The use of phospholipid vesicles for in vitro studies on cholesteryl ester hydrolysis

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Abstract Radiolabeled cholesteryl oleate was incorporated into vesicles prepared from egg yolk lecithin and utilized as a substrate for studies of sterol ester hydrolases present in rat liver homogenates. The cholesteryl oleate was shown to be associated with vesicles (unilamellar liposomes) using Sepharose 4B chromatography. With this substrate, two different cholesteryl ester hydrolytic enzymes were demonstrated in subcellular fractions from the liver homogenates. In the lysosome-rich fraction an acid hydrolase was present, while in the cytosol fraction (150,000 *g* supernatant), hydrolytic activity was shown to occur with an optimum pH between 8 and 8.5.

The substrate was characterized by Sepharose chromatography both before and after incubation with the liver fraction and was not dramatically altered even by rigorous incubation conditions. The lysosomal enzyme preparation was capable of hydrolyzing almost all the cholesteryl oleate in the vesicles. Hydrolysis of the phospholipid was proportionately much less than that of the cholesteryl oleate. Comparisons were performed between the vesicle preparation and an alternate substrate preparation involving the direct addition of cholesteryl oleate in acetone solution. The vesicles appeared to be a better substrate for the lysosomal enzyme whereas the activity in the cytosol fraction did not distinguish between the two substrate preparations. Unsonicated suspensions of cholesteryl oleate and lecithin did not serve as suitable substrates for the enzymes. These studies demonstrate the applicability of cholesteryl ester-containing vesicles as a useful substrate for studying cholesteryl ester hydrolysis in vitro.

Supplementary key words sterol ester hydrolase · liposome · lysosome · rat liver

The presence of sterol ester hydrolase (EC 3.1.1.13) (cholesterol esterase) has been documented in many different tissues and cell types using a wide range of in vitro conditions. The properties of the enzymatic activity measured have differed with respect to requirements for cofactors, pH dependency, and subcellular localization (1–14). Various modes of presenting the substrate to the enzyme system have been used, although the physical state of the substrate was usually not clearly defined. The enzymes involved in sterol ester hydrolysis have been reviewed (15) with emphasis on the various modes of substrate presentation.

Earlier studies by Deykin and Goodman (1) demonstrated the presence of soluble liver cholesterol esterase, using as substrate acetone solutions of labeled cholesteryl esters that were forcibly injected into the aqueous assay system, presumably forming microdroplets of the ester. Under such conditions, activity was observed only at pH values above 5.0 with the optimum between 6.5 and 7.5. Nilsson, Norder, and Wilhelmsson (5) demonstrated an acid hydrolase in rat liver lysosomes that was active on cholesteryl ester substrate presented as emulsions or incorporated into lipoproteins. An acid hydrolase also has been found in human liver homogenates using a substrate containing cholesteryl ester dispersed in albumin and Triton X-100 (4).

In the current study, we have utilized sonicated preparations of radioactively labeled cholesteryl oleate and egg yolk lecithin to study sterol ester hydrolase activity in rat liver homogenates. Such preparations form stable suspensions in water and serve as useful substrates for at least two different enzymes present in rat liver subcellular fractions.

EXPERIMENTAL PROCEDURES

Materials

[4-^{14}C]Cholesteryl oleate (sp act 54 mCi/mmole) and cholesteryl [1-^{14}C]oleate (sp act 55 mCi/mmole) were obtained from New England Nuclear Corporation, (Boston, Mass.), and 1,2, di[1-^{14}C]oleoyl-*sn*-glycero-3-phosphorylcholine (sp act 30.0 mCi/mmole) was from Applied Science Laboratories, Inc. (State College, Penn.).

Egg yolk lecithin was obtained from Lipid Products (Nutley, U. K.), sodium taurocholate from Maybridge Research Chemicals (N. Cornwall, U. K.), Sepharose 4B was a product of Pharmacia (Piscataway, N. J.) and cholesteryl oleate was from Applied Science Laboratories, Inc.

Preparations of Vesicles

Most experiments in this study utilized vesicles containing lecithin and cholesteryl oleate at a molar ratio of 66:1. The standard preparation was routinely made by dissolving egg yolk lecithin (60 mg), cholesteryl oleate (600 μg), and [4-^{14}C]cholesteryl oleate (8.0 μCi) or cholesteryl [1-^{14}C]oleate (8.0 μCi) in chloroform–methanol 2:1. The solvent was evaporated under a stream of nitrogen, and the mixture was lyophilized overnight to remove all traces of solvent. The lipids were then resuspended in 6.0 ml of a solution of 0.1 M NaCl, 0.01 M Tris (pH 7.4), and 0.02% sodium azide. A suspension was made by stirring the solution in a vortex type mixer for 4 min, at which time no lipid could be seen adhering to the walls of the glass centrifuge tube. The mixture was then sonicated in a glass jacketed cell at a temperature just above the melting point of cholesteryl oleate (16), under a stream of nitrogen, for 10 min using a Branson W-350 sonifer with an output setting of 3 (about 85 watts) (Branson Instruments, Stamford Conn.). The resulting suspension was centrifuged at 12,000 *g* for 30 min to sediment small fragments of titanium released from the standard 0.5 inch disruptor horn. No lipid was observed to sediment, and recovery of phospholipid and radioactive cholesteryl oleate in the clear supernatant was greater than 95%. The preparation was stored at 4°C under nitrogen and used for enzymatic studies within two weeks of preparation.

To prepare vesicles containing labeled phospholipid, the above procedure was followed, except that 8.0 μCi of labeled dioleoyl lecithin was added. The labeled cholesteryl oleate (8.0 μCi) was omitted and replaced with an equivalent amount (90 μg) of unlabeled cholesteryl oleate.

To prepare vesicles containing greater amounts of cholesteryl oleate relative to lecithin (e.g., a 1:1 molar ratio), the procedure used was similar to that described above, except that increased amounts of unlabeled cholesteryl oleate (60 mg) were initially mixed with lecithin and labeled cholesteryl oleate. After sonication and centrifugation, negligible amounts of lipid sedimented with the titanium. However, the supernatant was opalescent and appreciable amounts of lipid floated to the top of the centrifuge tube. This lipid was resuspended with the opalescent solution and transferred to siliconized vials for future use. Recovery of phospholipid averaged 90% while only about 70% of the cholesteryl ester was recovered in the final preparation. The 1:1 molar ratio was based on the concentration of phospholipid and cholesteryl oleate present in the preparation after sonication, centrifugation, and resuspension. Phospholipid was determined using the method of Bartlett (17).

Sepharose Chromatography

Sepharose 4B columns (2.6 × 45 cm) pre-equilibrated with 0.1 M NaCl, 0.01 M Tris (pH 7.4), and 0.02% sodium azide were used for characterizing the vesicle preparations. 0.5 ml of the vesicle preparation was applied to the column and the sample eluted with the buffer used for pre-equilibration at a flow rate of 25 ml per hour. The fractions collected from the column were analyzed for phospholipid (17), and radioactivity was determined by adding 0.5 ml aliquots to a scintillation cocktail containing toluene (750 ml), Triton X-100 (250 ml), water (180 ml), 2,5, diphenyloxazole (5.0 g) and *p*-bis-[2-(5-phenoxazoyl)]-benzene (400 mg). To compare the chromatographic properties of the vesicles before and after incubation, small Sepharose 4B columns (0.9 cm × 40 cm) were employed and less sample was applied (0.2 ml). Procedures for pre-equilibration, flow rate, and fraction collection were similar to those described above, and chromatography was conducted at 2°C.

Tissue Preparation

Female Sprague-Dawley rats weighing 200–250 g were used for all studies. To prepare the liver lysosomal fraction, a modification of a procedure described by Teng and Kaplan (18) was followed. Four to five animals were killed by guillotine and the livers were removed and homogenized in 4 volumes of 0.25 M sucrose 0.01 M Tris (pH 7.5), 1.0 mM EDTA, using a Virtis homogenizer at a setting of 5,000 rpm for 20 sec. The homogenate was centrifuged at 1,000 *g* for 10 min, and the resulting supernatant was centrifuged at 3300 *g* for 20 min, and then the 3300 *g* supernatant was spun at 12,000 *g* for 35 min. The brown pellet was resuspended in the sucrose solution and recentrifuged at 12,000 *g* for 20 min. The resulting pellet, considered to be the lysosomal fraction, was resuspended in 0.25 M sucrose, 0.01 M Tris (pH 7.4) and used for enzymatic studies. For studies on the cytosol fraction, the original 12,000 *g* supernatant from the liver homogenate was recentrifuged at 150,000 *g* for 45 min to obtain the high speed supernatant (cytosol fraction).

For selected experiments the lysosomal fraction was disrupted by resuspending the washed pellet in 0.1 M NaCl, 0.01 M Tris, pH 7.4, vigorously homogenized in an all glass apparatus and subjected to repeated freeze-thawing (10 times). The preparation was then centrifuged at 150,000 *g* for 30 min and ammonium sulfate was slowly added to the re-

sulting supernatant to a final concentration of 30%. After centrifugation at 30,000 *g* for 20 min the ammonium sulfate precipitate was resuspended in a small volume of 0.1 M NaCl and passed through a Sephadex G-25 column to remove traces of ammonium sulfate. The resulting solution (solubilized lysosomal fraction) was used for enzymatic studies.

Enzyme Assay

Incubations were performed in siliconized 15-ml conical centrifuge tubes with glass stoppers. All determinations were performed in duplicate. For the lysosomal fractions, the standard incubation mixture usually contained 0.1 ml of 0.15 M acetate buffer (pH 5.0), 0.2 ml of the lysosomal fraction containing approximately 0.5 mg protein, and 10 μl of the standard vesicle preparation (molar ratio of 66:1). For the cytosol fraction, the incubation mixture routinely contained 0.1 ml of 0.1 M Tris buffer (pH 8.0), 0.2 ml of the cytosol fraction containing approximately 2–3 mg protein, and 10 μl of the standard vesicle preparation. Thus, the final concentration of vesicle-associated phospholipid and cholesteryl oleate in the incubation mixtures was 390 μM and 5.9 μM, respectively. Fifteen-min incubations at 37°C were routinely performed for studies with the lysosomal fraction, whereas 2 hr incubations were used when assaying activity in the cytosol fraction. Conditions utilizing the solubilized lysosomal fraction are described in the appropriate figure legends. Protein was determined according to the method of Lowry et al (19).

For experiments designed to determine the effect of incubation on the Sepharose elution patterns of the vesicles, incubation conditions were adjusted to increase the amount of vesicles present in the reaction mixture and to minimize the contribution of tissue extract. For the cytosol fraction, 200 μl of the substrate preparation was incubated with 100 μl of high speed supernatant (about 1.5 mg of protein) at pH 8.0 (0.1 M Tris) for 90 min at 37°C. For the solubilized lysosomal fraction, 200 μl of the substrate were incubated with 50 μl of tissue extract (40 μg of protein) at pH 4.5 for 30 min at 37°C. After incubation, 20-μl aliquots were taken to determine the extent of hydrolysis and 0.2 ml of the incubation mixture was applied to sepharose 4B columns. Controls were performed by adding a mixture of vesicles and tissue fraction at the appropriate pH to the Sepharose column after incubation at 0°C. Chromatography was conducted at 2°C in all studies. Aliquots of the column effluent were analyzed for radioactivity, lipid phosphorus, and extent of hydrolysis.

Two methods were used to determine the hydrolysis

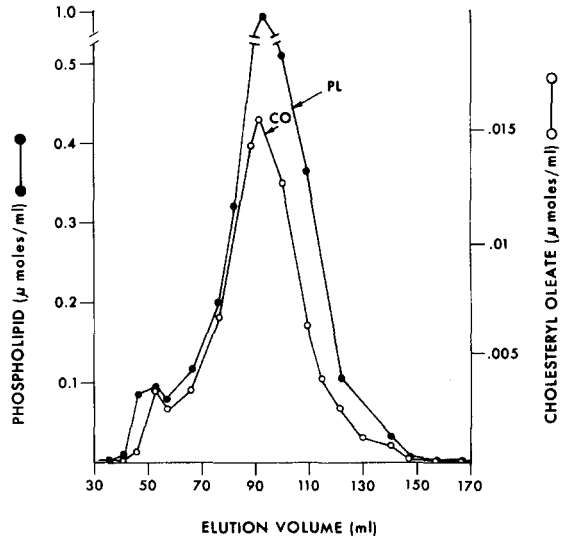

Fig. 1. Sepharose 4B chromatography of standard vesicle preparation (mole ratio 66:1). The specific activity of the labeled cholesteryl oleate in the preparation was 7.5 μCi/μmole. CO, cholesteryl oleate; PL, phospholipid.

of labeled cholesteryl oleate. When [4-^{14}C]cholesteryl oleate was used as labeled substrate, the reaction was terminated by the addition of 6.0 ml of chloroform–methanol 2:1 containing unlabeled cholesterol (10 μg/ml) and cholesteryl oleate (10 μg/ml). After the addition of 1.2 ml of 0.02% $CaCl_2$, the tubes were shaken and centrifuged at 3000 *g* for 10 min. The lower phase was transferred to 5-ml conical tubes, evaporated under nitrogen, redissolved in 20 μl of chloroform and 5 μl were applied to plastic-coated silica gel plates (Eastman Chromagram sheets). The plates were developed in petroleum ether–diethyl ether–glacial acetic acid 95:5:1; the lipid classes were isolated and the radioactivity was determined by scintillation counting.

When the incubation mixture contained cholesteryl ester labeled in the fatty acid moiety with [1-^{14}C]oleate, a minor modification of the method described by Pittman, Khoo, and Steinberg (14) was employed. The reaction was terminated by the addition of 3.0 ml of benzene–chloroform–methanol 1:0.5:1.2 containing unlabeled oleic acid (0.1 mM) as carrier. 0.6 ml of 0.3 M NaOH was then added, and the mixture was stirred for 20 sec and centrifuged at 1000 *g* for 10 min. 0.5 ml of the upper phase (containing free oleic acid) was added to a Triton–toluene scintillation cocktail and the radioactivity was determined. A comparison between the two methods using identical incubation conditions designed to obtain about 25% hydrolysis and conducted in sextuplicate were conducted. When the first procedure in which thin-layer chromatography was used, the percent hydrolysis was 26.1 ± 1.2 (mean ± SE). The corresponding value

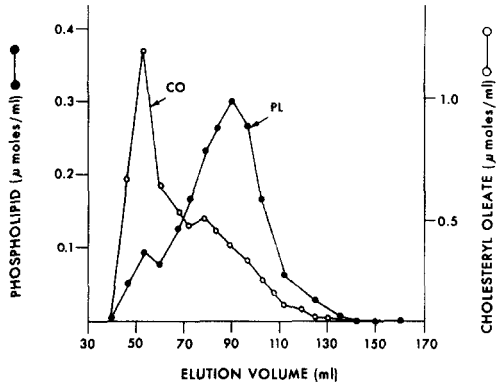

Fig. 2. Sepharose 4B chromatography of the cholesteryl ester-rich vesicle preparation (mole ratio 1:1). The specific activity of the labeled cholesteryl oleate was 0.087 μCi/μmole. CO, cholesteryl oleate; PL, phospholipid.

using the solvent partition method was 24.0 ± 0.4. Thus, both procedures produced comparable results, although the latter was easier, more sensitive, and more reproducible; it is now routinely used in our laboratory.

RESULTS

Data illustrating the distribution of phospholipid and radioactive cholesteryl oleate in the column effluent after chromatography of vesicle preparations on Sepharose 4B are shown in **Fig. 1.** When the mole ratio of lecithin to cholesteryl oleate was 66:1, essentially all the radioactivity was included within the Sepharose 4B and was eluted with the phospholipid as a symmetrical peak corresponding to a particle size of about 250 Å, characteristic of unilamellar vesicles (20). In contrast, when the mole ratio was 1:1, most of the radioactivity appeared immediately after the void volume, whereas the phospholipid was again eluted in a region corresponding to the unilamellar vesicles (**Fig. 2**). With both preparations, little adsorption of cholesteryl ester onto the Sepharose occurred since recovery of radioactivity off the columns was over 90%.

Other workers have reported the presence in rat liver of a lysosomal sterol ester hydrolase that was active at low pH (5) and a soluble enzyme with activity at pH values above neutrality (1). To determine whether these enzymes could be assayed using the vesicles as substrate, we attempted to separate the activities by subcellular fractionation. The liver lysosomal fraction was prepared and a high speed supernatant fraction was obtained from the same homogenate. Both fractions were assayed for hydrolase activity as a function of pH (**Fig. 3**). The lysosomal fraction exhibited greatest activity at pH values between 4 and 5.5 whereas the high speed supernatant fraction gave negligible hydrolysis in this range, the activity being greatest in the pH range between 7.5 and 8.5.

Fig. 4 illustrates both the effect of incubation time (Fig. 4A) and protein concentration (Fig. 4B) on the hydrolysis of cholesteryl ester by both tissue fractions. Activity was considerably greater in the lysosomal fraction. Of particular interest was the observation that under appropriate conditions, over 90% of the added cholesteryl oleate could be hydrolyzed by the lysosomal preparation (Fig. 4A), suggesting that most of the ester within the vesicle was accessible to the enzyme under these incubation conditions.

Hydrolysis in both the lysosomal and high speed supernatant fractions appears to be dependent on the

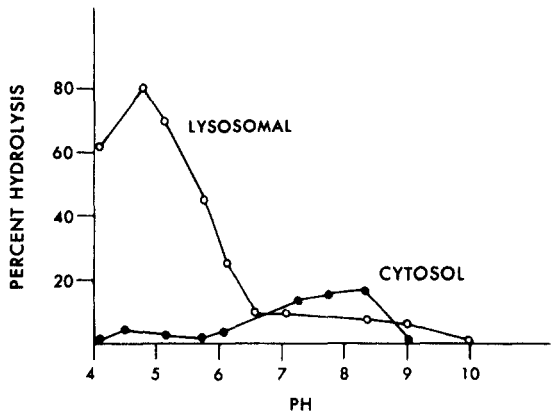

Fig. 3. Effect of pH on cholesteryl ester hydrolysis by lysosomal and cytosol liver fractions. Incubations were conducted by adding 0.2 ml of the appropriate tissue fraction to 0.1 ml of buffer containing 10 μl of the standard vesicle preparation. The buffers used were 0.15 M acetate (pH 3.5–5.5), 0.1M phosphate (pH 6.0–7.5), 0.05 M Tris (pH 7.5–8.5) and 0.1 M glycine (pH 9–10.5). The pH values on the abscissa represent the final pH of the entire incubation system. Incubations were at 37°C for 15 min for lysosomes and 120 min for the cytosol fraction.

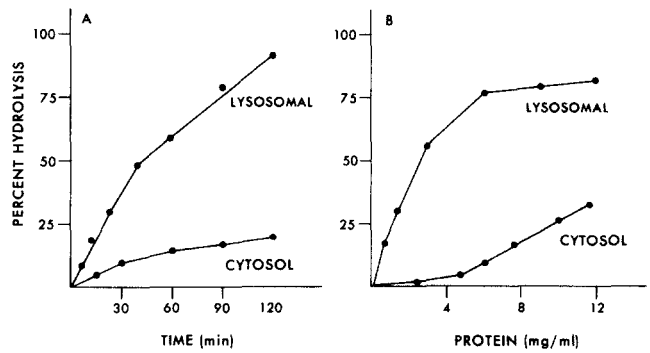

Fig. 4. Effect of incubation time (4A) and protein concentration (4B) on cholesteryl ester hydrolysis by the lysosomal and cytosol liver fractions. In Fig. 4B, incubations were conducted at pH 5.0 for 15 min and pH 8.0 for 120 min for the lysosomal and cytosol fraction, respectively.

physical state of the substrate. The data in **Table 1** show the hydrolysis of vesicle preparations containing different mole ratios of lecithin and cholesteryl ester. The percent hydrolysis was considerably lower using the preparation of lecithin:cholesteryl ester with a mole ratio of 1:1, as compared to the standard preparation with a mole ratio of 66:1. This might be explained by saturation of the enzyme by the much greater amount of cholesteryl ester present in the 1:1 preparation. However, if a preparation containing unlabeled cholesteryl oleate and phospholipid at a mole ratio of 1:1 was added to the standard 66:1 preparation (containing labeled cholesteryl oleate), the percentage of labeled material hydrolyzed was similar to the control incubation containing the 66:1 preparation alone. This suggests that the presence of the 1:1 preparation did not interfere with the hydrolysis of the labeled cholesteryl ester present in the 66:1 preparation. Table 1 also shows that addition of the suspension of lecithin: cholesteryl oleate (66:1) obtained just prior to sonication was relatively ineffective as a substrate for either enzymatic activity.

Further data on the properties of the incubation system are given in **Table 2**. *N*-Ethyl maleimide and *p*-chloromercuribenzoate inhibited enzymatic activity in both preparations at concentrations of 5.0 mM and 2.0 mM, respectively. Triton X-100 also reduced enzymatic activity while bovine serum albumin and sodium taurocholate enhanced the activity in both preparations. EDTA (5.0 MM), Ca^{2+}, Mg^{2+}, and Mn^{2+} (all at 5.0 mM), and unlabeled cholesterol (0.2 mM) or cholesteryl oleate (0.2 mM) added in a small volume of acetone, were all ineffective in altering hydrolysis of vesicle-associated cholesteryl oleate.

Experiments were performed to determine whether the incubation conditions altered the elution pattern of the vesicles from Sepharose columns. Incubation conditions were adjusted so that a relatively large amount of substrate was incubated with a relatively small amount of tissue protein (see Experimental Procedures). The solubilized lysosomal fraction was used for these studies since the ratio of the protein concentration (mg/ml) to the phospholipid concentration (mg/ml) of this fraction was considerably greater

TABLE 1. Effect of substrate presentation on cholesterol ester hydrolysis in rat liver fractions

Substrate	Lysosomal	Cytosol
	% hydrolysis	
Vesicles (66:1)[a]	55.2	16.4
Vesicles (1:1)[a]	14.4	2.1
Combination (66:1)[a] + (1:1)	51.1	16.2
Unsonicated suspension (66:1)[a]	2.1	0

[a] Contains [4-^{14}C]cholesteryl oleate.

Lysosomal and cytosol fractions were incubated under standard conditions with vesicle preparations of 66:1 or 1:1 molar ratio of phospholipid to labeled cholesteryl ester. A 1:1 preparation containing no radioactive lipid was used in combination with the labeled 66:1 preparation. The unsonicated suspension was an aliquot of the 66:1 preparation taken just prior to sonication. The experiments were performed on the same day the vesicles were prepared.

TABLE 2. Effect of various substances on cholesteryl ester hydrolysis by rat liver fractions

Substance	Concentration	Lysosomal	Cytosol
		Hydrolysis (% of control)	
N-Ethyl maleimide	5.0 mM	6.2	2.1
p-Chloromercuribenzoate	3.0 mM	3.8	0
Triton X-100	1.0%	62.1	4.2
Bovine serum albumin	0.1%	112.0	150.5
Sodium taurocholate	1.6 mM	128.4	230.5

Lysosomal and cytosol fractions were incubated under standard conditions. All incubation tubes contained 10 μl of the vesicle preparation (66:1) and the appropriate amount of the substance being tested. The hydrolysis of [4-^{14}C]cholesteryl oleate in the samples was expressed as a percent of the hydrolysis observed in control incubations lacking any additive.

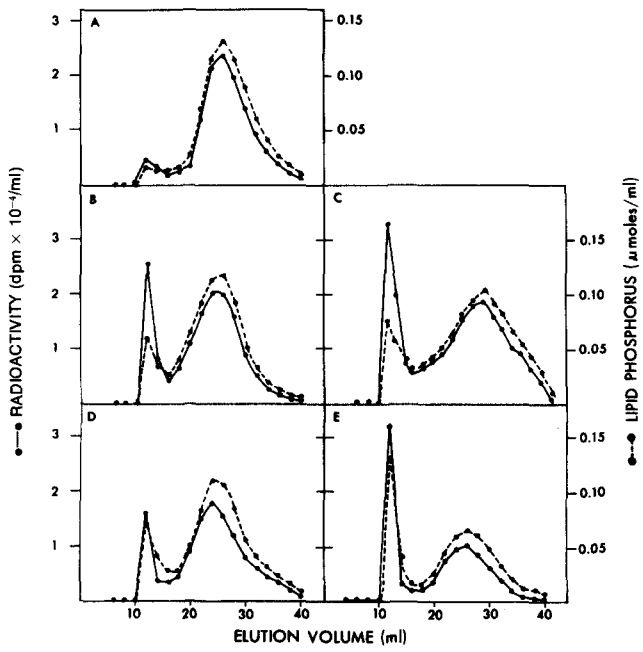

Fig. 5. Effect of incubation with the solubilized lysosomal fraction or the cytosol fraction on the Sepharose elution pattern of vesicles (66:1) containing [4-^{14}C]cholesteryl oleate. Incubations and Sepharose 4B chromatography were performed as described in the experimental section. A, vesicles alone, pre-incubated at 37°C for 2 hr; B, vesicles and cytosol fraction 0°C/2 hr; C, vesicles and cytosol fraction 37°C/2 hr; D, vesicles and solubilized lysosomal fraction 0°C/30 min; E, vesicles and solubilized lysosomal fraction 37°C/30 min. The percent hydrolysis of cholesteryl oleate in the total incubations was 14% for the incubation of Fig. 5C and 48.4% for Fig. 5E.

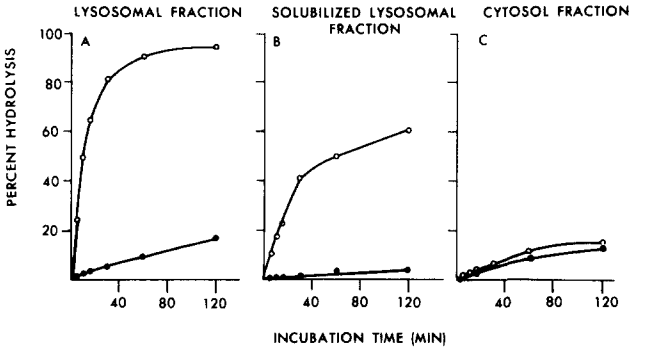

Fig. 6. The hydrolysis of lecithin and cholesteryl oleate contained in vesicles by liver subcellular fractions. Each subcellular fraction was incubated for different times with a vesicle preparation containing unlabeled egg yolk lecithin and cholesteryl [1-^{14}C]oleate ((66:1) or [1-^{14}C]dioleoyl lecithin and unlabeled cholesteryl oleate (66:1). All incubations were conducted at 37°C in a total volume of 0.3 ml and contained 0.55 mg of protein, 0.08 mg of protein and 2.8 mg of protein for the lysosomal, solubilized lysosomal, and cytosol fractions, respectively. The lysosomal fractions were incubated at pH 5.0 and the cytosol fraction at pH 8.0. ○ —— ○, cholesteryl ester hydrolysis; ● —— ●, phospholipid hydrolysis.

(35:1) than that of the original lysosomal fraction (3:1). Only trace amounts of phospholipid were present in the cytosol fraction. As controls, vesicles were incubated in the absence of the tissue fractions or incubated with the tissue fractions at 0°C.

Fig. 5 shows the Sepharose elution patterns for the incubation mixtures, using both the cytosol and the solubilized lysosomal fractions. Increased amounts of phospholipid and cholesteryl oleate were found at the void volume (first peak) for all samples chromatographed in the presence of the tissue fraction even when the incubation was at 0°C. This change in the elution profile probably represents association of the vesicles with aggregated protein, which also elutes at the void volume. However, in all cases most of the lipid was included within the column (second peak) with the labeled cholesteryl ester in close association with the phospholipid, suggesting the maintenance of the vesicle structure throughout the incubation procedure. Further studies were performed in which the extent of cholesteryl ester hydrolysis was determined for the individual fractions. For the cytosol incubation, the percentage hydrolysis for the fractions at the void volume and the included region both were 14%, agreeing with the percent hydrolysis for the total incubation obtained from an aliquot prior to chromatography. In the incubation with the solubilized lysosomal fraction, the percent hydrolysis for the whole incubation was 48.4%; however, fractions in peak 2 averaged 52.9% whereas those in peak 1 were 38.2%. No hydrolysis was observed in the fractions from control incubations. The labeled cholesterol formed as a result of hydrolysis remained associated with the vesicles. Experiments similar to those shown in Fig. 5 were performed using vesicles containing cholesteryl oleate labeled in the fatty acid moiety. No quantitative differences were seen in the distribution of labeled free fatty acid in the eluted fractions, however considerable adsorption of free fatty acid onto the Sepharose did occur when the vesicles were incubated with the solubilized lysosomal fraction of pH 4.5. Thus, the cholesteryl ester and the free cholesterol remained associated with the vesicles even with the prolonged incubations used for these studies and the vesicles were not dramatically altered. Furthermore, hydrolysis appeared to occur in the material eluting either at the first or second peak, although a greater percentage of the reaction product was found in the second peak for the lysosomal incubation.

We also determined the relationship between the hydrolysis of cholesteryl ester and the phospholipid within the vesicles by the tissue fractions (**Fig. 6**). Vesicles were prepared containing either labeled lecithin or labeled cholesteryl oleate and incubated under identical conditions with the lysosomal fraction, the solubilized lysosomal fraction, and the cytosol

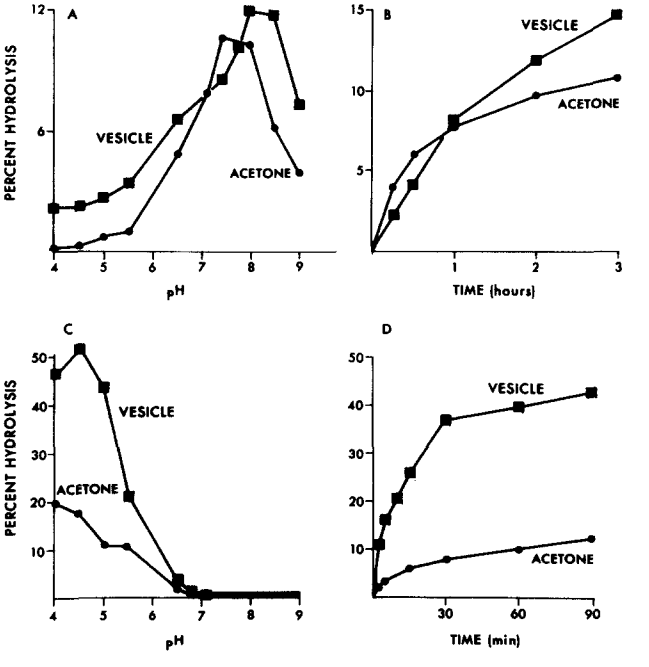

Fig. 7. Hydrolysis of cholesteryl [1-^{14}C]oleate contained in vesicles or dissolved in acetone. Labeled cholesteryl oleate was added to incubation tubes containing cytosol fraction or the solubilized lysosomal fraction and hydrolysis was measured as a function of pH or incubation time. All incubations were conducted at 37°C in a total volume of 0.3 ml and the final cholesteryl oleate concentration was 5.9 μM. A, cytosol fraction, incubation time 2 hr; B, cytosol fraction, pH 8.0; C, solubilized lysosomal fraction, incubation time 60 min; D, solubilized lysosomal fraction, pH 5.0.

fraction. In both lysosomal preparations the percent hydrolysis of cholesteryl oleate was considerably greater than that of the lecithin whereas in the soluble fraction similar values were obtained for both lipids. The data for the lysosomal fraction showed that, at a time when 50% of the cholesteryl oleate was hydrolyzed, only 2–3% of the phospholipid was altered.

An alternate procedure for measuring cholesteryl ester hydrolysis in liver tissue has been to introduce the labeled ester from an acetone solution directly into the tissue fraction. We compared the hydrolysis of equivalent amounts of cholesteryl ester contained either in vesicles or introduced directly from an acetone solution. Hydrolysis was studied both in the cytosol fraction and the solubilized lysosomal fraction with respect to reaction rate and pH (**Fig. 7**). In the cytosol fraction only slight differences were observed between the two substrates. Both the pH activity profile and the rate of hydrolysis were similar for both substrate preparations (Fig. 7A & B). Using the solubilized lysosomal fraction (Fig. 7C & D), the preparation was several-fold more effective in hydrolyzing cholesteryl oleate contained in vesicles than when added directly from an acetone solution. With both substrate preparations, greatest hydrolysis was seen at acid pH with no activity seen at pH values above neutrality (Fig. 7C). At all pH values in the acid region the cholesteryl ester in the vesicles was hydrolyzed more effectively than if it were added directly from an acetone solution.

An additional advantage of the vesicle preparation over the acetone addition was the precision of the assay technique. Small volumes of the liposome preparation can be added to individual incubation tubes and the hydrolysis can be determined accurately. However, when small volumes of acetone were added, the results were less reproducible. In a comparative study performed in sextuplicate, using the solubilized lysosomal fraction and adding equivalent amounts of cholesteryl oleate in acetone or in the vesicles, the hydrolysis after incubation at 37°C for 15 min was 2.98 ± 0.85% and 24.0 ± 1.0%, (mean ± SD) respectively. The coefficient of variation was appreciably less using the vesicles as substrate than for the acetone addition.

DISCUSSION

The phospholipid vesicles used for these studies (mole ratio 66:1) were shown to consist predominantly of unilamellar liposomes (vesicles) and they were a suitable substrate for the assay of two different enzyme systems in rat liver capable of hydrolyzing cholesteryl ester. The vesicles were readily suspendible in the assay systems and enzymatic activity could be demonstrated solely in the presence of lecithin and cholesteryl ester, requiring no additional detergents, lipids, proteins, or organic solvents for substrate stabilization. The cholesteryl ester in the preparation was associated with the lecithin after Sepharose 4B chromatography. The fact that small amounts of cholesteryl ester were associated with the vesicles is consistent with recent phase equilibrium studies of egg lecithin and cholesteryl esters which show that only very small amounts (i.e. 1 cholesteryl ester to 25–50 lecithin molecules) can be incorporated into the lecithin bilayer in aqueous systems (21, 22). Since the lysosomal incubation system could be adjusted to hydrolyze over 90% of the cholesteryl oleate in these vesicles, one can conclude that the ester is incorporated within the phospholipid bilayer in such a manner as to be accessible to the enzyme. In contrast, when the molar ratio of phospholipid to cholesteryl oleate was 1:1, most of the cholesteryl oleate was not associated with phospholipid as determined by Sepharose chromatography, suggesting different physical states for the cholesteryl ester in the two preparations. Phase equilibrium studies (21, 22) indicate that most of the cholesteryl ester in the 1:1 preparation would be present as a separate phase of pure ester. Since the melting point of cholesteryl oleate is about 50.5°C (16), at 37°C, which is the temperature of the standard incubation in these studies, the cholesteryl oleate not incorporated into the liposome would be in a crystal or metastable liquid crystal state (16). Work in progress involves the characterization of cholesteryl ester–lecithin liposome preparations by physical-chemical techniques and future studies will involve detailed analysis of the effect of the physical state of the substrate on the kinetics of hydrolysis.

The lysosomal fraction contained far more hydrolytic activity than the soluble fraction, consistent with the findings of Nillson et al (5). The dissimilar pH activity profiles of both types of enzymatic activity and the separation of such activity into two separate subcellular fractions strongly suggest that two different enzymes are involved. The lysosomal fraction used for our studies was obtained by differential centrifugation and almost certainly contains mitochondria, peroxisomes, and other organic fragments, making it difficult to state with certainty that the acid hydrolase is exclusively localized within lysosomes.

We found that the activities in both the lysosomal and soluble fractions were stimulated by albumin or sodium taurocholate. Although other workers have

utilized these substances in substrate preparations to study cholesteryl ester hydrolysis (4, 5, 7, 11, 12), it is not clear from any study whether the enhanced activity is due to an effect on the substrate or the enzyme. Such questions would best be answered by using purified enzyme preparations, and we are currently attempting to purify both enzymes.

Our studies are the first to utilize liposomes as a "carrier" for cholesteryl ester for studies on sterol ester hydrolase. Kamp, Wirtz, and Van Deenen (23) incorporated labeled cholesteryl oleate into phospholipid vesicles for studies on phospholipid exchange between liposomes and biological membranes. In their studies the cholesteryl oleate incorporated into vesicles was not influenced by the phospholipid exchange process being investigated. Labeled cholesteryl palmitate was recently used as a nonreactive marker for liposomes in a study of the homing of liposomes to target cells (24). A major advantage in using cholesteryl ester-containing phospholipid vesicles for enzymatic studies is that they can be readily characterized by biophysical techniques, a fact which could make future studies on the mechanism of action of sterol ester hydrolases more meaningful.

The physical state of the substrate is rarely determined in most studies relating to cholesteryl ester hydrolysis. The preparation used as a substrate for the studies reported here was characterized by Sepharose chromatography both before and after incubation with liver fractions. The cholesteryl oleate contained in the vesicle remained associated with the lecithin even after extensive incubation. The Sepharose elution pattern indicated no dramatic change in the cholesteryl oleate–lecithin interaction, although aggregation in the presence of the tissue fractions did occur. Since only a small percentage of the total egg yolk lecithin was degraded during the incubation periods used for these studies, the integrity of the phospholipid bilayer was probably not altered appreciably.

The cholesterol esterase activity derived from the lysosomal fraction was more effective in hydrolyzing cholesteryl oleate associated with vesicles than when cholesteryl oleate was added directly in acetone. The physical state of cholesteryl oleate after being added in acetone to the aqueous system is not known, although this method of substrate presentation has been used frequently for studies of cholesteryl ester hydrolysis. Since the acid hydrolase may be involved in hydrolyzing cholesteryl esters contained in lipoproteins (13), it may be advantageous to investigate the effect of phospholipid–cholesteryl ester–apoprotein interactions on hydrolysis using a model system such as vesicles.

An understanding of the mechanism by which cholesteryl esters are hydrolyzed is necessary to fully understand the dynamics of cholesterol and cholesteryl esters within cells. If it is true that cholesteryl esters exist within biological membranes in a manner analogous to their presence in vesicles and phospholipid bilayers (21, 22), such membranes may serve as a stable "support" for the water-insoluble esters and thereby aid in the regulation of cholesteryl ester hydrolysis by either soluble or membrane-associated enzymes.

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