a standard assay the preincubation volume was 0.48 ml potassium phosphate buffer (0.2 M, pH 7.4) containing 2.5 mg of bovine serum albumin and about 150 μg of microsomal protein. The esterification of cholesterol was initiated by the addition of 16–17 nmol [1-^{14}C]oleoyl-CoA (usually 180–200,000 dpm per vial) in 0.02 ml sodium acetate buffer, making the final incubation volume 0.5 ml. The reaction was stopped in 10 ml of chloroform–methanol 2:1 (v/v) and lipids were extracted by the method of Folch, Lees, and Sloane Stanley (21) using a salt solution of pH 3 in order to keep the fatty acids undissociated and thereby render them soluble in the chloroform phase. In some experiments the radioactivity of the aqueous phase was tested using Unisolve (Koch-Light Laboratories Ltd., Colnbrooke, England) as liquid scintillator. Usually, however, the upper phase was removed and the chloroform was evaporated. The lipids were dissolved in a small volume of hexane and taken quantitatively for separation by thin-layer chromatography on silica gel H, using light petroleum (b.p. 60–70°C)–diethylether–glacial acetic acid 85:15:3 (by vol) as the developing system. A lipid extract of normal serum was used as carrier. The solvent front was run 10 cm, and the lipid fractions were visualized with iodine vapour. The areas containing the different lipid classes were scraped into vials containing 10 ml Scinthei I (Koch-Light Laboratories Ltd.) and the radioactivity was quantitated in a Packard Tri-Carb liquid scintillation spectrometer, model 3385 (Packard Instrument Co., Inc., Downers Grove, IL.). Corrections were made for counting efficiency (channels ratio method) and esterification rates were calculated as nmol cholesteryl [1-^{14}C]oleate formed · mg microsomal protein^{-1} · min^{-1}.

Recovery of radioactive cholesteryl oleate was more than 85% for the whole procedure. In all experiments described, incubations without microsomes were used as controls and no esterification was observed. Microsomes that had been heat-inactivated at 60°C for 30 min had no esterifying activity.

RESULTS

Optimization of the ACAT assay

The rate of cholesteryl [1-^{14}C]oleate formation was linear for 2 min only (**Fig. 1A**), and this was therefore used as the incubation time in subsequent experiments. Fig. 1B shows the linear relationship between ACAT activity and content of microsomal protein per incubation vial up to 400 μg. The formation of cholesteryl ester was maximal between pH 7.2 and pH 7.8 (Fig. 1C), comparable to the optimum found when an oleoyl-CoA-generating system was used for examination of ACAT activity in the small intestine of man (3) and guinea pig (2). It also compares well with optimal pH for ACAT in rat liver (9, 13) and adrenal (15) cells, Ehrlich cells (12), and rabbit aortic cells (17).

Variation in the potassium phosphate buffer concentration from 0.03 to 0.83 M gave approximately constant esterification rates (data not shown).

Fig. 2A shows the effect of increasing [1-^{14}C]oleoyl-CoA concentration on the ACAT activity at different concentrations of bovine serum albumin. Without albumin present the activity was very low at all concentrations of [1-^{14}C]oleoyl-CoA tested. Addition of albumin produced a 5-fold increase in the ACAT activity. Whether 5 or 10 g/liter was used, the ACAT activity increased with increasing concentration of [1-^{14}C]oleoyl-CoA to the same maximal rate. This was, however, obtained at different concentrations of [1-^{14}C]oleoyl-CoA, 25 and 35 μM, respectively. Further increase in [1-^{14}C]oleoyl-CoA concentration had no influence when the albumin concentration was 10 g/liter, whereas at 5 g/liter some inhibition was seen. When the experimental data with albumin concentrations 5 and 10 g/liter were plotted according to Lineweaver-Burk (Fig. 2B), a straight line was obtained corresponding to the initial parts of the curves suggesting that the ACAT reaction followed Michaelis–Menten kinetics in the lower concentration range of [1-^{14}C]oleoyl-CoA. Extrapolation of the straight lines gave apparent K_m values of 7 and 18 μM at albumin concentration 5 and 10 g/liter, respectively.

These data pointed to an important effect of bovine serum albumin on the ACAT activity that was closely connected to the concentration of [1-^{14}C]oleoyl-CoA. In the experiment shown in **Fig. 3,** we tested the effect of increasing albumin concentrations on the ACAT activity at two different concentrations of [1-^{14}C]-oleoyl-CoA. The ACAT activity was again very low without albumin present. The maximal rate of cholesteryl ester formation was the same with 13 and

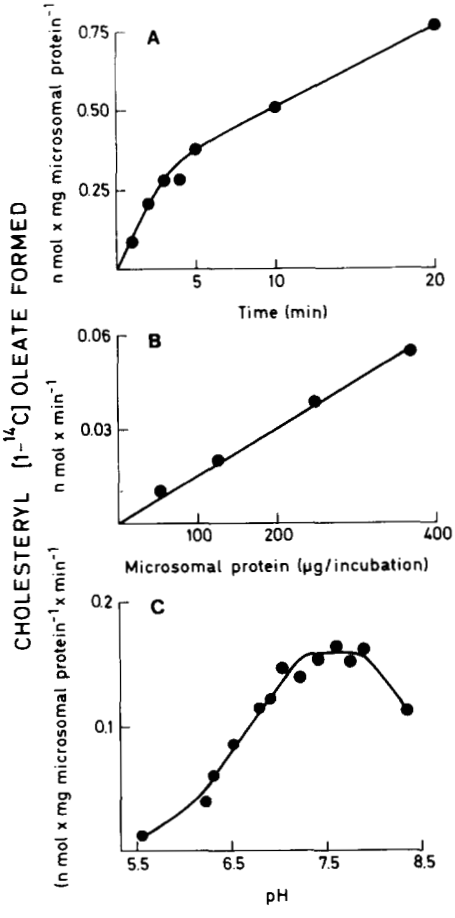

Fig. 1. The effect of incubation time, A; microsomal protein concentration, B; and pH, C, on the ACAT reaction with [1-^{14}C]-oleoyl-CoA as the labeled substrate. The incubation conditions were as described under Methods except for the variations shown. Each value in B is the average of two closely agreeing determinations. The pH, C, was varied by changing the ratio of KH_2PO_4 and K_2HPO_4 in the 0.2 M potassium phosphate buffer. The final pH was measured in duplicates of the incubation mixtures and no change was observed after the incubation period of 2 min.

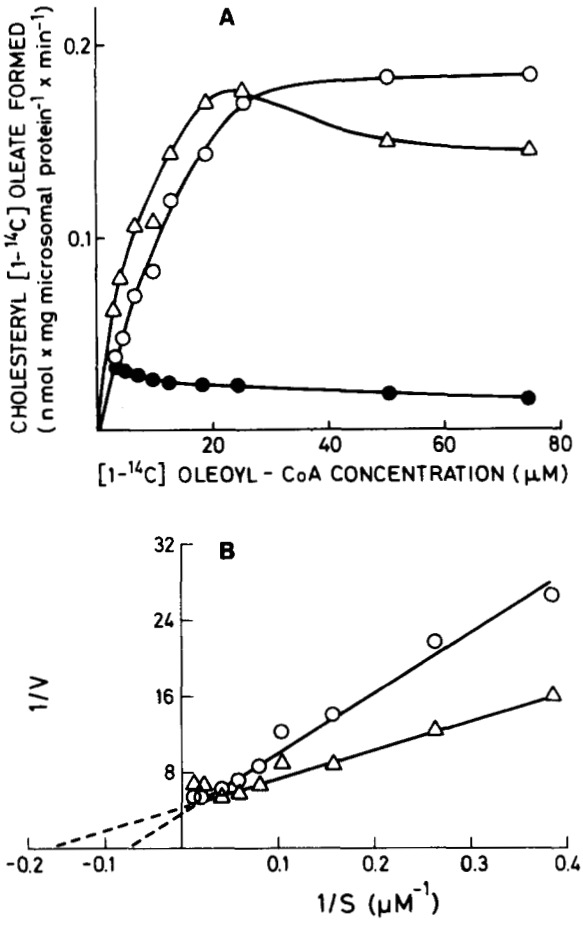

Fig. 2. Effect of [1-^{14}C]oleoyl-CoA concentration on the ACAT activity. In A the concentration was varied from 2.6 to 75.6 μM at three different concentrations of fatty acid-free bovine serum albumin: (● —— ●) no albumin; (△ —— △) 5 g/liter; (○ —— ○) 10 g/liter. Incubation conditions otherwise as described in Methods except that the preincubation time was 10 min. B, Lineweaver-Burk plot of the experiments with albumin present. Rate v is in nmol·mg microsomal protein^{-1}·min^{-1}. The apparent K_m values were 7 and 18 μM at albumin concentrations 5 and 10 g/liter, respectively.

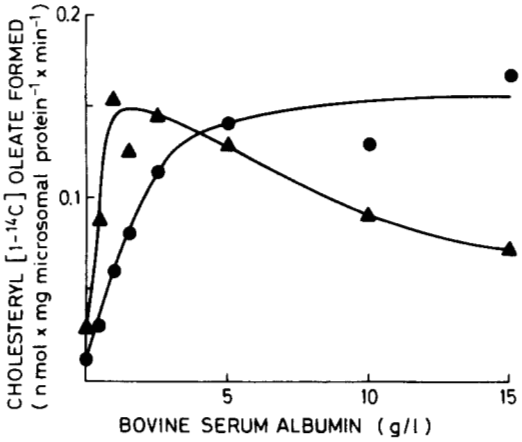

Fig. 3. Effect of bovine serum albumin on the ACAT activity. The concentration of albumin was, as shown, at two different concentrations of [1-^{14}C]oleoyl-CoA: (▲ —— ▲) 13 μM; (● —— ●) 32 μM. Incubation conditions otherwise as given in Methods.

32 μM [1-^{14}C]oleoyl-CoA. However when 13 μM was used, the ACAT activity was maximal at a low albumin concentration (1–3 g/liter) and inhibition occurred at higher concentrations. When 32 μM was used the ACAT activity was maximal in a broad range of albumin concentration from 5 to 15 g/liter. Other experiments with different microsomal preparations all confirmed that the same maximal ACAT activity was obtained with different concentrations of [1-^{14}C]-oleoyl-CoA (16–64 μM). At the higher concentrations of acyl-CoA, however, more albumin was necessary to obtain the optimal rate of esterification.

Availability of [1-^{14}C]oleoyl-CoA

Fig. 4 shows the recovery of lipid-soluble radioactivity with time after thin-layer chromatography. Most of the radioactivity was incorporated into the fractions corresponding to free fatty acid and phospholipid while incorporation into other lipids was small. These data suggested that after longer incubation periods lack of [1-^{14}C]oleoyl-CoA probably contributed to the reduced rate of cholesterol esterification. After the standard incubation time of 2 min, however, less than 10% of added radioactivity was recovered as lipid-soluble, indicating that about 90% of added [1-^{14}C]oleoyl-CoA was still unreacted. In separate experiments the unreacted [1-^{14}C]oleoyl-CoA was tested as water-soluble radioactivity after the lipid extraction. After 2 min of incubation, about 80% of the added radioactivity was recovered in the aqueous phase independent of variation in albumin concentration from 0 to 20 g/liter and whether 16 or 32 μM [1-^{14}C]oleoyl-CoA was used.

Acyl-CoA hydrolase activity

Hydrolysis of [1-^{14}C]oleoyl-CoA obviously was the most important reaction tending to reduce our substrate (Fig. 4) and, because free fatty acids are known to inhibit rat liver ACAT in concentrations from 1×10^{-5} M (13), the formation of [1-^{14}C]oleate was tested during our ACAT assay. **Fig. 5** shows that without albumin present in the incubation mixture the acyl-CoA hydrolase activity increased with increasing [1-^{14}C]oleoyl-CoA concentration up to 25 μM corresponding to a maximum rate of 4.4 nmol [1-^{14}C]-oleate formed per mg microsomal protein$^{-1}\cdot$min^{-1}. Thus if intestinal ACAT is inhibited by oleate this effect will probably be of importance only in prolonged incubations since about 7 min would be necessary to give a concentration of 1×10^{-5} M. With albumin present the recovery of [1-^{14}C]oleate was reduced at all concentrations of [1-^{14}C]oleoyl-CoA tested. **Fig. 6** illustrates the apparent inhibition of the hydrolase activity by increasing the concentration of albumin. Inhibition of acyl-CoA hydrolase by albumin has previously been described (16, 22, 23) and data presented from rat liver and Ehrlich cell microsomes are very similar to ours. The effect may have been only apparent however, and secondary to loss of [1-^{14}C]oleate bound to albumin during the lipid extraction inasmuch as an increase in albumin concentration did not increase the unreacted [1-^{14}C]-oleoyl-CoA (vide supra).

Endogenous fatty acid activation?

The hydrolysis of acyl-CoA (Fig. 4) led to increasing concentration of CoA and if free fatty acids in our microsomal preparation were activated during the assay this would give unlabeled cholesteryl ester and thus underestimation of the ACAT activity. To test this we performed experiments with [1-^{14}C]oleate. After 5 min of preincubation (146 μg microsomal protein, 2.5 mg bovine serum albumin with 16.5 nmol

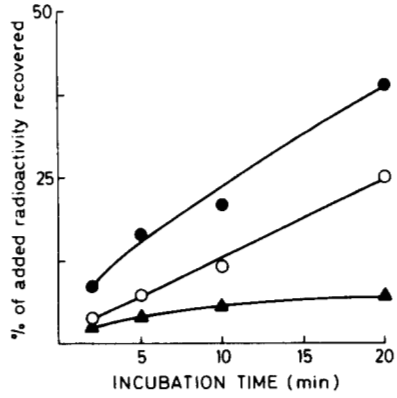

Fig. 4. Recovery of lipid soluble radioactivity from [1-^{14}C]oleoyl-CoA as a function of incubation time. Incubation conditions were as in Methods except for albumin concentration, 10 g/liter. Incorporation into free fatty acid (○ —— ○); phospholipids (▲ —— ▲) and total recovery after thin-layer chromatography (● —— ●) are given.

of [1-^{14}C]oleate in 0.2 M potassium phosphate buffer of pH 7.4) incubations were run for 2 min with unlabeled oleoyl-CoA or CoA (final concentrations 30 and 32 μM, respectively). No radioactive cholesteryl ester was detected, indicating that activation of free fatty acids was negligible in our assay system for ACAT.

ACAT activity in different parts of the small intestine

Table 1 shows that the mean ACAT activity was higher in microsomal preparations from jejunum than from duodenum and ileum. During optimization of the assay, we performed several determinations on the same specimens, and from these data we can conclude that most microsomal preparations are quite stable when kept at −70°C up to 1½ years. In others a variable, but small decrease was noted in the ACAT activity. A more pronounced variation, however, was found between the activity in microsomal preparations of jejunum from different individuals. While the mean specific activity was 0.21 nmol cholesteryl [1-^{14}C]-oleate per mg microsomal protein^{-1}·min^{-1}, the most active preparation had specific activity 0.71.

The ACAT activity obtained with whole homogenates of biopsy specimens from distal duodenum/proximal jejunum was 25% of the microsomal activity (Table 1). Included in this group are biopsies from normal men previously examined for ACAT activity by the [^{3}H]cholesterol assay system (3). These biopsies had been kept frozen in potassium phosphate buffer at −70°C for 3 years and their activity was not different from that of biopsies examined the same day or after only a few days at −70°C.

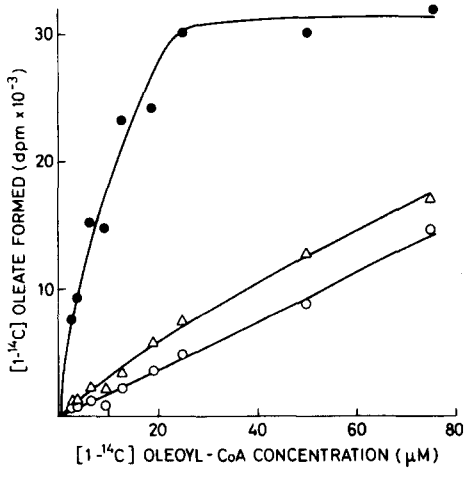

Fig. 5. Recovery of [1-^{14}C]oleate as a function of [1-^{14}C]oleoyl-CoA concentration at different concentrations of bovine serum albumin. (● —— ●), No albumin; (△ —— △), 5 g/liter; (○ —— ○), 10 g/liter. Incubation conditions otherwise as described in legend to Fig. 2. Specific activity of added [1-^{14}C]oleoyl-CoA was 23.4 × 10^{3} dpm·nmol^{-1}.

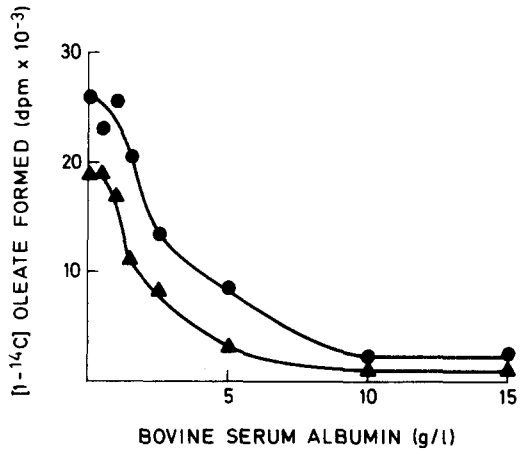

Fig. 6. Recovery of [1-^{14}C]oleate as a function of albumin concentration. Incubation conditions and experimental procedure as described in Methods except that two different concentrations of [1-^{14}C]oleoyl-CoA were used: (▲ —— ▲), 13 μM; (● —— ●), 32 μM. Specific activity of added [1-^{14}C]oleoyl-CoA was 20.8 × 10^{3} dpm·nmol^{-1}.

DISCUSSION

Esterification of intracellular cholesterol in most tissues is catalyzed by acyl-CoA:cholesterol acyltransferase (23), but attempts to detect ACAT in the intestine using ATP, CoA, and fatty acids have been unsuccessful (24) and the mechanism of cholesterol esterification in the mucosal cell has been poorly understood (23, 25). Using an efficient acyl-CoA generating system, however, we have recently been able to demonstrate CoA-dependent esterification in different species (1, 2, 3). With preformed [1-^{14}C]oleoyl-CoA as substrate we have in this report proved the existence of ACAT in human small intestine.

The maintenance of adequate concentrations of acyl-CoA is essential to any ACAT assay and this might have been one reason why the enzyme was difficult to detect. The generation of acyl-CoA from fatty acids, CoA, and ATP may be a rate-limiting step (26, 27), but probably more important is the rapid hydrolysis of acyl-CoA (Fig. 4, and ref. 16, 17, 23, 28). This probably contributed to the short linear period of cholesteryl ester formation. Furthermore, the concomitant formation of oleate which inhibits ACAT may also reduce the rate of cholesterol esterification with time (13).

Based on data from other tissues (12, 29–31), the pool of endogenous acyl-CoA is probably insignificant when compared to the exogenously added oleoyl-CoA. This is supported by our observation that without exogenous CoA very little [7α-^{3}H]cholesterol was esterified (3). A direct activation of endogenous free fatty acids is probably also insignificant. Non-radioactive acyl-CoA may, however, be produced during the incubation by direct transfer of acyl groups from

TABLE 1. ACAT activity and mucosal composition in different parts of human small intestine

	Number of Patients	Unesterified Cholesterol/ Protein	ACAT Activity
Microsomal preparations			
Duodenum	3	114.3 (25.8)	0.062 (0.036)
Jejunum	8	122.4 (27.3)	0.210 (0.190)
Ileum	5	150.2 (40.7)	0.053 (0.039)
Whole homogenate of biopsies from distal duodenum-proximal jejunum	15	36.1 (7.2)	0.050 (0.020)

The enzyme assay and chemical determinations were performed as described under Methods. Unesterified cholesterol/protein is given as nmol·mg protein^{-1}. ACAT activity is given as nmol cholesteryl [1-^{14}C]oleate formed·mg protein^{-1}·min^{-1}. Values are means (S.D.) of one to ten determinations from each preparation. Microsomal preparations and biopsies were stored at −70°C until assay maximally 1.5 and 3 years later, respectively. Whole homogenate of biopsies was prepared on the day of the ACAT assay.

endogenous acylcarnitines to CoA catalyzed by carnitine palmitoyl transferase (32). Thus, in the [7α-^{3}H]-cholesterol assay system, addition of CoA alone will result in some cholesterol esterification (3). The error due to this mechanism will increase with the incubation time and in order to achieve true initial velocity it is mandatory that the incubation time is short.

Long chain acyl-CoAs are amphipathic and in the absence of albumin their critical micellar concentration is in the range 2–5 μM (33, 34). An increasing concentration of acyl-CoA micelles are inhibitory to a variety of enzymes in vitro (35–37) as also shown for ACAT (12, 17, 23). This is probably due to a detergent effect of the micelles and thus comparable to the inhibition of ACAT by taurocholate (3, 13). Since albumin has a strong acyl-CoA binding effect, it probably reduces the inhibition by lowering the micellar concentration (35, 38). It is possible that the monomer concentration of oleoyl-CoA is not linearly related to the total concentration of oleoyl-CoA when albumin is present in the medium. However, we find it important that the initial hyperbolic shape of the curves is suddenly broken at the same maximal esterification rate (Fig. 2). This strongly suggests that the monomeric form of oleoyl-CoA is the substrate for ACAT (33, 39, 40).

With albumin present (9, 17) or absent (12) from the incubation mixture, the same dependence of acyl-CoA has been found for ACAT in other tissues, i.e., initial hyperbolic curves that level off. The apparent K_m values vary, but are in the same range as our data, when albumin is present.

The use of [1-^{14}C]oleoyl-CoA in the current study confirms that the activity is higher in the microsomal fraction than in the whole homogenate and the specific activity also compares well with the results obtained using [7α-^{3}H]cholesterol as the radiolabeled substrate (3). Thus in this latter study we found, in biopsies from distal duodenum/proximal jejunum of normal men, activities 3.6 ± 1.37 as compared to 3.0 ± 1.2 nmol cholesteryl ester formed·mg protein^{-1}·hr^{-1} in the present paper.

The specific activity in microsomal preparations was highest in jejunum. The number of patients was small and their conditions before surgery differed, but when different segments of the small intestine were from the same individual the results always showed highest activity in jejunum. Using the [7α-^{3}H]-cholesterol assay system we also found that the activity was higher in jejunum than in duodenum (3) and, in the rat, the activity was highest in proximal jejunum compared to the middle and distal one-third of the small intestine (1). The mean specific activity in human jejunum (0.21 nmol·mg microsomal protein^{-1}·min^{-1}) corresponds to the esterification of more than 100 mg of cholesterol a day by one gram of microsomal protein, indicating a high capacity of intestinal ACAT. Cholesteryl esters in intestinal lymph are transported in variable concentrations in the core of the chylomicrons (41). The intestinal ACAT activity may therefore be important in regulation of cholesterol absorption or at least in chylomicron formation (42).

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