Bile Salt-Dependent and Bile Salt-Independent Cholesteryl Ester Hydrolase Activities in Rat Liver Cytosol

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Abstract. **These studies report on the relationship between the bile salt–dependent and –independent cholesteryl ester hydrolase (CEH) activities found in rat liver cytosol. The two activities show very similar Michaelis-Menten substrate kinetics and pH dependence. After gel filtration of cytosol, the bile salt–independent activity elutes much earlier than the bile salt–dependent activity, suggesting that the two activities are associated with entities of different molecular size. However, when gel filtration is carried out in the presence of bile salt, the bile salt–dependent activity elutes as a large aggregate, similar to the bile salt–independent activity's behavior in the absence of bile salt. Both activities coelute after cytosol is passed through an ion exchange column. After each chromatographic procedure the recovery of the bile salt–dependent activity was substantially higher than the recovery of the bile salt–independent activity. When cytosol is incubated with anti-rat pancreatic CEH in the absence of cholate, the bile salt–dependent activity is inhibited more than 90% whereas bile salt–independent activity remains unaffected even at high antibody concentrations. When cytosol is incubated with anti-rat pancreatic CEH in the presence of cholate both CEH activities remain unaffected. The prevention of immunoinhibition by cholate seems to be specific for this detergent since CHAPS, a cholate analog, does not prevent immunoinhibition of the bile salt–dependent activity by anti-CEH. The experimental results are consistent with a model for CEH activity in liver cytosol in which there is only one enzyme that can exist in a monomeric, inactive form (that can be activated by addition of cholate to the assay and represents the bile salt–dependent activity) and in an active complex comprising several enzyme monomers as well as cholate micelles (that accounts for the bile salt–independent activity).**

[P.S.E.B.M. 1994, Vol 206]

The formation, transport, and hydrolysis of cholesteryl esters form an integral part of hepatic cholesterol metabolism. Hydrolysis of cholesteryl esters is carried out by lysosomal acid lipase and by cholesteryl ester hydrolases with neutral pH optima. While the lysosomal enzyme has been purified from several sources and is well characterized (1), there remain unresolved questions about the enzyme(s) responsible for the hydrolysis of cholesteryl esters at neutral pH.

Hepatic, neutral cholesteryl ester hydrolase activity assayed in the absence of exogenous bile salts has been reported in microsomal and cytosolic fractions (2). Studies with rat liver homogenates have demonstrated the presence of neutral cholesteryl ester hydrolase activity that requires millimolar concentrations of trihydroxy bile salts for optimal activity (3). The bile salt–dependent enzyme activity varies widely among homogenates from livers of individual rats (4). Bile

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Received October 12, 1993. [P.S.E.B.M. 1994, Vol 206]
Accepted January 25, 1994.

0037-9727/94/2061–0060$10.50/0

salt–dependent activities are also found in intestine, serum, and bile. The properties of these enzyme activities as determined in crude preparations are all similar and they are in turn similar to the properties exhibited by purified rat pancreatic cholesteryl ester hydrolase (4). Hui, Brockman, and coworkers have reported the purification of a cytosolic, bile salt–dependent neutral cholesteryl ester hydrolase from rat liver (5). In order to purify the enzyme, they used an immunoaffinity chromatography column constructed with antibodies prepared against the porcine pancreatic cholesterol esterase. They also showed that the first eight amino acids in the N-terminal sequence of the rat pancreatic and rat liver enzymes are identical. These results indicate that the pancreatic and the liver enzymes are closely related. However, a comparison between the turnover numbers of the purified hepatic (5) and pancreatic (4) enzymes shows the pancreatic enzyme is much more efficient at catalyzing the hydrolysis of cholesteryl esters *in vitro*.

Ghosh and Grogan reported the purification of a hepatic, neutral cholesteryl ester hydrolase which is not the pancreatic enzyme (6). Enzyme activity is only observed in the presence of taurocholate; highest activity is observed when using 0.5–10 m*M* taurocholate. Higher concentrations of bile salt strongly inhibit the enzyme in sharp contrast to purified pancreatic CEH which exhibits optimal activity at 75 m*M* cholate (4). The liver and pancreatic enzymes show different amino acid compositions and they exhibit different elution patterns from cation exchange and chromatofocusing columns. The liver enzyme isolated by Ghosh and Grogan does not cross react with antipancreatic CEH. Finally, liver CEH is activated by protein kinases, whereas pancreatic CEH is not. Clearly, these observations indicate significant differences between this particular hepatic enzyme and pancreatic CEH at the molecular and regulatory levels. The enzyme isolated by Ghosh and Grogan also appears to be distinct from the cytosolic CEH active in the absence of bile salts.

The present studies were undertaken to explore the possible relationship between the hepatic, bile salt–dependent CEH (the enzyme identical or closely similar to pancreatic CEH) and the CEH activity that can be assayed in the absence of any exogenous bile salts. The experimental data presented here are consistent with the suggestion that these two activities are due to the same enzyme, namely the hepatic CEH that is closely similar to pancreatic CEH.

Materials and Methods

Animals. Sprague-Dawley rats were purchased from Ace Animal Inc. (Byertown, PA) and housed in our animal facility where they were fed *ad libitum* a commercial diet. Livers from adult animals, weighing 500–650 g, were used in the preparation of cytosol.

Materials. Cholesteryl [1-^{14}C] oleate was purchased from Amersham (Arlington Heights, IL). Nonradioactive cholesteryl oleate, sodium cholate, and normal rabbit immunoglobulin G (IgG) were purchased from Sigma (St. Louis, MO), 3-(3-cholamidopropyl) dimethylammonio-1-propane sulfonate (CHAPS) was bought from Calbiochem (La Jolla, CA). A prepacked Superose 12 column and chromatography media (DEAE Sepharose and Sephacryl S-200) were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). Polyclonal rabbit anti-rat pancreatic CEH IgG was kindly provided by Dr. Linda Gallo of the George Washington University Medical School. The preparation of the antibody has been described previously (7). Common chemicals and reagents were obtained from either Sigma or Fisher.

Preparation of Rat Liver Cytosol. Rats were sacrificed by inhalation of carbon dioxide. The liver was excised, weighed, and minced with scissors. Sucrose solution (0.25 *M*, 30 ml) was added to the minced tissue, and the suspension was homogenized with a Polytron Homogenizer (Brinkman Instruments, Westbury, NY). The resulting homogenate was fractionated by differential centrifugation to obtain the cytosol. We used the method developed by de Duve *et al.* (8) with minor modifications. In the first step, low speed centrifugation (1000 rpm, SS34 rotor, 10 min) of the homogenate separates the nuclear material (pellet) from the cytoplasm (supernatant). The pellet is washed (i.e., resuspended and recentrifuged) twice with sucrose solution and the supernatant from each wash is added to the cytoplasmic extract. In the second step, centrifugation of the cytoplasmic extract (12000 rpm, SS34 rotor, 13 min) separates the mitochondrial/lysosomal fraction (pellet) from the microsomal/cytosolic fraction (supernatant). Again, the pellet is washed twice and the supernatant from each wash is added to the microsomal/cytosolic supernatant. In the final step, the microsomes are precipitated out of the cytosol by high-speed centrifugation (50,000 rpm, Ti50 rotor, 30 min).

Assay of Cholesteryl Ester Hydrolase (CEH) Activity. The radiometric assay of cholesteryl ester hydrolase (CEH) activity has been described previously (4). Reaction mixtures were prepared in capped test tubes in a final volume of 0.2 ml. The reaction mixture contained buffer (Tris or Tris-Maleate, pH 7, 50–100 m*M*), sodium cholate (20 m*M*) when determining bile salt–dependent activity and appropriately diluted enzyme source (cytosol or aliquots from eluting fractions from chromatographic columns). The hydrolysis reaction was initiated by addition of cholesteryl [1-^{14}C]

oleate dissolved in ethanol (2 nanomoles and 0.05 μCi in 5 or 10 μl). The tubes were incubated in a water bath at 37°C for 1 hr. The hydrolysis reaction was terminated by the addition of 3.25 ml of methanol/chloroform/heptane (1.41:1.25:1.00). After this, borate buffer (pH 10, 0.05 *M*, 1.05 ml) was added. The contents of the tube were mixed and then centrifuged for 15 min at 1000*g* at room temperature. An aliquot of the aqueous phase containing the released labeled fatty acid was mixed with 15 ml of biodegradable Scintiverse and the radioactivity determined. The amount of oleic acid released was determined from the partition coefficient for oleic acid (70%) and the specific radioactivity of substrate. Enzyme activity is given in units per milliliter of enzyme solution or milligram of total protein. A unit of enzyme activity is the number of picomoles of fatty acid released in 1 hr. All samples were assayed in duplicate or quadruplicate, and the activities reported are the means of these determinations. Variation in the multiple measurements was generally less than 15% of the mean.

Results

Table I confirms an earlier report (4) on the levels of the bile salt–dependent and –independent CEH activities among individual rat liver homogenates. The level of bile salt–dependent activity varies widely from rat to rat whereas the levels of bile salt–independent activity remain essentially constant. Bile salt–dependent activity can be anywhere from being very low (Rat 4) to levels that are many times the level of bile salt–independent activity (Rat 2). Other animals show comparable amounts of the two neutral CEH activities (Rat 1).

Figure 1 shows the effect of antipancreatic CEH antibody on the bile salt–dependent and –independent CEH activities. Bile salt–dependent activity is inhibited by more than 90% at an antibody concentration of 5 μg/ml. On the other hand, bile salt–independent activity remains unchanged even at antibody concentrations of 25 μg/ml. This experiment was repeated with a different cytosol, and the results were the same. At first sight, these results are consistent with the idea that the two activities are probably due to two different enzymes. However, the results are also consistent with the presence of only one enzyme as discussed later.

Table I. Cholesteryl Ester Hydrolase (CEH) Activities in Cytosol Fractions Prepared from Different Rats[a]

Rat	CEH Activity (units/mg protein)	
	(−) Bile salt	(+) Bile salt
1	1120	910
2	860	9080
3	910	330
4	1000	180

[a] CEH activity was determined in reaction mixtures containing Tris-Maleate (pH 7, 50 m*M*) and 10 u*M* cholesteryl [1 − ^{14}C] oleate. Bile salt, when present, was sodium cholate (20 m*M*). Hydrolysis reaction was carried out for 1 hr at 37°C.

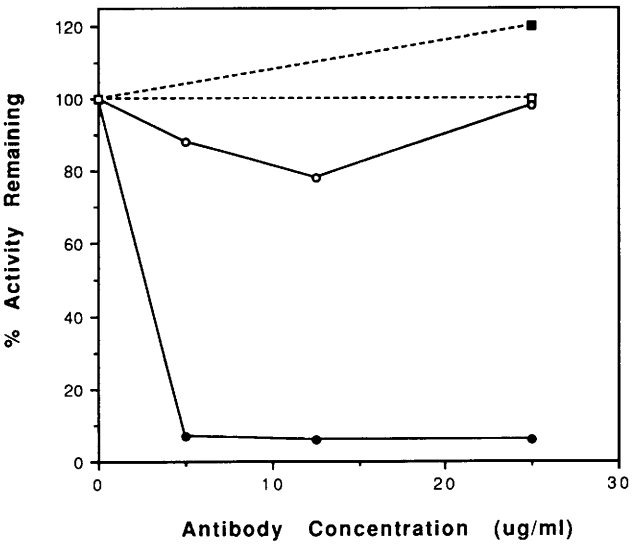

Figure 1. Effects of antipancreatic CEH antibody on the bile salt–dependent (●) and –independent (○) CEH activities. Rat liver cytosol was incubated overnight at 4°C with various amounts of rabbit anti-rat pancreatic CEH IgG in a volume of 70 μl. In control samples, enzyme was incubated with normal rabbit IgG at 25 μg/ml and then assayed in the presence (■) and absence (□) of bile salt. After incubation, CEH activities were determined and related to the percent of activity in control samples where no antibody was added. Absolute activities corresponding to 100% in the presence and absence of bile salt were 1950 and 1400 units/mg protein respectively. For the determination of CEH activities Tris-Maleate buffer (pH 7, 0.1 *M*, 100 μl), sodium cholate (0.2 *M*, 20 μl, + bile salt) or water (20 μl, − bile salt), and cholesteryl [1-^{14}C] oleate as substrate dissolved in ethanol (2 nanomoles, 0.05 μCi, 10 μl) were added. The hydrolysis reaction was allowed to proceed for 1 hr at 37°C.

A preliminary comparison of the kinetics of the two CEH activities shows similar characteristics (Fig. 2a). Apparent K_m values determined from the corresponding Lineweaver-Burk plots were 11 μ*M* (− cholate) and 8 μ*M* (+ cholate). Figure 2b shows the effect of pH on the two CEH activities. Maximal rates are reached in the range 7–8 for both activities. Hence the characterization of these activities as neutral CEH activities.

Figure 3 shows the elution profile from a DEAE Sepharose anion exchange column of the two neutral CEH activities found in cytosol. Both activities coelute and they are separated from the bulk of the cytosolic proteins which elute slightly earlier than the CEH activities. The cytosolic preparation used in this chromatographic procedure was that from Rat 1 (Table I) which contained comparable amounts of bile salt–

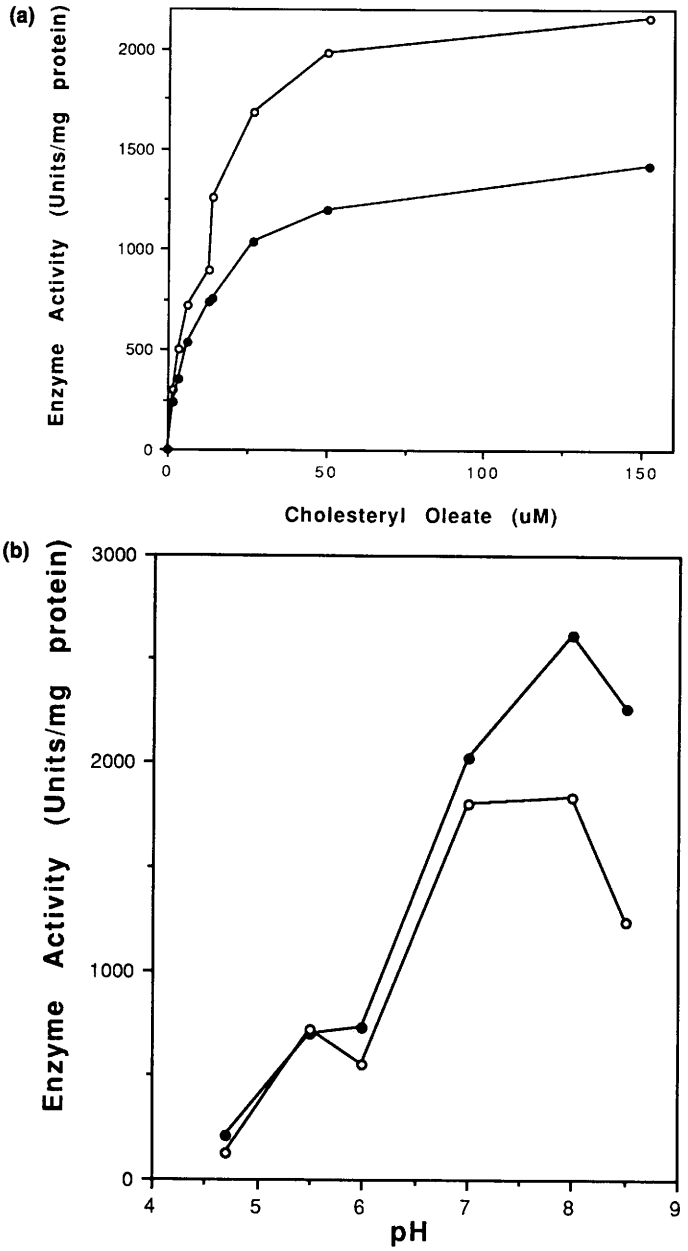

Figure 2. (a) Substrate concentration dependence of bile salt–independent (○) and –dependent (●) CEH activities of rat liver cytosol. Reaction mixture contained Tris-Maleate buffer (pH 7, 0.1 *M*, 170 μl), sodium cholate (0.2 *M*, 20 μl, + bile salt) or water (20 μl, − bile salt), enzyme source (cytosol, 7.8 mg/ml, 5 μl) and different concentrations of cholesteryl [1-^{14}C] oleate dissolved in 5 μl of ethanol. The cytosol used as enzyme source contained roughly comparable amounts of the two CEH activities as screened initially in the presence of 10 μ*M* final substrate concentration. Reaction was carried out for 1 hr at 37°C. (b) Effect of pH on the bile salt–dependent (●) and –independent (○) CEH activities of rat liver cytosol. Buffers used at a final concentration of 0.1 *M* were sodium acetate for pH 4.7 and 5.5, Tris-Maleate for pH 6, 7, 8, and 8.5. Reactions were conducted in the presence of 100 μ*M* cholesteryl [1-^{14}C] oleate (+) Bile salt assays contained 20 m*M* sodium cholate. Other conditions as in Figure 2a.

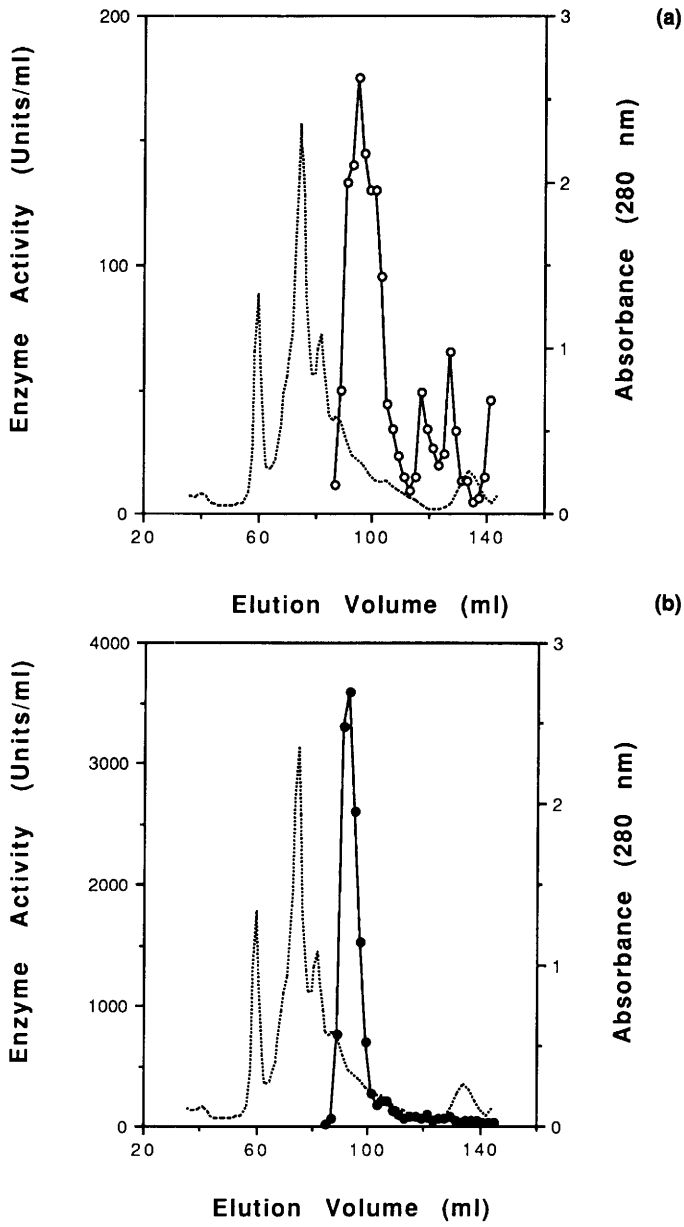

Figure 3. DEAE-Sepharose anion exchange chromatography of neutral CEH activities in rat liver cytosol. A cytosolic fraction (32 mg protein in 4 ml) containing similar amounts of bile salt–dependent (●) and –independent (○) CEH activities was applied to a DEAE Sepharose column (18 × 0.8 cm). The column had been previously equilibrated with Tris buffer (pH 7, 10 m*M*). Column flow rate was 60 ml/hr. After sample application the column was washed with 36 ml of the same buffer. The unbound fraction contained protein but no CEH activity. At that point, a linear gradient (0–0.5 *M* KCl, 100 ml in Tris buffer pH 7, 10 m*M*) was applied at fraction 36 to elute the bound protein and CEH activities. Samples were collected in 1-ml fractions. Protein absorbance at 280 nm is shown by the dotted line in both panels. (a), Bile salt–independent activity (○); (b) bile salt–dependent activity (●).

independent and –dependent activities. Interestingly, the percent recovery of the bile salt–dependent activity was 100%, whereas the percent recovery of the bile salt–independent activity was only 7%. This experiment was repeated three more times, and the recoveries of bile salt–dependent activity were always high (85%–100%) while those for bile salt–independent activity were low (15%–27%).

Figure 4 shows the elution profile from a Sephacryl S-200 gel filtration column of the two neutral CEH activities. The bile salt–independent activity coelutes with the major protein peak, but it is separated from the bile salt–dependent activity which elutes later. This result suggests that the enzyme responsible for the bile salt–independent activity has a higher molecular weight than the enzyme responsible for the bile salt–dependent activity. Again, the cytosolic preparation applied to this column contained comparable amounts of the two activities. The recovery of the bile salt–dependent activity was 57% whereas the recovery of the bile salt–independent activity was 4%.

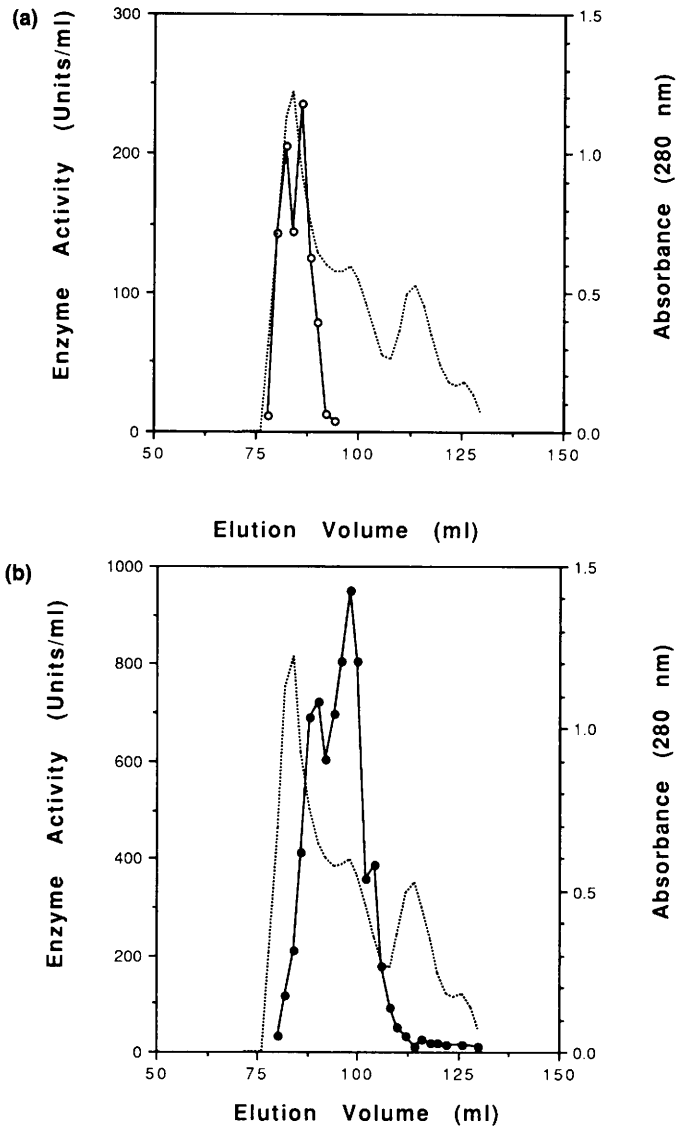

Figure 4. Sephacryl S-200 gel filtration chromatography of neutral CEH activities in rat liver cytosol. A cytosolic fraction (32 mg in 4 ml) containing similar amounts of bile salt–dependent and –independent CEH activities was applied to a Sephacryl S-200 column (90 × 1.5 cm). The column had been previously equilibrated with Tris buffer (pH 7, 10 m*M*). Column flow rate was 6 ml/hr. After sample application, additional buffer was applied (300 ml) to the column to elute the proteins present in the sample. Eluant was collected in 1-ml fractions. Protein absorbance at 280 nm is shown by the dotted line in both panels. (a), Bile salt independent activity (○); (b), bile salt dependent activity (●).

In order to determine the effect of cholate on the immunoinhibition of the bile salt–dependent CEH activity by antipancreatic CEH we preincubated cytosol and antipancreatic CEH in the absence of bile salt and in the presence of 20 m*M* sodium cholate. In this case, concentrations of antibody in the range 0–2 μg/ml were used. As controls, normal rabbit IgG at 2 μg/ml was incubated in the absence and in the presence of 20 m*M* cholate. Figure 5a shows the results; when enzyme was incubated with antibody at 2 μg/ml in the absence of cholate, percent of enzyme activity remaining was 20%. In contrast, when enzyme was incubated with the same concentration of antibody in the presence of 20 m*M* cholate, percent of enzyme activity remaining was about 80%. No loss of enzyme activity was observed in control incubations with normal rabbit IgG. Other experiments demonstrated that preincubations with as little as 5 m*M* cholate prevented immunoinhibition. To determine that any effects observed in the presence of cholate are not due to nonspecific detergent interactions, we also ran a control series where we used 5 m*M* CHAPS in the preincubation of cytosol and various amounts of antibody. Figure 5b shows that the bile salt–dependent activity is consistently less inhibited in the presence of several concentrations of antibody when cholate is present in the preincubation of cytosol and antibody than when there is no cholate during preincubation. The prevention of immunoinhibition by cholate seems to involve a specific interaction since the same effect is not observed when CHAPS is present during preincubation. In the presence of CHAPS, the immunoinhibition by various amounts of antipancreatic CEH follows the same pattern as when there is no bile salt present during preincubation.

The effect of cholate on the elution of bile salt–dependent CEH activity from a Superose 12/6 gel filtration column is shown in Figure 6. Figure 6a shows the elution profile of bile salt–independent and bile salt–dependent activities when cholate is not present. Clearly, the two enzyme activities elute separately; the molecular entity or complex responsible for bile salt–independent activity elutes first, confirming the result obtained with the Sephacryl S-200 column. As in the case of the Sephacryl column, the levels of bile salt–independent activity eluting from the column are much lower than the levels of bile salt–dependent activity even though the cytosol applied to the column had comparable amounts of both activities. Figure 6b shows the elution profile of the bile salt–dependent activity in the presence of 20 m*M* cholate. Under these conditions, the peak containing the bile salt–dependent activity elutes earlier than it does when

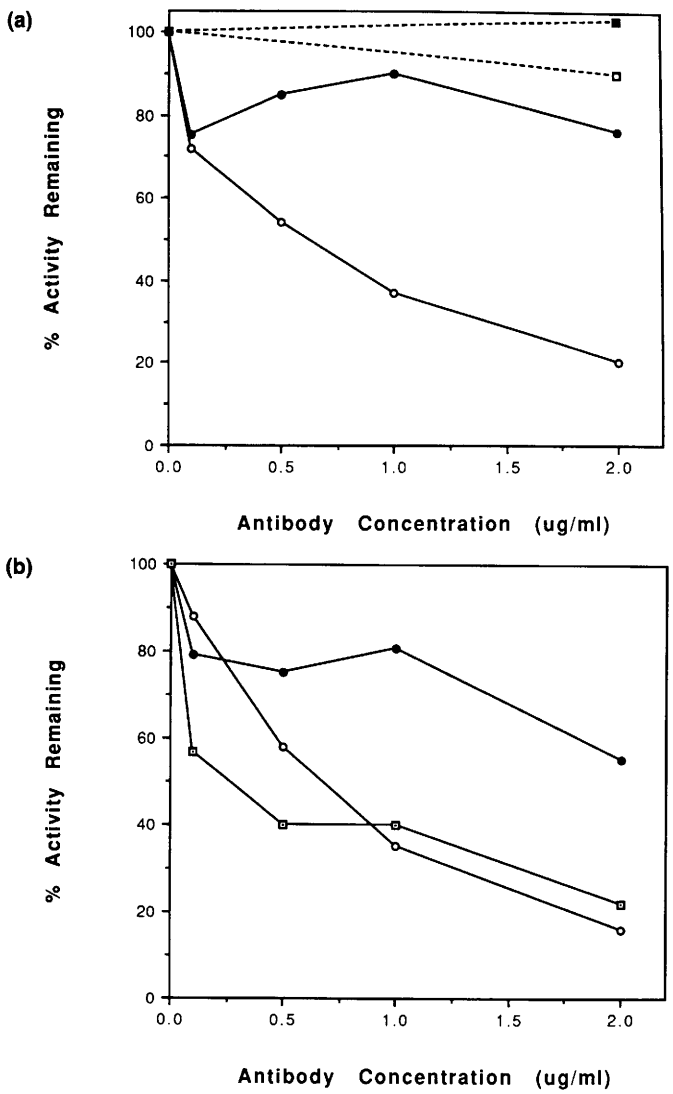

Figure 5. (a) Effect of cholate on the immunoinhibition of bile salt–dependent CEH activity by antipancreatic CEH. Tris-Maleate buffer (pH 7, 0.1 *M*, 120 μl), cytosol (9.0 mg/ml, 5 μl) and various amounts of antipancreatic CEH adjusted to 30 μl with saline were incubated for 5 hr (4 hr at 4°C and 1 hr at room temperature) in the absence of bile salt (○) and 20 m*M* cholate (●). Control samples were incubated with 2 μg/ml of normal rabbit IgG in the presence (■) and absence (□) of 20 m*M* cholate. The volume of incubation mixtures was adjusted with water to 175 μl. After incubation, the final concentration of sodium cholate was adjusted to 20 m*M* and cholesteryl [1-^{14}C] oleate dissolved in ethanol (2 nanomoles, 0.05 μCi, 5 μl) was added to determine the bile salt–dependent CEH activity. Bile salt–dependent activities corresponding to 100% activity were 5782 and 6091 units/mg protein for samples preincubated without cholate and 20 m*M* cholate, respectively. (b) Specificity of effect of cholate. Same experiment as in (a) except that preincubations included tubes with no cholate (○), 5 m*M* sodium cholate (●) and 5 m*M* CHAPS (⊡). The volume of incubation mixtures was adjusted with water to 175 μl. After incubation, the final concentration of sodium cholate was adjusted to 20 m*M* and cholesteryl [1-^{14}C] oleate dissolved in ethanol (2 nanomoles, 0.05 μCi, 5 μl) was added to determine the bile salt–dependent CEH activity.

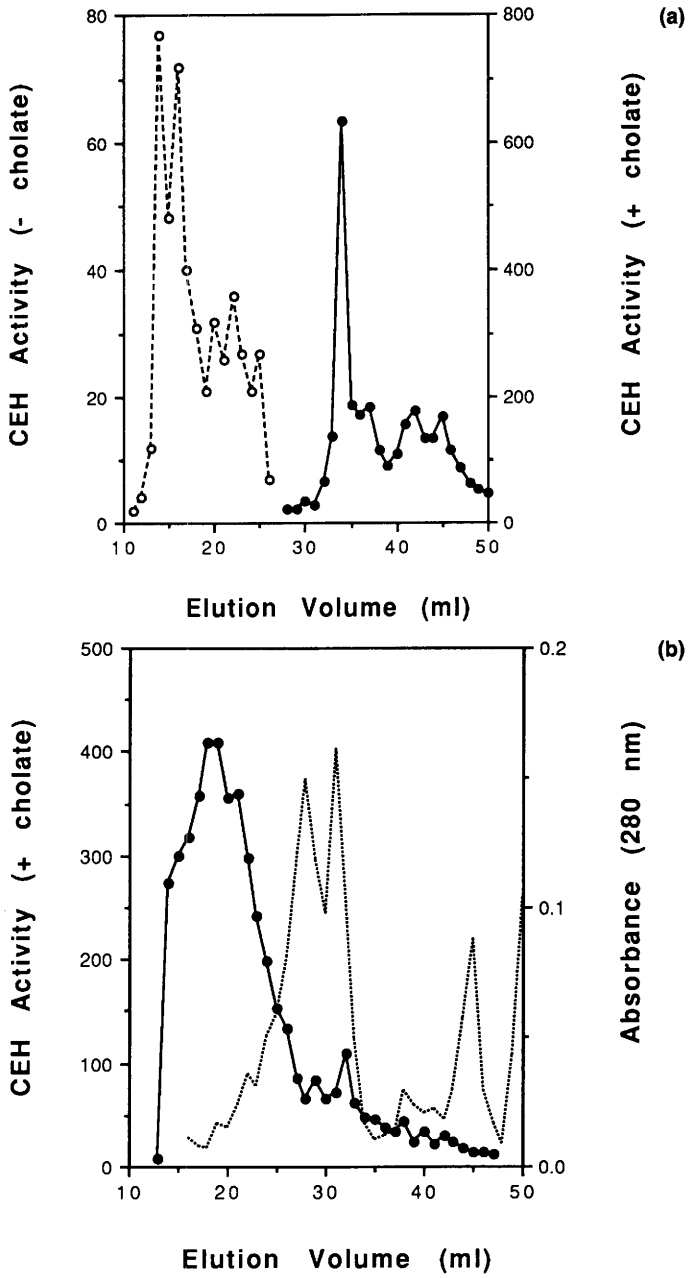

Figure 6. Superose 12/6 gel filtration chromatography of neutral CEH activities in rat liver cytosol. (a) Cytosol (200 μl, 7.8 mg/ml) containing comparable amounts of bile salt–dependent and –independent activities was applied to a Superose 12 and 6 (HR 10/30) columns connected in tandem and equilibrated with Tris buffer (pH 7, 50 m*M*, 0.5 *M* NaCl 1 m*M* EDTA). Column flow rate was 0.1 ml/min. Bile salt–independent (○) and –dependent (●) activities are given in units/ml. They were determined from aliquots (+ bile salt, 175 μl; − bile salt, 195 μl) taken out of every fraction. (b) Effect of cholate on the elution of bile salt–dependent CEH activity. Cytosol (200 μl, 7.8 mg/ml) was applied to the same Superose column, this time equilibrated with 20 m*M* cholate in addition to the buffer used in (a). Protein absorbance at 280 nm is shown by the dotted line. Elution of protein was the same in the presence and absence of cholate. Bile salt–dependent activity (●) was determined from an aliquot (195 μl) taken out of each fraction.

there is no cholate present. Moreover, the bile salt–dependent activity in the presence of cholate elutes at the same elution volume as the bile salt–independent activity in the absence of cholate. The elution profile for protein (shown in Figure 6b only) is the same in the presence and absence of cholate suggesting that the effect of cholate is specific for the enzyme responsible for bile salt–dependent activity.

Discussion

Previous studies on cholesteryl ester hydrolase activities in crude liver preparations have reported the presence of bile salt–dependent and bile salt–independent cholesteryl ester hydrolase activities. These terms are operational in that they are used to indicate whether or not trihydroxy bile salts are added to the reaction mixture. In any case, cytosolic preparations from liver contain significant amounts of cholate (9). Consequently, it is possible that the operationally defined bile salt–independent activity is made possible by endogenous cholate present in cytosol. The results of the present study are consistent with, but by no means prove, the idea that the two activities may originate from the same enzyme.

Figure 7 illustrates a model for CEH activity in liver cytosol consistent with all the experimental results reported here. The basic features of this model are: (i) the enzyme can exist in a monomeric, inactive form and in an active complex comprising several enzyme monomers as well as cholate micelles; (ii) Enzyme activity is regulated by the complexation between enzyme monomers and bile salt. The extent of complexation between enzyme and cholate is dictated by limiting amounts of cholate and it remains constant under normal dietary conditions; and (iii) The amount of free monomeric enzyme varies widely, and it might be related to different metabolic states of the cell. In light of this model, the operationally defined bile salt–independent CEH activity is a measure of constant levels of endogenous enzyme-cholate complex therefore it will appear constant (4). On the other hand, bile salt–dependent CEH activity reflects the widely variable amount of inactive monomer that is activated when exogenous bile salt is added during the *in vitro* assay. Since the levels of monomeric enzyme vary over a wide range, the bile salt–dependent activity will also vary over a wide range (4).

Chromatographic Behavior of Bile Salt–Independent and –Dependent Activities. In line with our proposed model, the enzyme-cholate complex is bigger than enzyme monomers. To the extent that the complex will withstand dissociation, we will observe it as a different entity in chromatographic separations based on size. Figures 4 and 6a illustrate the results of gel filtration chromatography of cytosol using Sephacryl S-200 and Superose 12/6 respectively. In

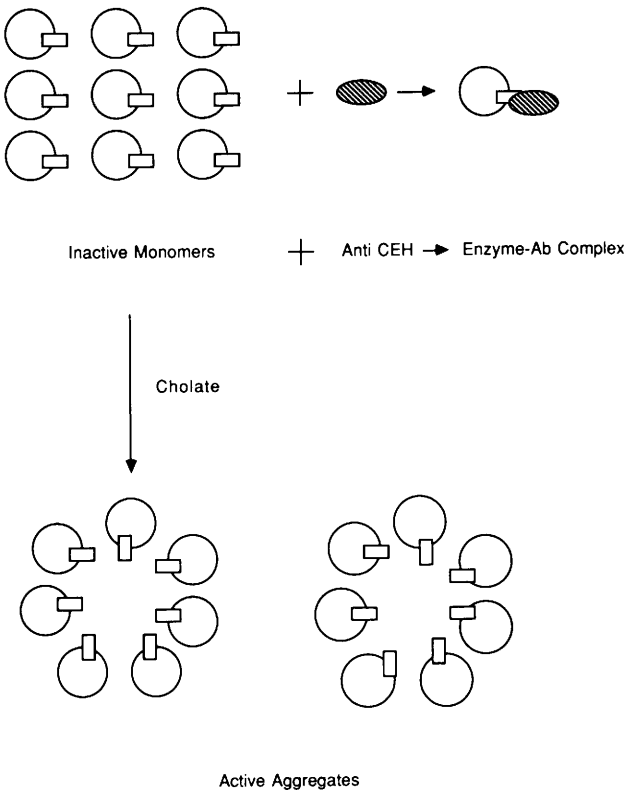

Figure 7. Schematic illustration of a hypothetical model for CEH activity in rat liver cytosol. No stoichiometry is implied in this illustration. The enzyme can exist as an inactive monomer or as an active aggregate in the presence of cholate. Anti-CEH (Ab) reacts only with the monomeric form of the enzyme to form an enzyme-antibody complex that does not react with cholate. Bile salt–independent CEH activity is the activity from aggregates due to endogenous amounts of cholate. Bile salt–dependent CEH activity is the activity from aggregates formed after cholate is added to the reaction mixture.

both cases bile salt–independent activity (endogenous enzyme-cholate complex) elutes earlier than the bile salt–dependent activity (enzyme monomer).

Ghosh and Grogan reported that in the presence of 0.5 m*M* taurocholate, CEH activity elutes from a gel filtration column with an apparent molecular weight of 60–70 kilodaltons. In the absence of taurocholate, CEH activity is partitioned into two peaks which elute at 400 and 60–70 kilodaltons; bile salt dissociates enzyme aggregates into a monomeric active form (6). In the case of pancreatic CEH, 70 kilodalton inactive monomers aggregate in the presence of bile salt to form a 400,000 molecular-weight active enzyme (10, 11). The chromatographic behavior and the bile salt activation pattern of the enzyme activities we report in the present study exhibit characteristics similar to that of pancreatic CEH. It appears that under their conditions of assay, Ghosh and Grogan detect a different hepatic CEH than that studied by us (4) and by Camulli *et al.* (5). This suggestion is supported by the markedly different properties of the purified enzymes isolated by the two groups of investigators (5, 6).

Figure 3 shows the elution profile of DEAE anion exchange chromatography of cytosol where both the monomeric and oligomeric forms of the enzyme coelute. This is consistent with the idea that cholate does not affect the charge on monomeric enzyme molecules. Consequently, the enzyme-cholate complex exhibits similar affinity for DEAE Sepharose as the enzyme monomer.

Since the very nature of the bile salt–independent activity assay precludes the addition of bile salt, detection of bile salt–independent activity after cytosol is subjected to a chromatographic procedure is contingent on the preservation of the enzyme-cholate complex. We suggest that the low recoveries of bile salt–independent activity observed after anion exchange and gel filtration chromatography are simply due to a dissociation of the enzyme-cholate complex and not to enzyme instability. Bile salt–dependent activity recoveries are high because the monomeric form is stable and it is activated by exogenous sodium cholate during the *in vitro* assay.

Immunoinhibition by Antipancreatic CEH. The results of the immunoinhibition experiment illustrated in Figure 1 suggest, at first sight, that the two CEH activities must be due to separate enzymes since bile salt–dependent activity is inhibited by antipancreatic CEH and bile salt–independent activity is not. However, these results are also readily explained in terms of our model which invokes the presence of only one enzyme. The bile salt–independent activity is not inhibited by the antibody even at high concentrations, because the antibody does not bind to the enzyme-cholate complex, the complex responsible for bile salt–independent activity. On the other hand, bile salt–dependent activity is inhibited by the antibody even at low concentrations, because the antibody can bind to the enzyme monomers responsible for the observed bile salt–dependent activity. When sodium cholate is added after incubation of cytosol and antibody in order to assay for bile salt–dependent activity, sodium cholate will not activate the enzyme monomer–antibody complex, thus resulting in the observed inhibition of bile salt–dependent activity. The possible validity of this interpretation was tested in one of the experiments discussed below.

Predictions Born Out from the Model. Two independent experiments suggested by the model were carried out. The first experiment was to add cholate to a cytosolic mixture known to contain bile salt–dependent cholesteryl ester hydrolase activity during the preincubation with the antibody. If enzyme monomers and cholate micelles associate before the antibody has a chance to bind to the monomer, the prediction from the model is that the bile salt–dependent activity will not be inhibited by the antibody. Figure 5 shows the results of this experiment; bile salt–dependent activity is significantly less inhibited by several concentrations of antibody when cholate is present during the preincubation than when it is absent. The cholate interaction with enzyme monomers seems to be specific rather than a general detergent effect since a structurally related detergent, CHAPS, does not prevent immunoinhibition by anti-CEH (Fig. 5).

The second experiment was to determine whether the presence of cholate could bring a shift in the elution of the bile salt–dependent activity from a gel filtration column. In line with the model, in the absence of cholate during gel filtration, bile salt–dependent activity will be detected where the monomer elutes; in the presence of cholate, complexation of monomers with cholate micelles will produce a molecular aggregate that is much bigger than enzyme monomers alone, and therefore it will elute earlier than enzyme monomers. Figure 6 shows the elution profiles from a Superose 12 gel filtration column of bile salt–dependent activity in the absence and presence of cholate. As predicted, there is a significant shift in the elution of the bile salt–dependent CEH activity consistent with the idea that cholate brings about the formation of a large macromolecular aggregate.

In conclusion, the data presented here are consistent with the suggestion that the CEH activity dependent on high bile salt concentration and the bile salt–independent CEH activity found in rat liver cytosol may originate from the same enzyme. The purification of a bile salt–dependent hepatic CEH has been reported (5), and the biochemical properties of the purified protein support the idea that this hepatic CEH is identical to or closely related to pancreatic CEH. Definitive proof for the suggestion made here, namely that this protein is also responsible for the operationally defined bile salt–independent CEH activity, will have to await the purification and biochemical characterization of this larger and more labile aggregate.

We thank Kathleen Popoff for her expert technical assistance. This research was supported by NIH Grant HL22633 and by funds from the Howard Heinz Endowment. CR was the recipient of a postdoctoral fellowship awarded by the American Heart Association Southeastern Pennsylvania Affiliate.

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