Cholesterol Metabolism in the Rat Lactating Mammary Gland: The Role of Cholesteryl Ester Hydrolase

Kathleen M. Botham*
Department of Veterinary Basic Sciences, The Royal Veterinary College, London, NW1 OTU, United Kingdom

An acid cholesteryl ester hydrolase activity associated with a fraction containing mitochondria and lysosomes from rat lactating mammary glands was found to have a pH optimum of 5.0. Its sedimentation pattern was closely related to that of the lysosomal enzyme markers acid phosphatase and β-glucuronidase, suggesting that the activity is associated with the lysosomes. The enzyme was strongly inhibited by Cu^{2+}, but was inhibited little by other divalent metal ions. Acid cholesteryl ester hydrolase activity was almost completely abolished by *p*-hydroxymercuribenzoate, but this effect was reversed in the presence of an equimolar concentration of reduced glutathione (GSH), indicating that the enzyme requires free sulfhydryl groups for activity. These properties are similar to those of acid, lysosomal cholesteryl ester hydrolases found in other tissues. Acid cholesteryl ester hydrolase activity was 8–14 fold higher in mammary tissue from lactating as compared to virgin rats. Neutral cholesteryl ester hydrolase activities associated with the microsomal and cytosolic subcellular fractions were also increased in lactating glands, but to a lesser extent. In addition, a 2-fold increase in the activities of both the acid and microsomal neutral enzymes was seen during the first few days of lactation, while the cytosolic neutral activity remained constant. These results suggest that mammary gland cholesteryl ester hydrolases have a role in the regulation of cholesterol metabolism in mammary cells, and in the provision of cholesterol for secretion into milk. *Lipids 26,* **901–906 (1991).**

Cholesterol, an important component of milk fat, is secreted into milk during lactation in both esterified and unesterified form. Approximately 20–40% of milk cholesterol originates from *de novo* synthesis within the mammary gland, while the remaining 60–80% is derived from plasma lipoproteins (1–3). Cholesterol from both these sources, however, may be esterified and stored in the mammary gland as cholesteryl esters (4,5), which may in turn be hydrolyzed to unesterified cholesterol when required. In other tissues the immediate fate of lipoprotein cholesteryl esters after uptake is hydrolysis by an acid cholesteryl ester hydrolase located in the lysosomes (6). By contrast, intracellular stores of cholesteryl ester are hydrolyzed by enzymes with pH optima near neutral, located in the microsomal and soluble cell fractions (7,8). As both these types of cholesteryl ester hydrolase have regulatory roles in intracellular cholesterol metabolism in some tissues (6,7,9), similar enzymes in the mammary gland may play an important part in determining the cholesterol content of milk by regulating the amount available for secretion.

We have shown previously that the rat lactating mammary gland contains an acid cholesteryl ester hydrolase associated with the mitochondrial/lysosomal subcellular fraction, and two neutral activities found in the microsomal and cytosolic compartments (10). Tawil *et al.* (11) have also found cholesteryl ester hydrolases with pH optima near neutral in rat mammary tissue. Studies of the microsomal and cytosolic enzymes provide evidence for modulation of their activity by phosphorylation-dephosphorylation (12), suggesting that these enzymes are under hormonal control and have a regulatory role in mammary gland cholesterol metabolism.

In the current work, the properties of the acid cholesteryl ester hydrolase found in lactating glands in our earlier report (10) were investigated, and the activities of the acid and neutral enzymes in non-lactating tissue were compared to those of lactating tissue. In addition, changes in the activities of the three cholesteryl ester hydrolases, which occur during the early lactation period, were examined.

MATERIALS AND METHODS

Cholesteryl oleate, phosphatidylcholine (type XI-E), oleic acid, sodium taurocholate, *p*-hydroxymercuribenzoate (sodium salt), phenylmethylsulfonyl fluoride, glutathione (reduced form) (GSH), bovine serum albumin (essentially fatty acid free) and 4-morpholino-ethanesulfonic acid (MES) (free acid and sodium salt) were obtained from Sigma (Poole, Dorset, United Kingdom). Cholesteryl [1-^{14}C]oleate and [1-^{14}C]oleic acid were supplied by Amersham International (Aylesbury, Bucks., United Kingdom).

Animals. Abdominal and inguinal glands were excised from lactating Wistar rats 2–9 days post-partum, or from virgin rats 15–20 weeks old. The glands were cleaned of fat and connective tissue, finely minced with scissors and homogenized using a glass Teflon homogenizer in 5 vol of Tris-HCl buffer pH 7.2 (50 mM), containing 0.25 M sucrose (homogenizing buffer).

Preparation of subcellular fractions. The homogenate was centrifuged at 800 × *g* for 10 min (4°C) in a swing-out rotor and the floating fat layer was removed and discarded. The supernatant was then centrifuged at 12,000 × *g* for 10 min (4°C) using a fixed angle rotor. After careful removal of the floating fat layer and the supernatant, the pellet was washed with 5 mL homogenizing buffer and resuspended in MES buffer pH 5.0 (100 mM). This fraction containing the mitochondria and lysosomes was used in all experiments with the acid cholesteryl ester hydrolase, except where indicated otherwise. Microsomal and cytosolic subcellular fractions were prepared from the 12,000 × *g* supernatant as before (12). For partial separation of mitochondrial and lysosomal enzyme activities in some experiments, the supernatant from the first centrifugation (800 × *g*) was centrifuged sequentially at 2,500 × *g*, 8,000 × *g* and 12,000 × *g* (10 min, 4°C) using a fixed-angle rotor, and the resulting pellets were washed and resuspended as described above.

Assay of cholesteryl ester hydrolase. Cholesteryl ester hydrolase activities were determined by measuring the

*To whom correspondence should be addressed at Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College St., London, NW1 OTU, United Kingdom.

Abbreviations: CEH, cholesteryl ester hydrolase; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione (reduced form); MES, 4-morpholino-ethanesulfonic acid.

release of [1-^{14}C]oleic acid from cholesteryl [1-^{14}C]oleate. The substrate was used as a mixed micelle with phosphatidylcholine and sodium taurocholate in molar ratios of 1:4:2 respectively, essentially as described by Hajjar *et al.* (13). Cholesteryl [1-^{14}C]oleate (185 KBq, 1.92 GBq/mmol) was added to a toluene solution containing phosphatidylcholine (57.6 μmol) and non-radioactive cholesteryl oleate (14.4 μmol) and the solvent was removed under a stream of nitrogen. Lipids were then resuspended in 5 mL Tris-HCl buffer pH 7.0 (50 mM) containing sodium taurocholate (28.8 μmol, 5.76 mM) and sonicated using a 0.5-inch horn for a total time of 30 min at 46°C. The resulting solution was centrifuged at 1,500 × *g* (10 min, room temperature) to remove metal fragments from the sonicator horn. Cholesteryl oleate/phosphatidylcholine vesicles were prepared in the same way, except that sodium taurocholate was omitted. Recovery of radioactivity in the substrate preparations was routinely >95%. The activity of acid cholesteryl ester hydrolase was assayed as follows: 150 μL MES buffer (pH 5.0, 100 mM) containing bovine serum albumin (essentially fatty acid free, 7.5 mg) was added to 200 μL of the mitochondrial/lysosomal subcellular fraction (approximately 0.2 mg protein) and incubated at 37°C for 10 min. The substrate (50 μL, 144 nmol cholesteryl oleate, 12.95 KBq/μmol) was then added to start the reaction. The final volume in the assay was 400 μL and the final concentrations of cholesteryl oleate, phosphatidylcholine and sodium taurocholate were 360 μM, 1440 μM and 720 μM respectively. After 15-min incubation at 37°C, the reaction was stopped with 1.5 mL chloroform/methanol/toluene (2:2.4:1, v/v/v) containing 0.29 mM oleic acid as carrier. Sodium hydroxide (50 μL, 1 M) was then added. The mixture was vortexed for 20 sec and finally centrifuged at 1,500 × *g* (25 min, 20°C) to separate the phases. The amount of sodium [1-^{14}C]oleate released into the upper phase was determined by liquid scintillation counting. Blank tubes containing substrate but no enzyme protein were included in each experiment. The efficiency of extraction of sodium [1-^{14}C]oleate was also estimated in each experiment, using mixed micelles prepared in the same way as the substrate except that [1-^{14}C]oleic acid was substituted for cholesteryl [1-^{14}C]oleate. Extraction efficiency was >85% and was unaffected by the amount of enzyme protein present. Cytosolic and microsomal neutral cholesteryl ester hydrolase activities were also assayed by this method using MES buffer (100 mM) at pH 6.6 and Tris-HCl (100 mM) at pH 7.0, respectively. Endogenous cholesteryl ester in the mitochondrial/lysosomal and microsomal fractions represented <2% of the total amount of substrate present in the assays. No cholesteryl ester was detected in the cytosolic fraction. Mixed micelles were used as substrate in all experiments except where indicated otherwise.

Other assays. The activities of glutamate dehydrogenase (14), acid phosphatase (15) and β-glucuronidase (16) were determined as described previously. Proteins were estimated by the method of Lowry *et al.* (17). Significance limits were calculated using Student's t-test or Pearson's correlation coefficient.

RESULTS

Previous studies have shown that rat lactating mammary glands contain a cholesteryl ester hydrolase activity with a pH optimum of about 5 associated with the subcellular fraction containing mitochondria and lysosomes (10). The effect of pH in the range 4–6.5 on the activity of this enzyme was tested to determine the pH optimum more precisely (Fig. 1), and a clear, sharp pH optimum at 5.0 was found. The rate of hydrolysis of cholesteryl ester at pH 5.0 was linear with both the time of incubation, up to approximately 30 min (Fig. 2, A), and with the amount of subcellular fraction protein added, up to about 0.2 mg (Fig. 2, B).

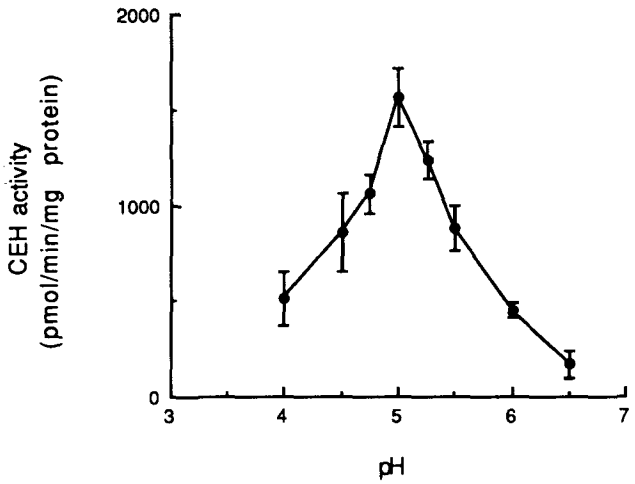

FIG. 1. The effect of pH on acid cholesteryl ester hydrolase activity. The activity of cholesteryl ester hydrolase in the subcellular fraction containing lysosomes and mitochondria prepared from rat lactating mammary tissue was assayed using sodium acetate (100 mM, pH 4.0–4.75) or MES (100 mM, pH 5.0–6.5) buffer. Each point is the mean from 3 experiments. Error bars show the standard error of the mean.

The low pH optimum and sedimentation pattern (10) of the acid cholesteryl ester hydrolase suggest that it may be a lysosomal enzyme. To test this possibility, three subcellular fractions containing mitochondria and lysosomes of increasing density were prepared from rat lactating mammary tissue and the activity of cholesteryl ester hydrolase in each fraction was compared to that of marker enzymes for mitochondria (glutamate dehydrogenase) and lysosomes (acid phosphatase and β-glucuronidase). The specific activities of the marker enzymes in each fraction relative to their specific activities in the whole mitochondrial pellet were consistent in preparations from four animals. Approximately 50% of the total activities (sum of the activities in the three fractions) of cholesteryl hydrolase (50.2 ± 2.1%), acid phosphatase (47.9 ± 1.5%) and β-glucuronidase (48.5 ± 15.4%) were associated with the pellet sedimenting at 8,000 × *g*. In contrast, only 27.0 ± 5.7% of the activity of glutamate dehydrogenase sedimented with this fraction. Over a number of experiments (data not shown), the percentage of the total activity of cholesteryl ester hydrolase associated with each fraction was much more strongly correlated with the percentage activities of acid phosphatase (Pearson's correlation coefficient, $r = 0.95$ $p<0.001$) and β-glucuronidase ($r = 0.95$ $p<0.001$) than with those of glutamate dehydrogenase ($r = 0.6$), suggesting

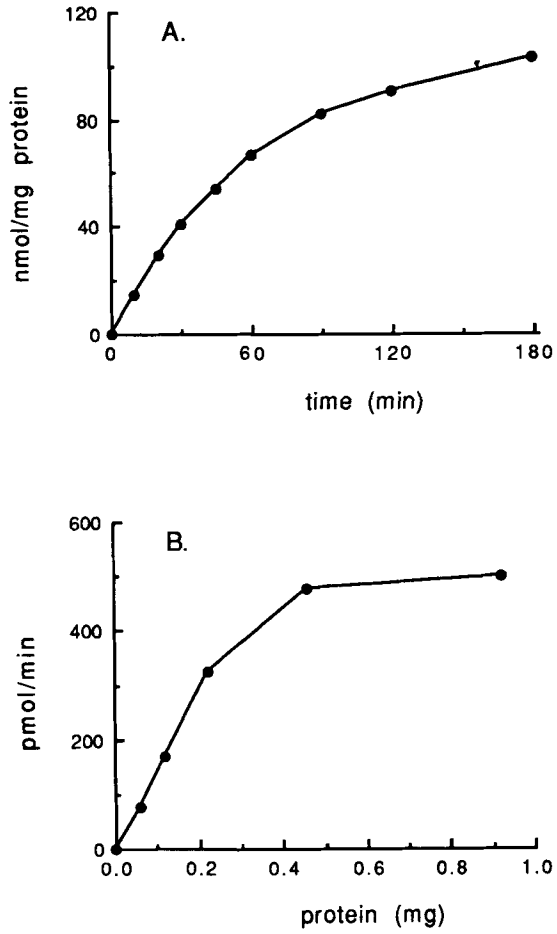

FIG. 2. The effect of time of incubation and enzyme protein concentration on acid cholesteryl ester hydrolase activity. The activity of cholesteryl ester hydrolase in the subcellular fraction containing lysosomes and mitochondria prepared from rat lactating mammary glands was assayed using the standard conditions described in Materials and Methods except that the time of incubation in the presence of the substrate (A), or the amount of subcellular fraction protein added (B), was varied as indicated. Each point is the mean from duplicate determinations and the experiment shown is typical of 4 performed in each case.

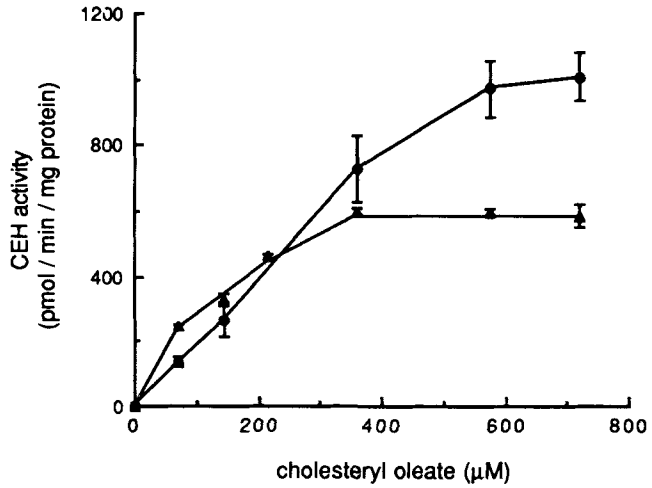

FIG. 3. The effect of substrate concentration on acid cholesteryl ester hydrolase activity. The activity of cholesteryl ester hydrolase in the subcellular fraction containing lysosomes and mitochondria prepared from rat lactating mammary tissue was determined using the concentrations of cholesteryl oleate indicated. Assays were carried out with the substrate in the form of mixed micelles (●) or cholesteryl oleate/phosphatidylcholine vesicles (molar ratio 1:4) in the presence of sodium taurocholate (720 μM) (▲). Each point is the mean from 3 experiments. Error bars show the standard error of the mean.

that the acid cholesteryl ester hydrolase is associated with the lysosomes in rat mammary tissue.

The activity of acid, lysosomal cholesteryl ester hydrolases in some tissues is influenced by the mode of presentation of the substrate (18). The highest activity is usually found when the cholesteryl ester is dispersed with phospholipid in micelles or vesicles with or without sodium taurocholate and/or digitonin (18,19). Previous experiments have shown that mammary gland acid cholesteryl ester hydrolase activity is decreased considerably when sodium taurocholate is omitted from the mixed micelle substrate, but can be substantially restored by separate addition of the bile salt (20). Addition of digitonin (50–250 μg/mL) to the assays did not significantly alter the observed activity in the presence or absence of sodium taurocholate (data not shown). In the experiment shown in Figure 3, the activity of acid cholesteryl ester hydrolase was assayed using different concentrations of cholesteryl oleate dispersed either as mixed micelles, or with phosphatidylcholine only (molar ratio 1:4) in the presence of sodium taurocholate (720 μM) added separately. The kinetics of the enzyme were markedly different with the two substrate presentation modes. These differences may be explained by the variation in the concentrations of sodium taurocholate which are added with the different cholesteryl oleate concentrations when the mixed micelle substrate is used. The detergent effect of the bile salt is likely to influence the size of the lipid/water interface between the enzyme and the substrate and thus modify the activity (18).

The effect of divalent metal ions (1 mM) on the activity of the acid cholesteryl ester hydrolase is shown in Table 1. The enzyme was strongly inhibited by Cu^{2+}, but was inhibited little by other divalent metal ions at the concentration used. Furthermore, ethylenediaminetetraacetic acid (EDTA) (1 mM) did not change the enzyme activity significantly (Table 2). The enzyme was inhibited by NaCl (10–100 mM) and NaF (10–50 mM) in a concentration-dependent way (Table 2). A concentration of 1 mM *p*-hydroxymercuribenzoate almost completely abolished the activity, while 1 mM GSH had only a small inhibitory effect. In the presence of equimolar concentrations of *p*-hydroxymercuribenzoate and GSH, however, activity of the enzyme was restored to the level found with GSH alone (Table 2). Finally, the serine esterase inhibitor phenylmethylsulfonyl fluoride (1 mM) caused a decrease in acid cholesteryl ester hydrolase activity of approximately 25% (Table 2).

Figure 4 shows the changes in the activities of the mam-

TABLE 1

The Effect of Divalent Metal Ions on Acid Cholesteryl Ester Hydrolase Activity[a]

Additions	Cholesteryl ester hydrolase activity (% control value)
None	100
$MgCl_2$	88.6 ± 2.2(5)
$MnCl_2$	83.3 ± 6.1(5)
$NiCl_2$	84.8 ± 5.2(5)
$CaCl_2$	74.7 ± 3.4(5)
$ZnCl_2$	80.9 ± 4.8(4)
$CuCl_2$	10.8 ± 1.5(4)

[a]The subcellular fraction containing lysosomes and mitochondria was prepared from rat lactating mammary tissue as described in Materials and Methods. Acid cholesteryl ester hydrolase activity was determined in the presence and absence of the metal ions indicated. The metal salts were added to the assays in the MES buffer prior to the preincubation period. Data are expressed as a percentage of the values found in the absence of added metal ions, and are the mean ± standard error of the mean from 4 or 5 experiments (indicated in brackets). Control values (pmol/min/mg protein) were 1054 ± 115 (4 experiments) and 1010 ± 99 (5 experiments).

TABLE 2

The Effect of Enzyme Inhibitors on Acid Cholesteryl Ester Hydrolase Activity[a]

Additions	Cholesteryl ester hydrolase activity (% control value)
None	100
NaF (10 mM)	46.0 ± 4.6(4)
NaF (50 mM)	16.1 ± 0.9(5)
NaCl (10 mM)	79.3 ± 4.1(4)
NaCl (100 mM)	52.8 ± 7.4(4)
EDTA (1 mM)	98.2 ± 2.5(5)
Phenylmethylsulfonyl fluoride (1 mM)	75.4 ± 1.3(4)
p-Hydroxymercuribenzoate (0.1 mM)	79.2 ± 2.1(5)
p-Hydroxymercuribenzoate (1 mM)	0.2 ± 0.1(5)
GSH (1 mM)	84.8 ± 1.5(4)
p-Hydroxymercuribenzoate (1 mM) + GSH (1 mM)	79.7 ± 3.2(4)

[a]The subcellular fraction containing mitochondria and lysosomes was prepared from rat lactating mammary tissue as described in Materials and Methods. Acid cholesteryl ester hydrolase activity was determined in the presence and absence of the compounds shown. Additions were made to the assays in the MES buffer prior to the preincubation period. Data are expressed as a percentage of the values found without additions, and are the mean ± standard error of the mean from 4 or 5 experiments (indicated in brackets). Control values (pmol/min/mg protein) were 1192 ± 87 (4 experiments) and 1132 ± 90 (5 experiments).

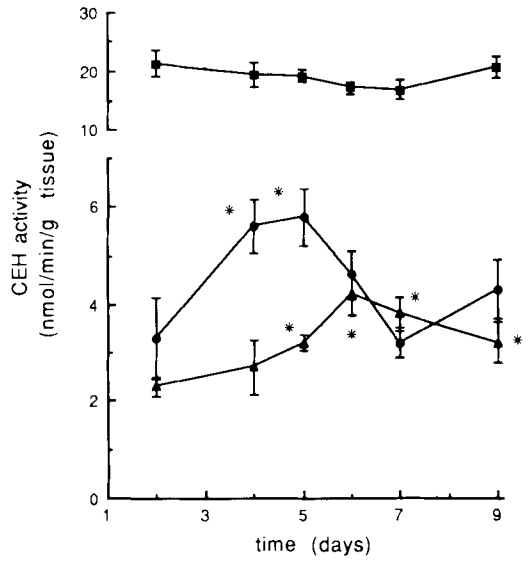

FIG. 4. The effect of time of lactation on acid and neutral cholesteryl ester hydrolase activities. The activities of acid and neutral cholesteryl ester hydrolases (CEH) in the subcellular fractions containing mitochondria and lysosomes (●), microsomes (▲) and cytosol (■) prepared from mammary glands taken from rats after the period of lactation shown were determined as described in Materials and Methods. Each point is the mean from at least 4 experiments. Error bars show the standard error of the mean. *Value is significantly different ($p<0.05$) from value at 2 days.

mary gland acid and neutral cholesteryl ester hydrolases which occur during the first 9 days of lactation. The results are expressed as nmol/min/g tissue so that their relative activities in whole tissue can be compared. The activity of the cytosolic neutral cholesteryl ester hydrolase was higher than that of the other two enzymes at all time points, and did not change significantly during the period studied. Acid cholesteryl ester hydrolase activity, however, increased significantly during the first few days of lactation, reaching a peak after 5 days, then declined to a value close to that seen at the start of the experiment after 7 days. The activity of the microsomal neutral enzyme also increased steadily during early lactation, reaching a maximum after 6 days. The relative specific activities of marker enzymes for lysosomes (acid phosphatase, 1.88 ± 0.15), microsomes (NADPH:cytochrome C reductase, 3.55 ± 0.21) and cytosol (lactate dehydrogenase, 1.71 ± 0.07) varied little in subcellular fractions of glands from seven rats between 2 and 9 days post-partum. No relationship between the relative specific activities of the enzymes and the number of days lactating was detected. Recovery of the marker enzymes in the appropriate fractions as a percentage of the total activity found in the homogenate was also consistent (± 5%). These results indicate that the changes in the activities of the three cholesteryl ester hydrolases during lactation are not caused by differences in purity or recovery of the individual preparations.

When subcellular fractions were prepared from mammary tissue from virgin rats, the activities of the three cholesteryl ester hydrolases under investigation were decreased considerably compared to the values obtained with lactating glands. The greatest difference, 8–14 fold, was seen in the activity of the acid cholesteryl ester hydrolase (non-lactating, 0.42 ± 0.11 nmol/min/g; peak lactating at 5 days, 5.8 ± 0.6 nmol/min/g) followed by the

microsomal enzyme, 3–5 fold (non-lactating, 0.75 ± 0.25; peak lactating at 6 days, 4.2 ± 0.4 nmol/min/g), while the cytosolic activity showed the smallest change, 2-fold (non-lactating, 9.3 ± 0.9; lactating at 2 days, 21.3 ± 2.3 nmol/min/g). The non-lactating values were obtained using tissue pooled from two or three rats because very much less mammary tissue is present in virgin rats. The data given are the mean ± standard error of the mean from three experiments using a total of eight animals.

DISCUSSION

Cholesteryl ester hydrolase activities with pH optima between 4.0 and 5.0 are associated with the lysosomes in a wide variety of tissue and cell types including liver (21,22), heart (23), aorta (24), fibroblasts (25,26), adrenal cortex (27) and adipose tissue (23). Our earlier experiments had suggested that a similar activity may be present in the rat lactating mammary gland (10). The results reported here confirm the presence in such tissue of an acid cholesteryl ester hydrolase with a pH optimum of 5.0 which sediments with lysosomal enzyme markers. Similar to the acid, lysosomal cholesteryl ester hydrolases in other tissues, the mammary gland enzyme is inhibited by Cu^{2+} (27–29), NaCl, and NaF (19,21,25). Furthermore, the inhibition of the mammary gland activity by *p*-hydroxymercuribenzoate, and its almost complete reversal by GSH (Table 2) indicates that the enzyme requires free sulfhydryl groups for activity, as has been found with similar enzymes in other cell types (21,22,24,25,30).

In a number of previous studies the activity of lysosomal cholesteryl ester hydrolase, even after extensive purification, could not be separated from that of lysosomal acid lipase, which is responsible for the hydrolysis of triglycerides, and it is believed that the same enzyme catalyzes both reactions (21,24,31). If this were the case with the mammary gland cholesteryl ester hydrolase, then the observed 25% inhibition by phenylmethylsulfonyl fluoride may indicate contamination of the preparation with other lipases, as lysosomal, acid lipases are not believed to be affected by serine esterase inhibitors. Imanaka *et al.* (32), however, have reported that the cholesteryl ester hydrolase activity of partially purified rabbit liver lysosomal lipase was inhibited by about 20% by 0.2 mM phenylmethylsulfonyl fluoride. The subcellular fraction used in the experiments described here may contain lipoprotein lipase, which is present in rat mammary tissue (33). This enzyme, however, has an alkaline pH optimum at about 8.5, and has virtually no activity below pH 6 (34). The milk of some species contains a bile salt-stimulated lipase (35), but this has not been detected in rat milk (36). Although the presence of other lipases in rat mammary tissue has not been reported, the possibility of some contamination by unknown lipases cannot be ruled out.

The lysosomal cholesteryl ester hydrolase has an important function in the regulation of cholesterol metabolism in some tissues (6,37). Increased flux of cholesterol from the plasma leads to decreased cholesterol synthesis and increased storage as esters. Feeding lactating rats a hypercholesterolemic diet has been shown to decrease cholesterol synthesis in the mammary gland (38), suggesting that cholesterol taken up from the plasma and processed by the acid cholesteryl ester hydrolase may also regulate cholesterol metabolism in mammary cells. In addition, neutral cholesteryl ester hydrolases in the adrenal cortex (7,9), liver (39) and adipose tissue (40) have been shown to be hormonally controlled by a mechanism involving phosphorylation-dephosphorylation. Thus they are believed to be involved in the regulation of the supply of unesterified cholesterol for metabolism in these tissue. Recent experiments in this laboratory (12) have suggested that the microsomal and cytosolic neutral cholesteryl ester hydrolases from rat mammary glands may be modulated by similar phosphorylation-dephosphorylation mechanisms. These enzymes, therefore, may play a part in regulating mammary gland cholesterol metabolism.

If the mammary gland cholesteryl ester hydrolases were important in the provision of unesterified cholesterol for secretion into milk, their activities would be expected to be higher in lactating as compared to non-lactating tissue. This was found to be the case for all three enzymes studied. The acid activity, however, showed the greatest increase (8–14 fold). A large proportion (60–80%) of milk cholesterol is derived from plasma lipoproteins (1–3), and the uptake of cholesterol from this source has been shown to be increased approximately 8-fold at the onset of lactation (41). Cholesteryl esters from chylomicrons have been shown to be internalized by mammary cells without prior hydrolysis (42). In addition, Raphael *et al.* (3) have reported that radioactivity from low density lipoprotein and very low density lipoprotein cholesteryl esters is transferred into milk. The results of the current work, therefore, suggest that the mammary gland acid cholesteryl ester hydrolase has a role in hydrolyzing lipoprotein cholesteryl ester to provide unesterified cholesterol for secretion into milk.

The experiments reported here demonstrate that the acid cholesteryl ester hydrolase activity found in the mammary gland has characteristics similar to those of acid, lysosomal cholesteryl ester hydrolases found in other tissues. In addition, the findings support the idea that the mammary gland acid and neutral cholesteryl ester hydrolases, like those found in other tissues, play a part in regulating intracellular cholesterol metabolism, and thus help control the availability of cholesterol for secretion into milk.

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