

Radiation inactivation analysis of acyl-CoA:retinol acyltransferase and lecithin:retinol acyltransferase in rat liver

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Abstract Microsomes from liver and several other tissues esterify retinol through both fatty acyl-CoA-dependent and -independent reactions. Two activities, acyl-CoA:retinol acyltransferase (ARAT) and lecithin:retinol acyltransferase (LRAT) activities, have been characterized enzymatically but neither has yet been purified and characterized biochemically. We have used the method of radiation inactivation to determine the target sizes of ARAT and LRAT in intact microsomal membranes from rat liver. After exposure of frozen liver microsomes to ionizing radiation, the activity of ARAT decayed exponentially yielding a target size of 73 ± 18 kDa (mean \pm SD, $n = 6$). The activity of LRAT was assayed both by monitoring the esterification of retinol bound to the cellular retinol-binding protein (CRBP) and of solvent-dispersed retinol. With both assays a single exponential was observed with radiation doses of 9 to 150 Mrads. The slopes obtained with both LRAT assays were similar, yielding target sizes of 52 ± 10 kDa ($n = 10$) for the LRAT assay with CRBP-retinol and 56 ± 7 kDa ($n = 6$) for the LRAT assay with dispersed retinol. These target sizes did not differ from each other but were significantly smaller than that of ARAT. These data provide the first physical evidence of the independent entities which catalyze the ARAT and LRAT reactions of liver microsomes.—Ross, A. C., and E. S. Kempner. Radiation inactivation analysis of acyl-CoA:retinol acyltransferase and lecithin:retinol acyltransferase in rat liver. *J. Lipid Res.* 1993. 34: 1201-1207.

Supplementary key words vitamin A • microsomes • target size • acyltransferase

Esterification with fatty acid is central to the transport of retinol from the intestine and to its storage in liver and many extrahepatic tissues. Shortly after the uptake of chylomicron remnants into liver, intestinal retinyl esters are hydrolyzed and new hepatic retinyl esters are formed (1, 2). In the rat and most species that have been examined, approximately 95% of liver vitamin A is esterified with long-chain fatty acids (3), principally palmitate and stearate (1, 3).

In a number of tissues, two retinol esterification reactions have been identified. An acyl-CoA-dependent reaction was first identified in microsomes from the lactat-

ing rat mammary gland (4) and subsequently was found in intestine (5) and liver (6). Microsomes from these tissues readily esterified solvent-dispersed retinol and the reaction velocity was increased substantially in the presence of fatty acyl-CoA. This activity has commonly been referred to as acyl-CoA:retinol acyltransferase (ARAT). An acyl-CoA-independent reaction was subsequently identified in microsomes of intestine (7), liver (8, 9), and the retinal pigment epithelium of the eye (10). In contrast to liver ARAT, this reaction, referred to as lecithin:retinol acyltransferase (LRAT), did not require fatty acyl-CoA (7-11) but utilized fatty acid from phosphatidylcholine (8, 11) and also was able to esterify retinol bound to the cellular retinol-binding protein, CRBP (8, 9). The K_m of the LRAT reaction for retinol was lower than that of the ARAT reaction (8, 12) and was generally below the cytoplasmic concentration of CRBP-retinol (9). The capacity (V_{max}) of the ARAT reaction, however, was substantially greater (6, 12) than that of the LRAT reaction (8, 9, 12).

There is very little information about the characteristics of the enzymes that catalyze either the LRAT or ARAT reactions. Recently, LRAT activity from bovine retina (11) and rat intestine and liver microsomes (13) was solubilized with detergents; solubilized LRAT was dependent on exogenous phosphatidylcholine. The recovery of activity in the supernatant varied from 5 to 196% (13). Purification has not yet been reported and, therefore, no direct physical evidence yet exists concerning the similarities or differences in size or other properties between LRAT and ARAT or other retinol-esterifying enzymes.

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; ARAT, acyl-CoA:retinol acyltransferase; CRBP, cellular retinol-binding protein; LCAT, lecithin:cholesterol acyltransferase; LRAT, lecithin:retinol acyltransferase.

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The method of radiation inactivation has been applied to study the target size (functional mass) of a large number of membrane-associated receptors and enzymes (14, 15). Particular strengths of this analysis are, first, that proteins can be studied in their membrane environment without purification and undisturbed by solvents or dissociation from lipids; and, second, that the entire active population can be monitored. An analysis of the target size of an enzyme or receptor by radiation inactivation is based on the observations that the absorption of ionizing radiation by protein causes extensive breakage of covalent bonds leading to complete inactivation, and that the probability of collision is related, through the Poisson distribution, to the molecular mass of each independent target. The target size of enzymes or receptors predicted by radiation analysis has generally agreed very well with their size as later determined independently after isolation or cloning (15). In this work, we have used radiation inactivation to characterize the target sizes of the ARAT and LRAT activities in intact rat liver membranes.

MATERIALS AND METHODS

Materials for microsomal preparation and assay

The preparation of [³H]retinol and CRBP[³H]retinol for substrates has been described previously. Briefly, 15(N)-[³H]retinol (DuPont) was mixed with unlabeled retinol to a specific activity of approximately 2×10^6 dpm/nmol for the LRAT assay using dispersed retinol and approximately 6×10^4 dpm/nmol for ARAT. [³H]retinol was purified before each assay by aluminum oxide column chromatography (4). Cellular retinol-binding protein (CRBP) complexed with purified [³H]retinol was isolated as previously described (9) and used to assay LRAT. Other materials were those used previously in studies on the activity of ARAT and LRAT (6, 9, 12).

Quantitative analytical subcellular fractionation

To determine the subcellular and submicrosomal location of LRAT, rat liver was quantitatively separated by differential centrifugation into four main fractions. The analysis of these fractions for marker enzymes and retinyl palmitate hydrolase activity was described previously (16). The microsomal fraction was further separated by sucrose density gradient into four subfractions which were also characterized by the activities of marker enzymes (16). LRAT activity was assayed in each fraction, as described below using CRBP-[³H]retinol as substrate, under conditions that were linear with respect to incubation time and the amount of protein assayed for each sample.

Microsome preparation and irradiation

Microsomes were prepared from homogenates of fresh liver (1–3 pooled livers per radiation experiment) from

normal rats fed a stock diet by methods that have been described in detail previously (6, 17). Briefly, livers were homogenized in 0.25 M sucrose and subjected to centrifugation to remove the nuclear and the mitochondrial-lysosomal fractions. The postmitochondrial supernatant was then centrifuged at $\geq 105,000 g$ for 30 min to prepare the microsomal pellet which was resuspended in a Dounce homogenizer in 0.25 M sucrose. This crude microsomal fraction was washed by re-centrifugation under the same conditions. The washed microsomal pellet was resuspended in 0.25 M sucrose at ~ 10 – 20 mg protein/ml and was dispensed in aliquots of 0.5 ml in washed 2-ml glass ampules (Kimble, Thomas Scientific, Philadelphia). The samples were frozen rapidly in a dry ice/methanol bath and ampules were sealed quickly with an oxygen-gas torch. Samples did not melt during sealing, shipping, or radiation procedures.

Protein assays were conducted on the control microsome preparations and on each individual sample after irradiation² using the modified Lowry et al. (18) procedure described by Markwell et al. (19). With this assay, total protein concentration decreased very little as result of irradiation.

Microsomes in ampules were shipped in dry ice from Philadelphia to Bethesda where they were irradiated in the frozen state at -135°C as described previously (20). Control ampules (0 Mrad) were subjected to the same sealing and shipping procedures but were kept at -80°C during the irradiation of other ampules. All samples were returned to Philadelphia in dry ice and were kept at -70°C until the time of enzyme assay.

In certain experiments, controls were subjected to -135°C or to even lower temperature (liquid nitrogen, -196°C) without irradiation and were compared to other control ampules kept frozen either at -80°C while in Bethesda or continuously at -70°C in Philadelphia. These tests showed that freezing to low temperature without irradiation did not alter ARAT or LRAT activities. Other tests showed that the activities of ARAT and LRAT in fresh microsomes and microsomes frozen and thawed once (comparable to the samples assayed after irradiation) did not differ significantly. The activities of two enzymes, nonspecific esterase and alkaline phosphodiesterase, which are associated with rat liver endoplasmic reticulum and plasma membrane, respectively, were measured in several microsome preparations; both activities declined exponentially with radiation dose as expected (E. H. Harrison and E. S. Kempner, unpublished observations).

²We found that the Bradford (32) dye-binding assay (Bio-Rad) could not be used with irradiated samples because the amount of color produced fell significantly with the dose of radiation, presumably due to destruction of the "dye-binding unit" of protein structure.

Assays for ARAT and LRAT

Six individual microsomes preparations were assayed for both ARAT and LRAT activities and an additional four preparations were assayed for LRAT activity. Microsomal ARAT activity was assayed with a saturating [³H]retinol concentration [100–120 μM (6)] in the presence of exogenous palmitoyl-CoA (100 μM), 20 μM bovine serum albumin, and 5 mM dithiothreitol under conditions previously shown to be linear with incubation time and amount of protein assayed (6, 9, 12). The activity of LRAT was assayed with two forms of substrate: CRBP-[³H]retinol [5 μM final concentration, near saturation ($K_m = 2 \mu\text{M}$, ref. 12)] or [³H]retinol [2–5 μM final concentration dispersed in dimethylsulfoxide, final concentration of ≤5% (12)]. Exogenous phosphatidylcholine was not added because in separate experiments no difference was observed in the rate of the LRAT reaction by rat liver microsomes in the presence and absence of 50 μM dilauryl phosphatidylcholine. For both assays, microsomes in 0.15 M potassium phosphate buffer, pH 7.45, with 1–2 mM dithiothreitol were incubated with substrate for 4–5 min under conditions previously shown to be linear with microsomal protein addition. Each sample for ARAT and LRAT assay was tested in duplicate and the activity of a boiled control from the same ampule was subtracted from the mean activity of each sample (6). Boiled controls and controls without microsomes gave similar low values. Each experiment included control microsomes from two or three non-irradiated ampules whose activities were averaged together to obtain the control value, A_o , for each experiment. The fraction of activity remaining, A/A_o , was calculated for each radiation dose.

Radiation inactivation analysis

The surviving enzymatic activity (A) in samples irradiated with radiation dose, D (in Mrads), over the range of 9 to 150 Mrads was analyzed according to $A = A_o e^{-kD}$ using a least squares fit. The slope of the inactivation curve, k, gave the target mass, M (in kDa) according to $M = 1792 k$.

These equations take into account the temperature at which the irradiation was performed (–135°C).

Data are reported as the mean ± 1 SD for the number of independent experiments indicated. Statistical comparisons were made by a two-tailed unpaired *t*-test.

RESULTS

Subcellular localization of LRAT activity in rat liver

The microsomal localization of ARAT had been demonstrated in previous studies using quantitative subcellular fractionation (17). Although LRAT has been assayed in the crude membrane fraction of various tissues, quantitative studies of its subcellular localization have not been reported. Therefore, we first used analytical subcellular fractionation to determine the microsomal location of LRAT in rat liver and its distribution within membranes of the microsomal fraction. As is shown in **Table 1**, LRAT activity in the microsomal (P) fraction was enriched 3.3-fold as compared to the recovery of protein and was enriched 2.7-fold as compared to the LRAT specific activity in the whole homogenate (H). These data compare closely to those reported previously for enzyme markers for the P fraction, alkaline phosphodiesterase and glucose-6-phosphatase, that were measured on the same samples (16). No other fraction (nuclear, mitochondrial-lysosomal, or soluble) showed any enrichment in LRAT activity. When the P fraction was separated into four subfractions by sucrose density gradient centrifugation, essentially all (>98%) of the LRAT activity was in the fraction of highest density; over 90% of the activity of glucose-6-phosphatase, a marker of the rough endoplasmic reticulum, was found in the same subfraction.

Radiation inactivation of ARAT

Acyl-CoA:retinol acyltransferase was assayed by the conversion of dispersed [³H]retinol to [³H]retinyl ester in the presence of saturating concentrations of palmitoyl-CoA (6, 12). In irradiated microsomes, there was a decrease in measurable activity, but at very high doses

TABLE 1. Localization of LRAT activity in rat liver

Fraction	LRAT Activity	% of Recovery	Relative Specific Activity	
			% Activity/% Protein	Specific Activity of Fraction/Specific Activity of H
	<i>pmol retinyl ester/min/mg</i>			
Homogenate (H)	13.3	100.0		
Nuclear (N)	11.0	23.5	0.9	1.0
Mitochondrial-lysosomal (M-L)	0.5	0.8	0.1	0.1
Microsomal (P)	31.6	73.7	3.3	2.7
Soluble (S)	0.65	2.0	0.1	0.1

(above 180 Mrads) there was a small residual activity (approximately 1% of the unirradiated control) which was resistant to further radiation exposure. Similar observations have also been made with the enzyme acyl-CoA:cholesterol acyltransferase (ACAT) (21). As in that study, this residual activity was interpreted as a non-enzymatic background reaction that was subtracted from all samples. In unirradiated samples, the ARAT activity averaged 710 pmol/min per mg protein (Table 2). As shown in Fig. 1, the surviving enzymatic activity could be followed to very low levels ($<0.5\%$ of A_0 at 150 Mrads). A single exponential loss of activity is observed at ≥ 9 Mrads exposure. In six independent experiments, this simple exponential analysis yielded a y -intercept of approximately 0.75 and the slope gave a target size of 73 ± 18 kDa for ARAT activity. Thus, at least three-quarters of the original ARAT activity is due to an enzyme of 73 kDa.

Radiation inactivation of LRAT

The activity of LRAT was measured with two forms of retinol substrate. In ten experiments, microsomes were incubated with the CRBP-[3 H]retinol complex which was previously shown to be a substrate for LRAT and not ARAT (6, 9, 12). As in previous experiments with intact microsomes, no exogenous donor of fatty acid was added (see Methods) as endogenous phosphatidylcholine supported a maximal rate of retinol esterification. The LRAT activity averaged 69 pmol retinyl ester/min per mg of microsomal protein. In six experiments, LRAT activity was also measured with a low concentration (2–5 μ M) of dispersed [3 H]retinol under conditions that assay liver LRAT activity with little contribution from ARAT (12). In this assay, LRAT activity averaged 95 pmol retinyl ester/min per mg. Frozen samples for LRAT assays were irradiated to high radiation doses. It was possible to follow the activity to as little as 1% of the control value. With either CRBP-[3 H]retinol or dispersed [3 H]retinol as substrate, LRAT activity showed no radiation-independent background activity as seen for ARAT at high radiation exposures and no background correction was required. Fig. 2 shows the radiation inactivation of LRAT activity as measured with each of the substrates. As with ARAT

TABLE 2. Initial activities and target sizes of rat liver ARAT and LRAT

	ARAT	LRAT	
		CRBP-[3 H]Retinol	[3 H]Retinol
Activity (A_0) ^a	710 \pm 460 (6)	69 \pm 25 (9)	96 \pm 42 (6)
Target size (kDa)	73 \pm 18 (6)	52 \pm 10 (10)	56 \pm 17 (6)

^apmol retinyl ester/min per mg microsomal protein (n = number of independent assays).

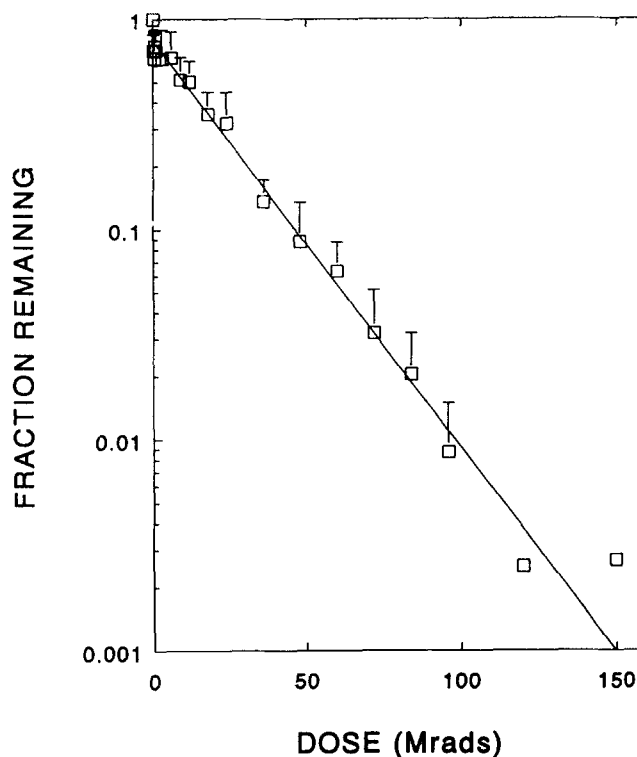


Fig. 1. Activity of ARAT as a function of radiation dose in six preparations of rat liver microsomes. Points show the mean \pm 1 SD except for doses of 120 and 150 Mrad which represent 1–2 points. The average size obtained from a least squares fit of doses (D) of 9–150 Mrads in each of the six experiments was used to obtain the curve $A/A_0 = 0.752e^{-0.0405D}$.

activity, there was a terminal slope at doses ≥ 9 Mrads at which the activity decayed exponentially. Similar curves were obtained in additional radiation experiments using microsomes that had been sealed in ampules gassed with nitrogen or to which 2 mM dithiothreitol was added before the ampules were sealed, whereas no decrease was found in unirradiated microsomes exposed to -196°C . For LRAT assayed with CRBP-[3 H]retinol (Fig. 2A), the simple exponential fit gave a y -intercept of 0.79 and a target size of 52 ± 10 kDa. The latter was significantly different ($P < 0.01$) from that for ARAT. The same analysis for LRAT activity assayed with dispersed [3 H]retinol (Fig. 2B), resulted in a y -intercept of 0.57 and a target size of 56 ± 7 kDa which is marginally different ($P = 0.06$) from the ARAT target size. For both substrates the extrapolated activity at zero dose was significantly less than that observed in the unirradiated controls. Nevertheless, most of the LRAT activity was due to enzymes with these target sizes.

An experiment was conducted to compare the K_m for retinol esterification in control and irradiated microsomes. The Michaelis constant, K_m , was determined in unirradiated microsomes and microsomes exposed to 18 Mrads (i.e., so that less than 50% of the control LRAT

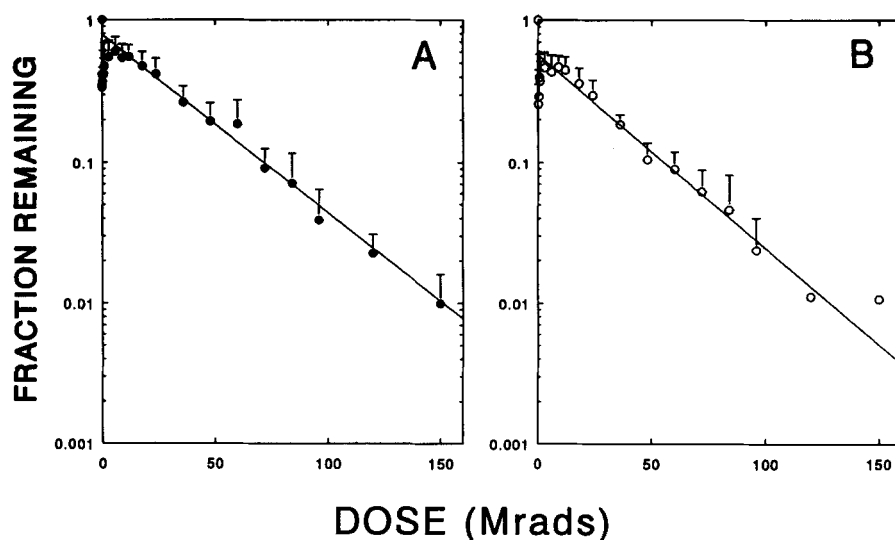


Fig. 2. Activity of LRAT in rat liver microsomes as a function of radiation dose. LRAT activity was assayed with CRBP- ^{3}H retinol in ten experiments (Fig. 2A) or with dispersed ^{3}H retinol in six experiments (Fig. 2B). Curves are from a least squares fit of doses (D) from 9–150 Mrads as described in Fig. 1. In Fig. 2A, $A/A_0 = 0.789e^{-0.0289D}$ and in Fig. 2B, $A/A_0 = 0.572e^{-0.0315D}$

activity remained). The K_m values of $1.8\ \mu\text{M}$ (0 Mrads) and $2.2\ \mu\text{M}$ (18 Mrads) were close to each other and both were within the range we have found previously with various microsomes preparations. This further supports the idea that a single LRAT reaction was measured across the range of radiation doses used in these studies.

DISCUSSION

Two major principles of radiation inactivation target analysis are fundamental to the present study: 1) direct damage to macromolecules by ionizing radiation occurs randomly, depending only on the mass of the molecule; and 2) no biological activity remains in a hit molecule, while un-hit molecules retain all original properties. In the simplest cases, these postulates predict that the surviving activity in irradiated samples will decrease as a simple exponential function of radiation dose, and the surviving molecules will be normal. With all three enzymatic assays reported here, a simple exponential loss of activity was observed in samples irradiated at ≥ 9 Mrads exposure. Each exponential curve extrapolated to $\sim 70\%$ of the original activity. Thus a small component of each activity is not defined in the present study. This could be due to some unknown factor which is expressed only in the un-irradiated samples or to a more complex situation. Further studies of these observations are in progress. In irradiated samples, we observed acyltransferase activity decreases to nearly 1% of control as a simple exponential function of dose, with no significant change in K_m . The data from >9 Mrad led to a target size of 52 ± 10 kDa

for LRAT (with CRBP- ^{3}H retinol), whereas the target size of ARAT was significantly greater, 73 ± 18 kDa. These represent the maximum size of protein structures, either one or several polypeptides, required for these acyltransferase activities.

The physical relationship of ARAT and LRAT, both located in the microsomal fraction of rat liver, had not previously been resolved. Based on the inactivation of these acyltransferases after radiation exposure, liver ARAT and LRAT differ significantly in target size. Other biochemical lines of evidence supporting two distinct activities include kinetic differences (12), the ability of alkylating agents to inhibit LRAT selectively (7, 12, 13), and differences in regulation in response to nutritional status (22). A number of arguments favor the hypothesis that LRAT is the enzyme responsible for the physiological esterification of retinol. These include a K_m for CRBP-retinol (8, 12) that appears appropriate for the concentration in liver cytosol (8, 9, 17); a pattern of retinyl ester products similar to the saturated *sn*-1 fatty acid of lecithin (8, 9, 13) and the retinyl esters of intact liver (3); and changes in rat liver LRAT activity, but not of ARAT, in response to changes in vitamin A status (22). Although the physiological role of liver ARAT is unclear, its higher capacity in comparison to LRAT implies it might function mainly when the influx of retinol is high or the binding capacity of CRBP is exceeded. Differences in the mechanism of retinol esterification among tissues was implied from a comparison of retinol esterification by microsomes from the lactating rat mammary gland and rat liver (12). In the mammary gland, in contrast to liver, LRAT activity was barely detectable in microsomes and the cyto-

solic CRBP concentration was very low, whereas ARAT activity was strong even in the presence of very low concentrations of palmitoyl-CoA (12).

The present study showed that LRAT, like ARAT, is enriched in microsomal membranes to the same extent as the enzyme markers glucose-6-phosphatase and 5'-nucleotidase (16). The activity of LRAT was further localized to the endoplasmic reticulum fraction of rat liver. This location is similar to that for other enzymes of lipid esterification such as ACAT (21, 23). Studies by Goodman, Huang, and Shiratori (1) of chylomicron retinyl ester metabolism had shown that intestinal retinyl esters are hydrolyzed and new hepatic retinyl esters are formed within a few hours of remnant uptake into liver. Harrison and Gad (16, 24) have partially characterized a retinyl ester hydrolase located in rat liver plasma membranes and have suggested that this may be an initial site of chylomicron retinyl ester metabolism. If so, and if the LRAT activity in the liver endoplasmic reticulum is responsible for the re-esterification of retinol, then the retinol molecule must move from the site of its formation in the plasma membrane to LRAT in the endoplasmic reticulum. Previously, we and others have shown that liver LRAT but not ARAT is able to recognize and esterify retinol associated with CRBP (8, 9, 12, 13). These data suggest a model in which one function of CRBP is to shuttle retinol from the plasma membrane to LRAT for esterification. This model is consistent with current thinking that CRBP serves both to control the concentration of unesterified retinol in cells and to direct ligand to specific enzymes of metabolism (25).

Similarities and differences between the esterification of retinol and cholesterol have been noted previously. For both lipids, the free alcohol form is maintained at a rather constant concentration in cells while the esterified form varies and generally represents reserves or excess lipid deposits. The intracellular esterification of cholesterol is catalyzed by ACAT (26), while the esterification of plasma lipoprotein free cholesterol is catalyzed by lecithin:cholesterol acyltransferase (LCAT) (27). Although both ARAT and ACAT are fatty acyl-CoA-dependent enzymes, their activities are distinct by biochemical and physical criteria. The activity of ACAT in both microsomes and intact cells was blocked selectively by inhibitors that did not reduce ARAT activity (28). The target size of rat liver ACAT, 170–180 kDa as determined by radiation inactivation (21), is also distinct from that of liver ARAT (~73 kDa). Neither of these activities has been solubilized and therefore the extent to which other similarities or differences exist is not known.

Both LRAT and LCAT are CoA-independent enzymes that transfer the *sn*-1 or *sn*-2 fatty acids from phosphatidylcholine to retinol or cholesterol, respectively. The activities of both LRAT and LCAT are inhibited by alkylating reagents such as phenylmethylsulfonyl fluoride (7, 12, 13,

27, 29) but differences in their sensitivity to other inhibitors implies that their catalytic centers differ with respect to active serine versus cysteine residues (13). A major difference between LRAT and LCAT is the location in which each is active. Although LCAT is synthesized in liver (30), it is thought to esterify cholesterol only in plasma. In contrast, LRAT is tightly associated with the liver endoplasmic reticulum and, as far as is known, is not secreted. The target size we have determined for LRAT in these studies (52 ± 10 kDa) is close to the molecular mass of LCAT (47 kDa for the protein component³) which is known precisely from purification and molecular cloning (29, 31). Based on similarities in size and in some of their catalytic properties, is it tempting to speculate that there may be additional relationships between LRAT and LCAT, as well as interesting distinctions that account for their cellular trafficking to the endoplasmic reticulum or to the secretory pathway, respectively. ■

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³The approximate molecular weight for LCAT of ~63,000 *M*, includes 25% of carbohydrate (31). Only the protein component of glycoproteins is detected by radiation inactivation (33).

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