Functional sizes of hepatic enzymes of cholesteryl ester metabolism determined by radiation inactivation

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Abstract Cellular cholesteryl ester metabolism is regulated largely by the balance between intracellular esterification catalyzed by acyl-CoA:cholesterol acyltransferase and cholesteryl ester hydrolysis catalyzed by the cholesteryl ester hydrolases. The hydrolases include both cytosolic and membrane-associated activities; acidic and neutral activities have been described in both compartments. Esterification via the acyltransferase is membrane-associated. Neither the acyltransferase nor the membrane-associated hydrolases have been purified and characterized, and little is known about their genes. Thus, nothing is known about their sizes or structures. Radiation inactivation was used to determine the functional sizes in situ of acyl-coenzyme A:cholesterol acyltransferase, fatty acyl-CoA hydrolase, and acidic and neutral membrane-associated cholesteryl ester hydrolase activities. The functional $M_r \pm$ SD of the acyltransferase was 213 ± 35 kD; for the acidic membrane-associated hydrolase, 48 ± 2 kD; for the neutral membrane-associated hydrolase, 94 ± 15 kD; and for the fatty acyl-CoA hydrolase, 65 ± 15 kD. Monoexponential curves were observed in all cases using radiation exposures that inactivated enzyme activities to ≤ 10% of control values. Substrate specificity and inhibition studies suggested that the active sites of the acyltransferase and fatty acyl-CoA hydrolase were different, supporting the concept that the hydrolase is not part of the functional unit required for cholesterol esterification.—**Erickson, S. K., S. R. Lear, and M. J. McCreery.** Functional sizes of hepatic enzymes of cholesteryl ester metabolism determined by radiation inactivation. *J. Lipid Res.* 1994. **35:** 763–769.

Supplementary key words acyl-coenzyme A:cholesterol acyltransferase • acidic cholesteryl ester hydrolase • neutral cholesteryl ester hydrolase • acyl-coenzyme A hydrolase

The regulation of hepatic cholesteryl ester metabolism is accomplished in part by the balance between intracellular cholesterol esterification catalyzed by the enzyme acyl-coenzyme A:cholesterol acyltransferase (EC 2.3.1.26, ACAT) and cholesteryl ester hydrolysis catalyzed by the cholesteryl ester hydrolases (EC 3.1.1.13, CEH).

ACAT is localized to the endoplasmic reticulum (ER) with enrichment in the rough ER (1, 2). It is an intrinsic ER protein. Although ACAT has been solubilized (3–5), it has not been purified. Thus, little is known about its size or structure.

The CE hydrolases include both cytosolic (soluble) and membrane-associated activities (6). The cytosolic enzymes include an acidic activity believed to be of lysosomal origin (7) and a neutral activity (8) which may include a separate bile salt-dependent enzyme (9, 10). A number of the cytosolic enzymes have been purified and studied in some detail (11–14). The soluble human lysosomal CEH has been cloned (15). A liver neutral cytosolic CEH has been purified and characterized by Ghosh et al. (16, 17).

Little is known about the membrane-associated cholesteryl ester hydrolases. At least two membrane-associated activities have been described, one with maximal activity at pH 4.5, probably of endosomal origin (18), and an activity with a pH maximum about 8.0 thought to be associated with the ER (8). A neutral CEH has been reported to be enriched in the rough ER (19). Both neutral and acidic CEH activities also have been described in rat liver plasma membrane and endosomes (18, 20). A neutral membrane-associated CEH has been solubilized (21), but none of the membrane-associated CEHs have been purified, and little is known about their sizes and structures or whether they are similar to the cytosolic CE hydrolases. The gene for a microsomal carboxylesterase has been isolated (22); however, the relationship of this enzyme to the membrane-associated cholesteryl ester hydrolases is unknown.

Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; CEH, cholesteryl ester hydrolase; ER, endoplasmic reticulum; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; PMSF, phenylmethylsulfonyl fluoride; PHMB, *p*-hydroxymercuribenzoate; ACH, acyl-CoA hydrolase; CE, cholesteryl ester.

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Most techniques used to determine the size of proteins require that they be in a soluble form. Thus, intrinsic membrane proteins must first be solubilized and, in most cases, purified extensively before their size can be determined. Solubilization alone often leads to loss of activity, loss of native conformation of the protein, proteolytic clipping, separation of noncovalently linked subunits, or nonspecific association of extraneous proteins. Thus, purification of solubilized intrinsic membrane proteins tends to intensify the problems further. Obtaining information on the size and native form of intrinsic membrane proteins is a formidable task. For these reasons, the technique of radiation inactivation (23) was chosen to probe the functional sizes in situ of the membrane-associated liver enzymes of cholesteryl ester metabolism, ACAT, and the CEHs. This technique has been used successfully in the past to study a number of liver microsomal enzymes (24–28).

EXPERIMENTAL PROCEDURES

Materials

Animals. Male Sprague-Dawley rats, 180–200 g (Bantin and Kingman, Newark, CA) were housed under reverse illumination (lights on 3 PM, lights off 3 AM). They were allowed free access to Purina rat chow and water.

Chemicals. [1,2-^{3}H]cholesteryl oleate (60–100 mCi/mmol), [^{14}C]3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) (40–60 mCi/mmol), and [^{3}H]mevalonate (1–5 Ci/mmol) were from New England Nuclear (Boston, MA). [1-^{14}C]oleoyl coenzyme A (50–60 mCi/mmol) and [1-^{14}C]palmitoyl coenzyme A (50–60 mCi/mmol) were from Amersham Corp. (Arlington Heights, IL). Cholesterol, egg lecithin, cholesteryl oleate, oleoyl coenzyme A, glucose-6-phosphate, palmitoyl coenzyme A, NADPH+, and glucose-6-phosphate dehydrogenase (EC 1.1.1.49, bakers yeast, Type VII) were from Sigma Chemical Co. (St. Louis, MO). All other reagents were analytical grade.

Preparation of liver microsomes and their irradiation and assay

For each experiment, liver microsomes were prepared from pooled livers of three rats as described previously (29). The washed microsomes were suspended in buffer containing 0.1 M sucrose, 0.05 M KCl, 0.03 M EDTA, 0.04 M KH_2PO_4, pH 7.2, at approximately 10 mg protein/ml. Glucose-6-phosphate dehydrogenase was added and 0.5 ml/vial was transferred into 2-ml glass vials. The vials were frozen at −70°C except during radiation exposure which took place at −135°C. High energy electrons were used to deliver predetermined doses of radiation to the samples as described previously (24–28). HMG-CoA reductase was assayed as described previously (30). ACAT activity was determined either as described previously (2) or in the presence of cholesterol-phosphatidylcholine liposomes as described previously (31), using a 2-min assay time. Cholesteryl ester hydrolase activities were determined at pH 4.5 and at pH 8.0 based on the method of Brecher et al. (32) as described previously (30). Fatty acyl CoA hydrolase was assayed according to Berge (33), using [1-^{14}C]palmitoyl CoA, the preferred substrate for the enzyme.

All assays were in the linear range with respect to protein, time, and substrate concentration (data not shown). Thus, the activities were not substrate-limited over the assay period. Freezing and thawing the microsomes had no detectable effect on any of the enzyme activities. All assays were run in triplicate. Enzyme activities in irradiated samples were normalized to the activities determined in unirradiated samples. Inactivation curves were plotted as logarithm of the surviving fraction of activity as a function of radiation dose. In all cases the data most closely fit a single exponential. Curves were analyzed by least squares analysis constrained to 1.0 at zero radiation dose. R values for these curves were in all cases >0.95, and in most cases, were 0.99. Target sizes were calculated (34) and expressed as the average ± SD.

RESULTS AND DISCUSSION

In order to validate the techniques used in this study, glucose-6-phosphate dehydrogenase was added to the microsomes as an internal standard. The target size obtained for this enzyme was 114 ± 19 kDa, in agreement with previously published values (24, 27, 35) indicating that the experimental conditions had not introduced radiation artifacts. Estimates of the functional size of HMG-CoA reductase, an intrinsic ER enzyme, in these same samples averaged 239 ± 12 kD which is in the range previously reported (24, 25). Thus, it is unlikely that gross microsome-specific artifacts had been introduced by the procedure.

Cholesterol esterification was estimated by measuring ACAT activity using two different assay protocols. The first assay used the endogenous microsomal cholesterol as substrate, and the second used exogenous cholesterol in the form of cholesterol-phospholipid liposomes. Both assays used [^{14}C]oleoyl coenzyme A as the second substrate. Radiation inactivation curves for ACAT activity assayed by the two methods yielded similar results (**Fig. 1**). Using endogenous cholesterol the functional size obtained was 224 ± 41 kD, and using exogenous cholesterol, the functional size was 193 ± 25 kD (Fig. 1). These two values were not statistically significantly different and suggest that a functional unit of approximately 200 kD is required for expression of ACAT activity in liver microsomes from normal adult rats. This value is similar to that (170–180

kD) reported by others (36) using a different assay method. This unit may include one or more subunits which may be of the same or different size. It is a measure of the minimal size required for function. It represents the protein moiety and does not include any carbohydrate that might be present (37). Thus, this is an estimate of the functional size of ACAT in situ and provides an approximation of the mass of the structure required for expression of this enzyme activity in the intact cell.

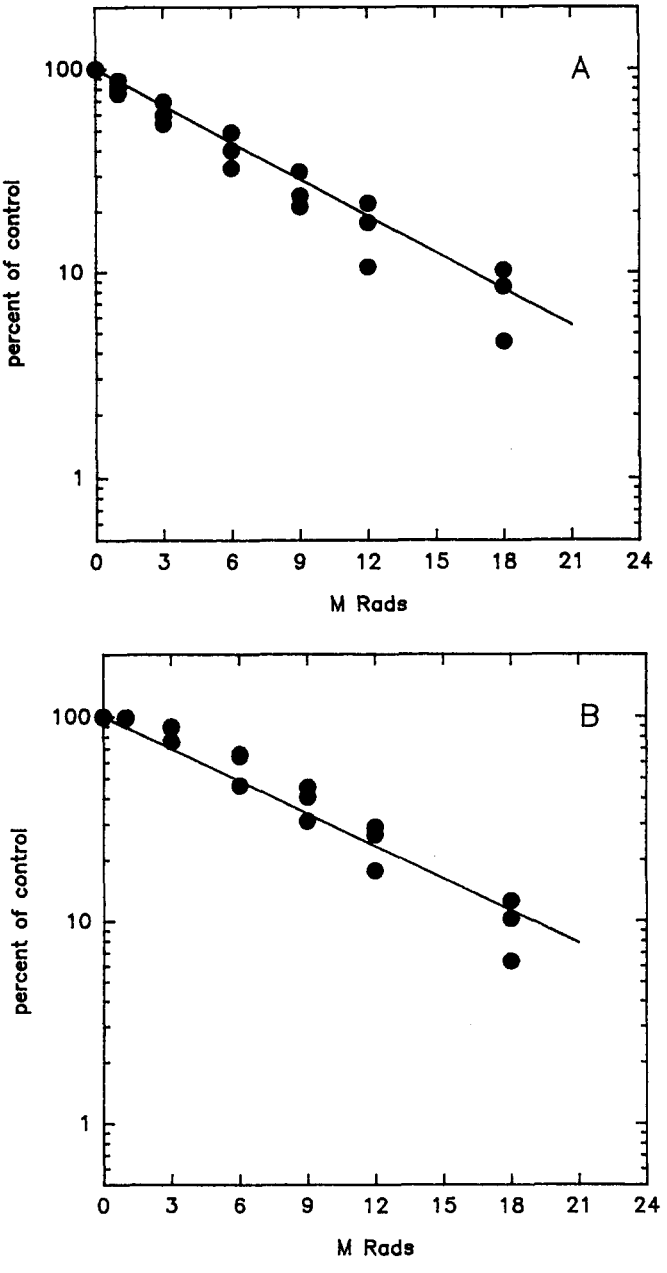

Fig. 1. Loss of ACAT activity from rat liver microsomes as a function of radiation dose. Liver microsomes were prepared and assayed as described in Methods. For each experiment, microsomes were prepared from livers pooled from three rats. Initial ACAT activities were 150–200 pmol CE/min per mg protein using the endogenous cholesterol substrate (panel A) and 600–700 pmol CE/min per mg protein using exogenous cholesterol substrate delivered as cholesterol–phosphatidylcholine liposomes (panel B). Each point is the average of triplicate determinations from each of three representative independent experiments.

There have been two recent reports describing cloning of a gene for ACAT (38, 39). Based on the gene structures, M_r values of about 60 kD were estimated. These data, together with the radiation inactivation data, suggest ACAT may be functionally active as a trimer.

It has been suggested (36) that the enzyme fatty acyl-CoA hydrolase (EC 3.1.2.2) is an intrinsic part of the ACAT functional unit. This thesis was derived from several observations. It was found that radiation inactivation of acyl-CoA hydrolase activity followed a complex, nonlinear decay curve. At low radiation doses, ACH activity increased above control levels, whereas a more characteristic decrease in enzyme activity occurred at higher doses. By first assuming that a larger protein inhibited ACH, functional unit sizes were calculated for both ACH and for the larger inhibitor protein by fitting the data to a model having two exponentials. Average M_r values for fatty acyl-CoA hydrolase of 46 ± 15 kD and 180 ± 92 kD for the inhibitor protein were calculated (36). It was reported that exogenous cholesterol inhibited ACH activity while simultaneously stimulating ACAT; however, it was also reported in the same study that ACH was increased by this treatment (36). Nevertheless, based on these data, the authors concluded that ACH was the acyl-CoA binding component, and that the larger subunit inhibiting ACH was the cholesterol-binding component of ACAT (36).

In contrast to the above results, upon assaying the activity of ACH in liver microsomes after radiation exposure, we found that activity decreased monotonically with dose and could be fitted easily to a single exponential (**Fig. 2**). We observed an increase in enzyme activity above unirradiated control with no radiation exposure. The M_r of the functional unit of fatty acyl-CoA hydrolase using this technique was 65 ± 15 kD. In a limited series of experiments, radiation inactivation of ACH assayed with oleoyl-CoA was compared with that using palmitoyl-CoA. The functional M_r with palmitoyl-CoA was 58 ± 10 kD and with oleoyl-CoA, 41 ± 10 kD (n = 2). Both values were in good agreement with an M_r of about 59 kD reported previously by Berge (33) based on analysis by SDS gel electrophoresis, gel chromatography, and equilibrium sedimentation of the purified protein solubilized from rat liver.

Results of radiation inactivation target size analysis of fatty acyl-CoA hydrolase in the present study led us to investigate the proposed model (36) that ACH is the acyl-CoA binding component of ACAT.

To gain further insight into the validity of this model, the nature of the active site(s) of fatty acyl-CoA hydrolase and ACAT was studied. First, substrate preference of the

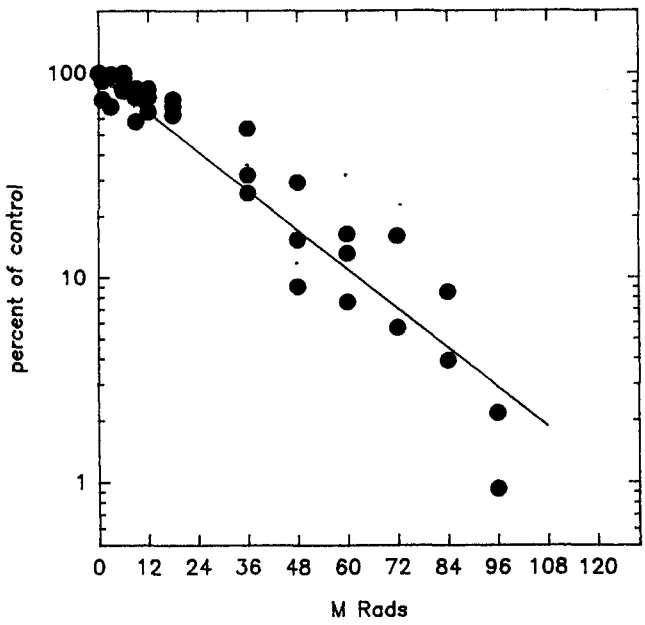

Fig. 2. Loss of fatty acyl-CoA hydrolase activity from rat liver microsomes as a function of radiation dose. Liver microsomes were prepared and assayed as described in Methods. For each experiment, microsomes were prepared from livers pooled from three rats. Initial fatty acyl-CoA hydrolase activity was 74.3 ± 16.0 nmol palmitate released/min per mg protein. Each point is the average of triplicate determinations for each of three independent experiments.

two enzymes was reexamined. If ACH is the fatty acyl-CoA binding component of ACAT, one would expect that the two enzymes would show similar substrate specificity. However, fatty acyl-CoA hydrolase is reported to show a preference for palmitoyl-CoA over oleoyl-CoA (33, 41) while ACAT shows a preference for oleoyl-CoA (40). These preferences were confirmed (**Table 1**). ACAT was 3 times more active with oleoyl-CoA as substrate assayed under the same conditions, while ACH was 5 times more active with palmitoyl-CoA. Thus, these data suggest that the two fatty acyl-CoA binding sites differ.

Next, sensitivity of the two enzymes to inhibitors was examined (**Table 2**). If the same active site is involved, response to one or more inhibitors would be expected to be similar. Further, if ACH is required for ACAT activity, its inhibition should result in ACAT inhibition.

TABLE 1. Fatty acyl-CoA preference of rat liver microsomal enzymes ACAT and fatty acyl-CoA hydrolase

Enzyme	+ Palmitoyl CoA	+ Oleoyl CoA
ACAT, pmol CE/min/mg protein	60 ± 5	180 ± 20
Acyl-CoA hydrolase, nmol FA/min/mg protein	74 ± 16	15 ± 3

Microsomes were prepared and assayed as described in Methods. The results are the means of values from three independent preparations each assayed in triplicate.

TABLE 2. Effect of inhibitors on rat liver microsomal ACAT and fatty acyl CoA hydrolase activities

Inhibitor	ACAT	Fatty Acyl-CoA Hydrolase
	% control	
PMSF, M		
10^{-9}	100	100
10^{-6}	100	100
10^{-3}	100	50
PHMB, M		
10^{-9}	100	100
10^{-6}	100	100
10^{-4}	10	250
Deoxycholate, M		
10^{-9}	100	100
10^{-6}	100	100
10^{-3}	10	100

Microsomes were prepared and assayed as described in Methods. Inhibitors were added at the concentrations indicated. The results are expressed as % change from control values. The results are representative of values from three independent experiments each assayed in triplicate.

Phenylmethylsulfonyl fluoride (PMSF) inhibited fatty acyl-CoA hydrolase by 50% but had no effect on ACAT activity at 10^{-3} M. *p*-Hydroxymercuribenzoate (PHMB) stimulated fatty acyl-CoA hydrolase activity by 250% but inhibited ACAT activity 90% at 10^{-4} M. At 10^{-3} M deoxycholate (DOC), the activity of fatty acyl-CoA hydrolase remained unchanged, but ACAT activity decreased by 90%. Taken together, the above data support the thesis that ACH and ACAT have different active sites and likely are separate and independent enzymes.

The effects of cholesterol-phospholipid liposomes on ACH and diacylglycerol-acyltransferase (DGAT, EC 2.3.1.20), a microsomal enzyme responsible for triglyceride synthesis that also uses fatty acyl-CoA as substrate (27), were examined. Both ACH and DGAT activities were inhibited about 80% by addition of phospholipid-cholesterol liposomes while ACAT was stimulated 4- to 6-fold (data not shown). Thus, the inhibitory effect of cholesterol was not specific to ACH, but rather affected both fatty acyl-CoA utilizing microsomal enzymes, suggesting that affinities for fatty acyl-CoA may have been altered in the three enzymes.

The functional sizes of the membrane-associated enzyme(s) responsible for cholesteryl ester hydrolysis were estimated by determining the activity of the hydrolases present in the microsomal fraction that were active at pH 4.5 and at pH 8.0. The acidic membrane-associated CEH activity is reported to be present in endosomes and plasma membranes (19, 20). The neutral membrane-associated CEH activity is reported resident in the endoplasmic reticulum, endosomes, and plasma membrane (18–20). Radiation inactivation curves of these two activities yielded a functional size of 48 ± 2 kD for the pH 4.5

(acidic) activity and of 94 ± 15 kDa for the pH 8.0 (neutral) hydrolase (**Fig. 3**). Both curves followed straightforward, first-order kinetics.

These radiation inactivation studies suggest that two different size functional units are required for expression of the membrane-associated acidic and neutral cholesteryl ester hydrolases. As these membrane-associated CEH activities have never been purified, these functional sizes obtained by radiation inactivation are the first approximation of the molecular masses required for their activity in situ. They likely reflect the minimal sizes of the protein structures in the intact cell that are required for expression of these hydrolase activities. The functional units might be single peptides or consist of multiple subunits.

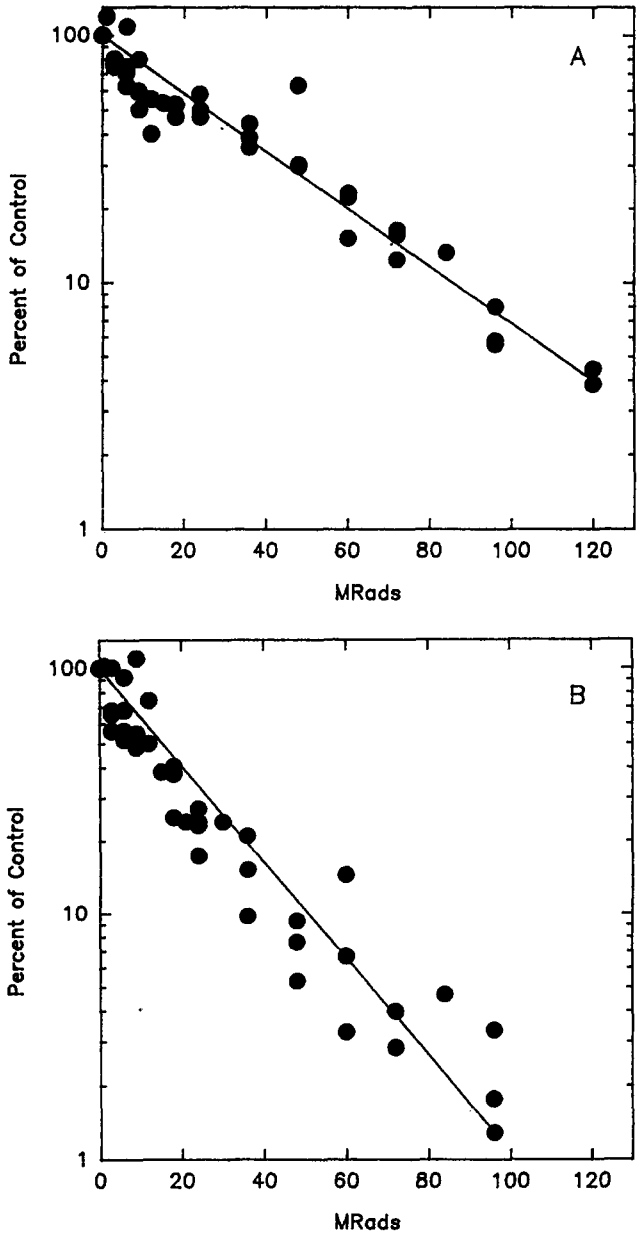

Fig. 3. Loss of cholesteryl ester hydrolase activities from rat liver microsomes as a function of radiation dose. Liver microsomes were prepared and assayed as described in Methods. For each experiment microsomes were prepared from livers pooled from three rats. Initial CEH activities were 0.2–0.4 pmol oleate released/min per mg protein at pH 4.5 (panel A) and 1.5–2.0 pmol oleate released/min per mg protein at pH 8.0 (panel B). Each point is the average of triplicate determinations from each of three representative independent experiments.

TABLE 3. Summary of functional molecular weights of internal standards, enzymes of cholesteryl ester metabolism, and fatty acyl CoA hydrolase as determined by radiation inactivation of rat liver microsomes

Enzyme	Functional Molecular Mass, kD
Glucose-6-phosphate dehydrogenase (7)	114 ± 19
HMG-CoA reductase (7)	239 ± 12
ACAT (endogenous cholesterol substrate, 7)	224 ± 41
ACAT (+ cholesterol-phospholipid liposomes, 4)	193 ± 25
CEH, pH 4.5 (4)	48 ± 2
CEH, pH 8.0 (4)	94 ± 15
Fatty acyl CoA hydrolase (3)	65 ± 15

Rat liver microsomes were prepared, glucose-6-phosphate dehydrogenase (internal standard) was added, the microsomes were irradiated, and enzymes were assayed as described in Methods. HMG-CoA reductase was assayed as a second internal standard reflecting general microsomal response to radiation. Values are the means ± SD. The number of individual experiments is in parentheses. For each experiment microsomes were prepared from the pooled livers of three rats.

Little is known about the relationship of the membrane CEHs with one another or to the cytosolic CEHs. Some of the cytosolic (or soluble) cholesteryl ester hydrolases have been purified. The cytosolic acidic CEH (lysosomal) purified from human liver has been reported to have an M_r of 29 kD on SDS gel electrophoresis and about 125 kD in the presence of Triton X100 (14), while that from human fibroblasts had M_r = 47–49 kD both by SDS gel electrophoresis and gel filtration (11). Although the radiation inactivation functional size of the rat liver membrane-associated acidic CEH activity at approximately 49 kD reported here is similar to that of the cytosolic enzyme isolated from human skin fibroblasts (11), relationship of the two activities is unclear. The soluble enzyme is believed to be of lysosomal and late endosomal origin (19), while the membrane-associated enzyme has been recovered in the plasma membrane and endosomal compartments (19, 20). However, the similar molecular weight values suggest that the protein responsible for the membrane-associated acidic activity may be related to the acidic cytosolic or soluble enzyme.

The M_r of a cytosolic neutral bile salt-stimulated CEH from rat liver analyzed by SDS gel electrophoresis and immunoblotting was about 67 kD (14). The M_r of a neutral rat liver cytosolic CEH shown to be immunologically

distinct from the bile salt-stimulated CEH was about 66 kD (17). As the functional size of the membrane-associated neutral activity was approximately 94 kD, the membrane enzyme likely is different from both hepatic neutral cytosolic enzymes.

A possible candidate for the membrane-associated cholesteryl ester hydrolase activity in rat liver is the microsomal nonspecific carboxylesterase. This activity has an M_r = 59 kD (22). However, the functional M_r of approximately 94 kD for the microsomal neutral CEH reported here suggests that CEH may be a different enzyme. Thus, the microsomal membrane-associated neutral CEH activity appears to have a unique functional size, suggesting it is distinct from other neutral CEH activities described to date.

The marked differences in the functional sizes for the CEH activities and ACAT (**Table 3**) also suggest it is unlikely that the same functional units are involved in expression of these enzymes, and that, in all likelihood, the different activities represent distinct proteins and gene products.

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