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Cholesterol Esterification by Mammary Gland Microsomes from the Lactating Rat (41840)

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Abstract. Cholesterol esterification by fatty acyl-CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26) has been demonstrated in microsomes prepared from breast tissue of the lactating rat, employing the incorporation of [1-^{14}C]oleoyl coenzyme-A into cholesteryl[^{14}C]oleate. The regulation of this activity *in vitro* was studied by supplementing microsomes with unesterified cholesterol supplied either as a dispersion in acetone or by incubating microsomes with cholesterol-enriched serum lipoproteins prior to enzyme assay. ACAT activity was increased by more than 80% when the ratio of unesterified cholesterol to microsomal protein was increased from 29 to 48 μg/mg protein, indicating that the normal cholesterol content of mammary gland microsomes does not saturate this enzyme. Cholesterol esterification could be inhibited nearly completely *in vitro* by addition of the polar steroid progesterone (93% decrease in ACAT activity with 75 μ*M* progesterone) and, to lesser extents, by estradiol and retinol. Enzyme assays were also performed after incubating microsomes with *N*-acylamides that inhibit cholesteryl ester synthesis in other systems. Under conditions where the mammary gland ACAT reaction was inhibited by more than 82%, the esterification of retinol was reduced by less than 30% and incorporation of [^{14}C]oleoyl-CoA into triglycerides was not inhibited at all. These studies indicate that the lactating mammary gland contains ACAT activity having properties similar to ACAT in other organs. The presence of ACAT activity in the lactating mammary gland provides a possible mechanism for the synthesis of cholesteryl esters found in milk. It can also be inferred that ACAT in mammary gland microsomes is likely to be distinct from the microsomal acyltransferases that catalyze the esterification of retinol and glycerides.

Among the many lipid constituents of milk are cholesterol and its esters. Considerable attention has been given to the determination of the cholesterol concentration in milks from various species and its relationship to diet (1–4). Of total milk cholesterol, about 10–15% is present in esterified form in bovine milk (5) and in rat's milk (6). Little is known of the origin of this esterified cholesterol; reasonable possibilities include uptake of esterified cholesterol from serum lipoproteins, and esterification of cholesterol within the lactating gland (7).

Most acyltransferases that catalyze lipid ester synthesis are membrane-bound, microsomal enzymes. As such, relatively little is known about the properties, specificities, and regulation of these enzymes. Recently, we described the esterification of vitamin A (retinol) by microsomal membranes prepared from the lactating mammary gland of the rat (8). During these studies we became interested in whether the lactating mammary gland can also esterify cholesterol and whether a unique enzyme catalyzes each esterification reaction. This report provides evidence for a microsomal fatty acyl-CoA:cholesterol acyl-transferase (ACAT) in this organ and examines the effects of membrane cholesterol content, hormones, and other lipids on this enzymic process.

Materials and Methods. *Materials.* [1-^{14}C]-Oleoyl-CoA from New England Nuclear or Amersham Radiochemicals was diluted with oleoyl-CoA from P-L Biochemicals to a specific activity of 14–24 dpm/pmole. Progesterone and other steroids were obtained from Steraloids, Inc., and retinol, dithiothreitol, and bovine serum albumin (BSA, essentially fatty acid free) were purchased from Sigma Chemical Company. Two *N*-acylamides, Sandoz drugs 58-035 and 57-118, were generously provided by Dr. John Heider of Sandoz, Inc., Hanover, New Jersey. Their chemical names, respectively, are 3-[decyldimethylsilyl]-*N*-[2-(4-methylphenyl)-1-phenylethyl] propanamide and *N*-(1-oxo-9-octadecenyl)-DL-trypto-

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phan(Z)ethyl ester. Each *N*-acylamide was dissolved in dimethylsulfoxide and appropriate amounts were added to microsomes so that the final concentration of dimethylsulfoxide was maintained constant at 1% (wt/vol).

Preparation of microsomes. Microsomes were prepared as described previously (8) from breast tissue of Sprague–Dawley rats lactating for 1–14 days and fed a standard laboratory diet (Wayne Lab-blox). Microsomes were suspended at 4–10 mg protein/ml in 0.15 *M* potassium phosphate buffer, pH 7.4, frozen in Dri-Ice/acetone, and stored at −65°C. Under these conditions, microsomal ACAT activity was stable for 6–8 weeks and essentially equal to that in fresh microsomal membranes. Cholesterol in microsomes was determined by gas–liquid chromatography (9) and protein was measured by the dye-binding method of Bradford (10).

Incubations. Microsomes in 0.15 *M* potassium phosphate buffer, pH 7.4, were pipetted into glass screw-cap tubes on ice and additions such as dithiothreitol and BSA were made in the same buffer so that the volume equalled 0.17 ml. To begin the esterification reaction, an appropriate amount of [1-^{14}C]oleoyl-CoA in 30 μl buffer was added and tubes were incubated at 37°C in a shaking waterbath for specified times. Each incubation condition was tested in duplicate or triplicate and a blank containing boiled microsomes was also incubated for each condition. Each incubation was stopped by addition of 1 ml ethanol and an internal recovery standard of cholesteryl[^{3}H]oleate (approximately 1500 cpm) was added along with 50 μg each of unlabeled cholesteryl oleate, methyl oleate, and triolein for detection after TLC. Neutral lipids were extracted by adding 4 ml hexanes and 1 ml water, with shaking after each addition. After a brief centrifugation, 3.5 ml of the hexane upper phase was taken to dryness under nitrogen and applied to plastic-backed thin-layer plates of silica gel (Baker-flex IB2) which were developed in hexane/diethylether/acetic acid, 85/15/1 by volume. Lipids were visualized by a brief exposure to iodine vapors and zones containing lipid esters were cut and placed in scintillation vials with 10 ml of ScintiLene (Fisher Scientific Co.) for determination of ^{3}H and ^{14}C in a Beckman 7500 scintillation spectrometer. After correction for isotope overlaps, counts per minute were converted to dissintegrations per minute by reference to calibrated [^{3}H]- and [^{14}C]toluene standards. Value for blanks containing boiled microsomes have been subtracted in all cases.

Progesterone and other lipids (Table I) were added in 2 μl acetone to 0.17 ml of microsomes in the presence of dithiothreitol and BSA and were incubated at 37°C for 10 min before addition of [^{14}C]oleoyl-CoA.

For incubation with lipoproteins, 1-ml portions of microsomes (4 mg protein) were incubated for 1 hr at 30°C with either 0.2 ml phosphate buffer, pH 7.4, or with 0.2 ml of a lipoprotein solution of density < 1.06 g/cm^{3} from a cholesterol-fed rabbit, added at 40 and 158 μg unesterified cholesterol/mg microsomal protein. After incubation, microsomes were reisolated by centrifugation (11) and suspended in phosphate buffer for measurements of cholesterol, protein content, and ACAT activity.

Results. *Demonstration of ACAT activity.* When mammary gland microsomes from normal lactating rats were incubated with [^{14}C]oleoyl-CoA, cholesteryl[^{14}C]oleate was detected after thin-layer chromatography. Incubation conditions were varied to determine how the esterification of microsomal cholesterol varied with the concentration of oleoyl-CoA, time of incubation, amount of microsomal protein, and presence or absence of a reducing agent (dithiothreitol) and of BSA. Figure 1A shows that cholesteryl ester synthesis reached a plateau with approximately 50 μ*M* [^{14}C]oleoyl-CoA; these incubations were conducted in the presence of 2 m*M* dithiothreitol and 80 μ*M* BSA. With a saturating

TABLE I. EFFECT OF LIPIDS ON MAMMARY GLAND ACAT ACTIVITY IN VITRO

Lipid addition	ACAT activity, percentage of control[a] ($\bar{x}$ ± SD, N = 3)	Significance (P)
Progesterone, 15 μ*M*	44.01 ± 1.21	<0.001
Progesterone, 75 μ*M*	7.33 ± 1.81	<0.001
17-β-Estradiol, 75 μ*M*	69.7 ± 2.55	<0.001
Retinol, 75 μ*M*	69.7 ± 7.11	<0.005
Cholesterol, 75 μ*M*	123.5 ± 6.08	<0.025

[a] Control incubations that included the acetone vehicle were run with each test substance or group. Control values equalled 15.9 ± 2.62 pmole/0.2 mg protein in an 8-min incubation period.

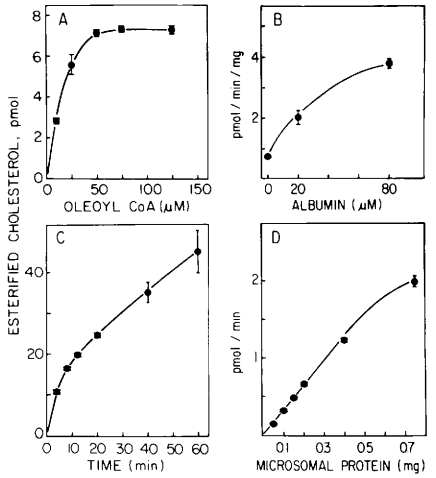

FIG. 1. Effects of concentration of oleoyl-CoA, time of incubation, microsomal protein, and concentration of BSA on yield of esterified cholesterol. (A) Incubation included mammary gland microsomes (0.2 mg protein), 2 m*M* dithiothreitol, 80 μM BSA, and the indicated concentrations of [^{14}C]oleoyl-CoA in 0.2 ml of 0.15 *M* potassium phosphate buffer, pH 7.4. Incubation time was 8 min. (B) Incubations included 2 m*M* dithiothreitol, 62.5 μM [^{14}C]oleoyl-CoA, 0.2 mg microsomal protein, and varying amounts of BSA. Incubation time was 30 min. (C) Conditions were the same as those for A, except that all incubations contained 100 μM [^{14}C]oleoyl-CoA and incubation times varied from 4 to 60 min. (D) Incubations included 2 m*M* dithiothreitol, 80 μM BSA, 100 μM [^{14}C]oleoyl-CoA, and the indicated amounts of microsomal proteins. A different preparation of microsomes having consistently higher ACAT activity was used for the time course. Data are the means ± SD of triplicate incubations corrected in each case for a blank that contained boiled microsomes.

concentration of oleoyl-CoA, exclusion of BSA reduced cholesterol esterification to 19.6% of the value observed with 80 μM BSA (Fig. 1B). Exclusion of dithiothreitol had little effect on ACAT activity. Subsequent studies generally employed 50 or 100 μM [^{14}C]oleoyl-CoA, 80 μM albumin, and 2 m*M* dithiothreitol. A time course (Fig. 1C) indicated that esterification continued for at least 60 min, although the rate of esterification decreased somewhat after a short time. An incubation period of 8 min or less was chosen for most routine incubations. Under these conditions, esterification increased linearly with amount of microsomes up to approximately 0.4 mg of protein (Fig. 1D).

Modulation of ACAT activity in vitro. Since the acyltransferase reaction that is measured with [^{14}C]oleoyl-CoA utilizes endogenous microsomal cholesterol, it is possible that the rate of esterification is limited by membrane cholesterol content. Two experiments were conducted to determine whether addition of cholesterol to rat mammary gland microsomes could increase the yield of esterified cholesterol. Addition of cholesterol as a dispersion in acetone (25 μg cholesterol/0.2 mg microsomal protein) significantly increased the yield of esterified cholesterol as compared to control incubations to which only acetone was added (17.5 ± 0.6 vs 10.8 ± 0.5 pmole cholesteryl ester/30 min, $P < 0.001$). Since the amount of cholesterol incorporated into the microsomal membrane under these conditions was unknown, a second experiment was conducted in which mammary gland microsomes were incubated with serum lipoproteins as a source of free cholesterol, then reisolated by ultracentrifugation before incubation with [^{14}C]oleoyl-CoA to measure ACAT activity. Lipoproteins of $d \leq 1.063$ (very-low- and low-density lipoproteins) from a cholesterol-fed rabbit were utilized since these are enriched in both esterified and unesterified cholesterol and have been shown previously by Rothblat *et al.* (11) to be more efficient donors of cholesterol than are normal lipoproteins. Figure 2 shows that prior incubation with lipoproteins did enrich mammary gland microsomes with unesterified cholesterol, and that subsequent

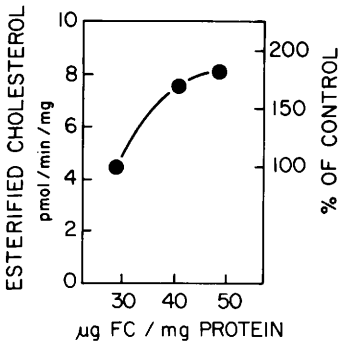

FIG. 2. Microsomal ACAT activity after enrichment of membranes with free cholesterol (FC) by exposure to serum lipoproteins. Points are the means of triplicates of 0.084–0.103 mg protein incubated for 8 min with 50 μM [^{14}C]oleoyl-CoA, 2 m*M* dithiothreitol, and 80 μM BSA.

synthesis of cholesteryl [^{14}C]oleate was also increased after this treatment. Enrichment from 29 to 41 μg cholesterol/mg microsomal protein resulted in 61% increase in ACAT activity, while further enrichment to 48 μg/mg protein resulted in an 81% increase. We may infer from these experiments that ACAT in normal rat mammary gland microsomes is not saturated with cholesterol since provision of extra cholesterol *in vitro* increased both microsomal cholesterol content and the rate of cholesteryl ester synthesis.

Experiments were also conducted to determine whether mammary gland ACAT activity can be modulated *in vitro* by polar steroids such as progesterone. Flint *et al.* (12) showed that progesterone can inhibit ACAT in ovarian extracts, a finding since confirmed in other microsomal systems (13–16). This inhibition by progesterone was reversed by removal of progesterone, but was not overcome by addition of exogenous cholesterol (14). It appears most likely that progesterone perturbs the microsomal membrane and, perhaps, limits the interaction of ACAT with cholesterol. Table I shows that addition of progesterone to rat mammary gland microsomes also inhibited cholesterol esterification: in the presence of 75 μ*M* progesterone, ACAT activity was reduced to only 7.3% of its control value whereas partial inhibition was obtained at a lower concentration of progesterone. Retinol (vitamin A_1), which has been shown to penetrate and expand phospholipid monolayers (17), also significantly reduced the esterification of microsomal cholesterol, as did 17-β-estradiol.

It has recently been found that various *N*-acylamides effectively reduce the esterification of cholesterol *in vitro* and in isolated microsomal membranes (18), as well as in cultured cells (19). We have asked whether these compounds also inhibit ACAT in mammary gland microsomes and, if so, whether the response of ACAT is similar to or different from that of other microsomal acyltransferases. Compound 58-035, a potent inhibitor of cellular cholesterol esterification, was added at concentrations from 2.5 ng to 1.28 μg per incubation (0.2 mg protein in 0.2 ml). At concentrations of 90 ng/incubation and greater, ACAT activity was reduced by at least 85% (Fig. 3). Compound 57-118 at 1.5 μg/incubation also inhibited ACAT by more than 92%

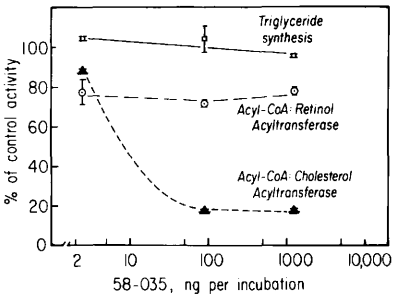

FIG. 3. Effect of compound 58-035 on mammary microsomal ACAT, acyl CoA:retinol acyltransferase, and triglyceride synthesis. Microsomes [0.2 mg protein having 7 μg free cholesterol per mg) from a rat on Day 4 of lactation were incubated for 15 min at 37°C with 58-035 (2.5 ng (5.5 pmole), 90 ng (197 pmole), or 1.28 μg (2.8 mmole)) prior to enzyme assay for 4 min. Control activities in microsomes to which only dimethylsulfoxide was added were: ACAT (▲), 36.5 pmole/min/mg protein; acyl CoA:retinol acyltransferase (⊙), 294 pmole/min/mg; and triglyceride synthesis (□), 75.4 pmole/min/mg. Points are the means (± range) of duplicate incubations.

(data not shown). Compound 58-035, at the same concentrations that inhibited ACAT activity, had no effect on the incorporation of oleate from oleoyl-CoA into triglycerides. When acyl-CoA:retinol acyltransferase was assayed under similar conditions, at least 70% of activity was retained at all concentration of 58-035 that were tested (Fig. 3). It appears, then, that these acylamides block ACAT nearly completely and selectively when added to isolated mammary gland membranes.

Discussion. Acyl-CoA:cholesterol acyltransferase, first described in rat liver membranes (20), has now been found in microsomes of numerous tissues and organs (21). However, ACAT has not been described previously in the lactating mammary gland. The presence of this enzyme suggests that esterified cholesterol in milk may indeed derive from the esterification of cholesterol within this tissue during milk fat biosynthesis.

The regulation of ACAT is still poorly understood. ACAT is an intrinsic membrane protein and most assays of its activity have utilized endogenous microsomal cholesterol as one substrate. Attention has been called to the availability of endogenous cholesterol as a potential factor limiting esterified cholesterol synthesis (11, 14, 21–26). Enrichment of cel-

lular membranes with cholesterol *in vitro* has been found to enhance esterified cholesterol synthesis (11, 14, 23–25) providing evidence that the membrane environment around ACAT does not always provide a saturating concentration of cholesterol. Using a solubilized, partially purified, reconstituted preparation of pig liver ACAT, Doolittle and Chang (26) have recently demonstrated dependence of esterified cholesterol production on the amount of cholesterol in the liposomal membrane. The present study has shown that the normal cholesterol content of the mammary gland microsomal fraction does not support a maximal rate of esterified cholesterol synthesis and that enrichment with free cholesterol *in vitro* can enhance ACAT activity significantly. Enrichment of liver microsomes with cholesterol and increased ACAT activities also have been found after cholesterol feeding (15). However, our experience with the lactating rat has been that feeding animals a diet containing 1% cholesterol–10% olive oil from days 4 to 14 of lactation produced very little change in either the cholesterol content of mammary gland microsomes or in their ACAT activity.[1] Thus, the membrane environment around mammary gland ACAT is likely to provide less than saturating concentrations of cholesterol over a considerable range of dietary cholesterol intakes.

Effects of exogenous steroids on ACAT in microsomal fractions from various tissues have been reported in the past few years. Inhibition by progesterone appears to be a consistent property of ACAT in ovary (12), placenta (27), liver (14), fibroblasts (13), and mammary gland. Other steroids at comparable concentrations have had lesser effects *in vitro.* Simpson and Burkhart (27) found partial (52%) inhibition of placental ACAT by 17-β-estradiol (100 μM), and our results with mammary gland membranes are quite similar. It is not yet known whether steroid hormones regulate ACAT activity acutely *in vivo* under physiological conditions, or whether their effects *in vitro* are due to the surface-active properties of these lipids at relatively high concentrations. Retinol, which is also surface active (17), had similar inhibitory effects on mammary gland, and liver,[1] microsomal ACAT.

Recent studies by Heider *et al.* (18) have shown the ability of the *N*-acylamide 57-118 to block esterified cholesterol synthesis in rabbit intestine and isolated microsomal membranes. The question of whether ACAT is selectively inhibited, or whether other fatty acyltransferases are also blocked, has been explored very recently using cultured hepatoma cells as well as isolated hepatoma or liver membranes (19). These studies have demonstrated essentially complete inhibition of ACAT under conditions where the esterification of glycerides, phospholipids, and retinol was reduced little, if at all. Although the mechanism of this selectivity is not yet understood, our observations permit the conclusion that membrane-bound ACAT is likely to be a distinct acyltransferase whose activity can be modulated independently from that of other lipid acyltransferases. Our comparison in the present report of ACAT, acyl-CoA:retinol acyltransferase, and triglyceride synthesis in rat mammary gland microsomes substantiates our previous results and supports the independence of cholesteryl ester and retinyl ester synthesis in this tissue.

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[1] A. C. Ross and J. F. Rowe, unpublished results.

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