Effects of sphingomyelin and cholesterol on lipoprotein lipase-mediated lipolysis in lipid emulsions

Itaru Arimoto,* Hiroyuki Saito,† Yasuko Kawashima,* Koichiro Miyajima,* and Tetsurou Handa[1,*]

Faculty of Pharmaceutical Sciences,* Kyoto University, Sakyo-ku, Kyoto 606-01, Japan, and Division of Drugs,† National Institute of Health Sciences, 1-1-43 Hoenzaka, Chuoh-ku, Osaka 540, Japan

Abstract Lipoprotein lipase (LPL) hydrolyzes triacylglycerol (TG) of TG-rich lipoproteins. We investigated the effects of sphingomyelin (SM) and cholesterol (Chol) on the lipolysis of lipid emulsions by LPL using human apolipoprotein C-II (apoC-II) or plasma as an activator. Kinetic studies of the lipolysis rates clearly demonstrated that the primary effect of the activator on the LPL reaction was not to increase the affinity of LPL for the emulsion surface, but to enhance LPL catalytic activity. Incorporation of SM into the emulsion surface caused increases in $K_{m(app)}$ and decreases in $V_{max(app)}$, indicating that SM inhibited lipolysis by decreasing both affinity for substrates and catalytic activity of LPL. SM was also found to affect possible factors related to the lipolysis rates; that is, SM increased TG solubility in surface layers and decreased apoC-II binding to the emulsion surface. Interestingly, Chol did not affect the lipolysis rates even though it decreased TG solubility and apoC-II binding. These results indicated that neither TG solubility nor amount of apoC-II binding were determinate factors in LPL-mediated lipolysis under physiological conditions. Our results suggest that the content of SM in the lipoprotein surface plays an important role by controlling lipoprotein lipase-mediated lipolysis, and that cholesterol enrichment in the lipoprotein surface has no influence on lipolysis, but may affect other metabolic processes such as uptake by the liver through the selectivity of apolipoprotein binding.—**Arimoto, I., H. Saito, Y. Kawashima, K. Miyajima, and T. Handa.** Effects of sphingomyelin and cholesterol on lipoprotein lipase-mediated lipolysis in lipid emulsions. *J. Lipid Res.* 1998. **39:** 143–151.

Supplementary key words lipoprotein lipase • lipolysis • lipid emulsions • sphingomyelin • cholesterol • apolipoprotein C-II

Lipoprotein lipase (LPL, EC 3.1.1.34) hydrolyzes triacylglycerols in chylomicrons or very low density lipoproteins (VLDL) to monodiacylglycerols and fatty acids in animal plasma. The latter are taken up by cells and utilized as a source of energy (1). During lipolysis, chylomicrons and VLDL become relatively enriched in apolipoprotein (apo)E (2, 3), and resulting remnants are subsequently taken up by the liver through apoE-specific receptors (4–6). LPL has only limited activity unless serum is added as a source of activator. Further studies have provided conclusive evidence that apoC-II, a 79 amino acid residue peptide present in chylomicrons, VLDL, and high density lipoproteins (HDL), acts as the activator of LPL. However, the activation mechanism of LPL by apoC-II has not been characterized in detail. On the other hand, the inhibitory effect on hepatic uptake through the apoE-specific receptors by apoC-II has also been reported (7, 8).

The differences in surface lipid composition of plasma lipoproteins are probably important factors influencing their metabolic fates in animal plasma. We have previously shown that the surface composition of lipid emulsions affects the binding of apolipoproteins to the particle surface (9, 10) and their metabolism in rats (11). The major lipid constituents of plasma lipoprotein surface are phosphatidylcholine (PC), sphingomyelin (SM), and unesterified cholesterol (Chol). The contents of SM and Chol vary widely among plasma lipoproteins (12–18). SM is a phospholipid that is present in cell membranes and plasma lipoproteins. The role of SM in cell membranes, especially with regard to cholesterol homeostasis, has been investigated at several laboratories (19–21). However, the influence of SM on lipoprotein metabolism is poorly understood, although SM is the second most abundant phospholipid next to PC in mammalian plasma. Further, the influence of Chol on lipoprotein metabolism remains to be fully clarified. We have shown that Chol regulates mobility of the surface layers and consequently influences apoli-

Abbreviations: LPL, lipoprotein lipase; SM, sphingomyelin; Chol, cholesterol; apoC-II, apolipoprotein C-II; TG, triacylglycerol; TO, triolein; FFA, free fatty acids; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase.

[1]To whom correspondence should be addressed.

poprotein binding to the surface (9, 10). We presumed that the composition of the emulsion surface plays a crucial role in lipoprotein metabolism, in particular, in LPL-mediated lipolysis.

The purpose of this study is to obtain information about the roles of SM and Chol in the metabolism of plasma lipoproteins. We evaluated the effects of SM and Chol on the apparent maximum velocity ($V_{max(app)}$) and apparent Michaelis-Menten constant ($K_{m(app)}$) of the LPL reaction in the presence of an activating factor, plasma, or apoC-II. Furthermore, binding of apoC-II to the emulsion particles and triolein solubility in the surface layers were estimated, and the physiological relevance of the results to lipoprotein metabolism was discussed.

EXPERIMENTAL PROCEDURES

Materials

Egg yolk phosphatidylcholine (PC) was kindly provided by Asahi Kasei Co. The purity (over 99.5%) was determined by thin-layer chromatography (TLC). Egg yolk sphingomyelin (SM), cholesterol (Chol), and triolein (TO) were obtained from Sigma Chemical Co. (St. Louis, MO). Each sample showed a single spot on TLC (solvent: chloroform–hexane, 4/1 (v/v)) and then was used without further purification. Lyophilized apoC-II from human plasma was purchased from Calbiochem (San Diego, CA). Fatty acid-free bovine serum albumin and sodium heparin were obtained from Sigma. Glyceryl tri[1-^{13}C]oleate ([1-^{13}C]TO) was purchased from Isotec Inc. (Miamisburg, OH). All other chemicals were special grade from Wako Pure Chemicals. Water was doubly distilled with a quartz still.

Preparation of emulsions

The lipid emulsions were prepared by the method described previously (22) using a high pressure emulsifier (Nanomizer; Nanomizer Inc., Tokyo, Japan). Briefly, PC (or PC + SM, PC + Chol) and TO in chloroform at a molar ratio of 1:3 were dried in vacuum, resuspended in 30 ml of 180 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, and 0.01% (w/v) NaN_3, and successively emulsified under a pressure of 700–900 kg/cm^2. The contaminating vesicles were removed by ultracentrifugation. The weight-averaged particle size of each emulsion was about 120 ± 20 nm determined from quasi-elastic light scattering measurements (Photal LPA-3000/3100: Otsuka Electronic Co.).

LPL and apoC-II

Purified bovine milk LPL (5,000 unit, Lot No. 45H8025), suspended in 3.8 M ammonium sulfate, 0.02 M Tris-HCl, pH 8.0, was purchased from Sigma Chemical Co. LPL suspension was centrifuged (10,000 *g*, 20 min, 4°C), and the resulting pellet was dissolved in 50% glycerol in 10 mM Tris-HCl buffer, pH 7.4, and stored at −40°C (final protein concentration, 40 μg/ml). The specimen showed a single band on SDS-polyacrylamide gel electrophoresis (molecular weight: 55 kDa). The molecular weight agreed with the reported value (23). The LPL solution remained fully active over a 3-month period.

The lyophilized human apoC-II was dissolved in 6 M guanidine hydrochloride solution and dialyzed extensively against Tris-buffer. The apoC-II preparation gave a single band of expected molecular weights on SDS-polyacrylamide gel electrophoresis. Concentrations of LPL and apoC-II were determined by micro bicinchoninic acid protein reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard.

LPL activity assay

Various amounts of lipid emulsions were preincubated at 37°C for 20 min with 4% fatty acid-free BSA, 180 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.01% (w/v) NaN_3, 66.7 μg/ml sodium heparin, pH 8.5, with and without human plasma or apoC-II at a total volume of 0.3 ml. Triolein lipolysis was started by addition of 165 ng LPL into incubation mixtures. Released free fatty acids (FFA) during the lipolysis were measured at the indicated times using an enzymatic colorimetric reagent, NEFA C (Wako Pure Chemicals). The rate of FFA release was linear for at least 20 min and the lipolysis rate was determined from the initial slope of the released FFA increase.

ApoC-II binding

Binding amounts of apoC-II to lipid emulsions were determined by an ultracentrifugation method as described (9). Human plasma was centrifuged for 1.5 h at 27,000 rpm in a Beckman SW 28 rotor to remove the top fraction containing chylomicrons. The mixtures of various amounts of emulsions and chylomicron-free plasma were incubated for 20 min at 37°C and centrifuged for 20 min at 32,000 rpm in a Beckman SW 50.1 rotor. The equilibrium concentration of apoC-II in the bottom fraction was determined in single radial immunodiffusion assay plates, purchased from Dai-ichi Pure Chemicals (Tokyo, Japan). The amount of bound apoC-II was calculated from the difference between the apoC-II concentrations before and after ultracentrifugation.

NMR studies

Triolein (TO) solubility in phospholipid vesicles was determined by ^{13}C NMR (24–26). Aqueous lipid dispersions for NMR analysis were prepared by the method

described previously (27, 28). Phospholipid (PC or PC + SM) and 5 mol% [1–^{13}C]TO were mixed in chloroform and the solvent was evaporated under reduced pressure. After drying in vacuum overnight, the lipid film was hydrated with Tris-HCl buffer, vortexed, and sonicated with a probe-type sonicator (UD-200, Tomy Seiko, Tokyo, Japan) for 30 min under a nitrogen stream.

^{13}C NMR spectra were obtained at 75.45 MHz with a Bruker AC-300 spectrometer under inverse-gated decoupling at 37°C. Internal $^{2}H_2O$ was used as a lock and shim signal. Chemical shifts are referenced from the terminal methyl carbon of the fatty acid chains at 14.10 ppm (26, 29). The extent of incorporation of TO into phospholipid vesicles was calculated from the peak intensity ratio The mole % of TO in surface layers (S) is given by

$$S = 5 \text{ mol\%} \times (Su_\alpha + Su_\beta)/(Su_\alpha + Su_\beta + O_\alpha + O_\beta) \quad Eq.\ 1)$$

Where Su_α, O_α, Su_β, and O_β are the signal peak areas of ^{13}C-TO from α-chain 'surface,' β-chain 'surface,' α-chain 'oil' phase, and β-chain 'oil' phase carbonyls, respectively (24, 26).

RESULTS

SM inhibits LPL-mediated lipolysis in lipid emulsions

Figure 1 shows typical time courses of free fatty acid (FFA) release during lipolysis in the presence of 30% plasma. The total phospholipid concentration was kept constant and the mol% of SM was varied from 0 to 100 mol%. The concentration of released FFA increased linearly for at least 20 min in all emulsions. Therefore, from the initial slope of FFA release, the lipolysis rates were calculated as released FFA (mmol)/h per mg LPL. Increase of the mol% SM in surface layers of TO-PC emulsions reduced the lipolysis rates, and about 90% inhibition of LPL activity was observed in TO-SM emulsions (Fig. 1, inset).

Activiation of LPL reaction by plasma and apoC-II

To evaluate the activation mechanism of LPL reaction by human plasma, we examined kinetic parameters of the LPL reaction for TO-PC/SM (2/1, molar ratio) emulsions; that is, 33 mol% of PC in TO-PC emulsions was replaced by SM, in the presence of 0–80% plasma. **Figure 2** shows relationships between the TO concentration of emulsions divided by lipolysis rates and the TO concentration of emulsions. The slope of the plot represents ($1/V_{max(app)}$) and the TO concentration axis–intercept gives ($-K_{m(app)}$). The emulsion TO concentration was the concentration throughout the reaction mixture

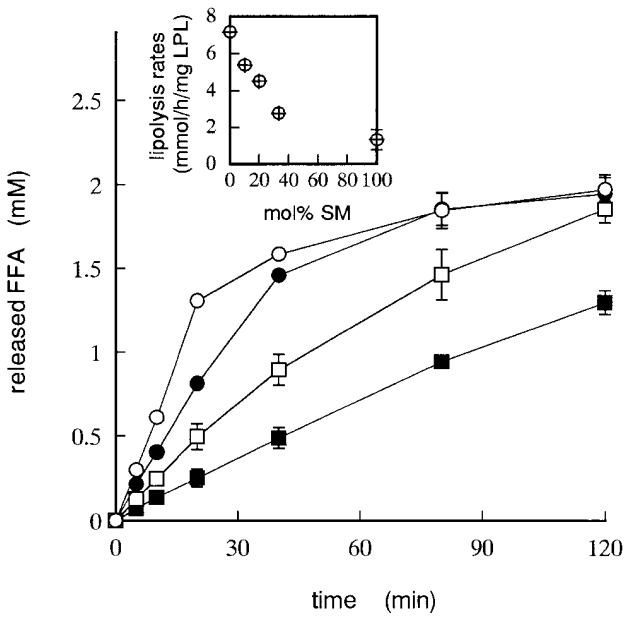

Fig. 1. Time courses of free fatty acid (FFA) release during lipolysis of lipid emulsions with different SM content in the presence of 30% plasma: (○) TO-PC; (●) TO-PC/SM(4/1, molar ratio); (□) TO-PC/SM(2/1); (■) TO-SM emulsions. Triolein concentration of emulsions was 1 mM. LPL was added at $t = 0$. Temperature was maintained at 37°C. Inset, the lipolysis rates of emulsions are represented as a function of the mol% of SM in the emulsion surface. Triolein concentration of emulsions was 1 mM. The rates were determined from the initial slopes of released FFA. Plots represent the averages ± SD of three experiments.

and proportional to the concentration of emulsion particles. The local concentration of TO at the emulsion surface is a constant for a defined emulsion preparation and differs from the TO concentration in the reaction mixture. Therefore, in spite of using pure enzyme, the results obtained in Fig. 2 were apparent kinetic parameters (apparent Michaelis-Menten constants, $K_{m(app)}$, and apparent maximal lipolysis rates, $V_{max(app)}$). **Figure 3** shows the resulting apparent kinetic parameters for TO-PC/SM (molar ratio, 2/1) emulsions as function of the content of plasma in the reaction mixture. Addition of plasma led to an increase in the $V_{max(app)}$ value, but gave negligible effects on the $K_{m(app)}$ value, suggesting that LPL activation by plasma was attributed to the enhanced catalytic ability of LPL and not to the increased affinity between LPL and substrates.

Isolated apoC-II of 10 μg/ml was also used as an activator in place of plasma. The peptide concentration corresponded to the concentration in 30% plasma. The similar activation effect of 10 μg/ml apoC-II with 30% plasma as seen in Fig. 3 suggested that the peptide, apoC-II, was responsible for the enhanced hydrolysis by LPL in plasma.

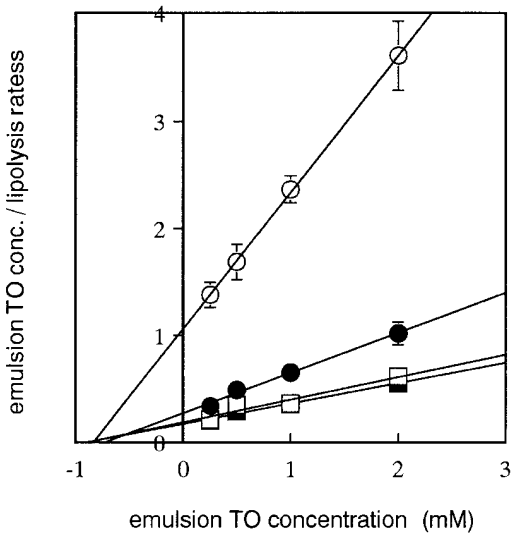

Fig. 2. Emulsion triolein concentration divided by lipolysis rate was represented as a function of emulsion triolein concentration for TO-PC/SM(2/1, molar ratio) emulsions; (○) without plasma; in the presence of 10% plasma (●), 30% plasma (□), and 80% Plasma (■). Lipolysis rates were measured at triolein concentrations ranging from 0.25 to 2 mM. Data represent the averages ± SD of three experiments. The $V_{max(app)}$ and $K_{m(app)}$ values obtained are presented in Fig. 3.

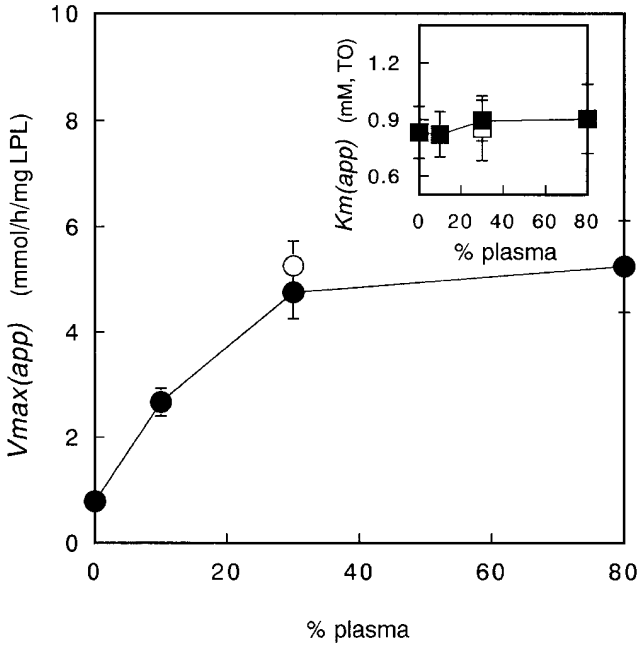

Fig. 3. Activation of LPL with increasing amounts of human plasma. The $V_{max(app)}$ values for TO-PC/SM(2/1) emulsions are represented as a function of % plasma (●); (○) in the presence of isolated apoC-II solution of 10 μg/ml in place of 30% plasma. Inset, the $K_{m(app)}$ value for TO-PC/SM(2/1) emulsions are presented as a function of % plasma (■); (□) in the presence of isolated apoC-II solution of 10 μg/ml in place of 30% plasma.

Effects of SM and Chol on the apparent kinetic parameters of the LPL reaction

The effects of replacement of PC by SM or Chol in TO-PC emulsions on the $K_{m(app)}$ and $V_{max(app)}$ of the LPL reaction in the presence of 30% plasma or isolated apoC-II or 10 μg/ml are summarized in **Table 1**. There were no significant differences in kinetic parameters between plasma and isolated apoC-II solution, suggesting that apoC-II in the plasma activates LPL catalysis. We found that SM in the emulsion surface increased $K_{m(app)}$ and decreased $V_{max(app)}$. For TO-PC/SM(2/1) emulsions, the $K_{m(app)}$ value was about 4.5-fold higher and the $V_{max(app)}$ value was about half that for TO-PC emulsions. SM inhibited LPL-mediated lipolysis by lowering the affinity between LPL and substrates and by decreasing catalytic activity. On the other hand, as shown in Table 1, Chol at the emulsion surface had no significant influence on the kinetic parameters. These results indicate that the key component in the emulsion surface regulating the lipolysis rate is not Chol but SM.

Binding of apoC-II to lipid emulsions

The $V_{max(app)}$ value of the LPL reaction in the presence of plasma is thought to be dependent on the amount of apoC-II bound to emulsions. We measured amount of apoC-II binding to emulsions to evaluate the concentration of apoC-II participating in LPL activation at the lipid particle surface. **Table 2** shows amounts of apoC-II bound to emulsions containing SM or Chol from human plasma. The amount of apoC-II binding per total surface lipid attained saturation in the emulsion concentration range from 0.1 to 0.4 mM of the surface lipid. As shown in Table 2, replacement of PC by SM or Chol in emulsion surface significantly reduced the binding of apoC-II. It was unexpectedly found that Chol had no influence on lipolysis rates in spite of the decrease in apoC-II binding. This would at least be consistent with early data showing that the amount of apoC-II required for maximal rates of LPL catalysis is about 10% of that present in VLDL (30).

Effects of SM on TO solubility in phospholipid surface layers

As substrate solubility at the emulsion surface may modulate lipolysis rate, solubilities of TO in phospholipid vesicles were determined by ^{13}C NMR spectroscopy. Decreased TO solubility in the surface layers by adding Chol has been reported (25). We evaluated the

TABLE 1. Apparent kinetic parameters of the LPL reaction for lipid emulsions containing SM or Chol

Emulsion Type	Surface Molar Ratio	$K_{m(app)}$		$V_{max(app)}$	
		+ 30% Plasma	+ 10 μg/ml ApoC-II	+ 30% Plasma	+ 10 μg/ml ApoC-II
		mM, TO		*mmol/h/mg LPL*	
TO-PC		0.194 ± 0.048	0.205 ± 0.059	8.38 ± 0.30	8.09 ± 0.35
TO-PC/SM	9/1	0.412 ± 0.113	0.441 ± 0.124	7.24 ± 0.51	7.18 ± 0.62
TO-PC/SM	4/1	0.541 ± 0.075	0.553 ± 0.094	6.95 ± 0.42	7.06 ± 0.62
TO-PC/SM	2/1	0.895 ± 0.108	0.853 ± 0.171	4.76 ± 0.50	5.26 ± 0.47
TO-PC/Chol	4/1	0.202 ± 0.029	0.209 ± 0.052	8.10 ± 0.67	8.29 ± 0.71
TO-PC/Chol	3/2	0.206 ± 0.041	0.196 ± 0.031	8.45 ± 0.61	9.12 ± 0.53

The kinetic parameters were determined from emulsion concentration versus emulsion concentration/lipolysis rates.

effects of SM on TO solubility using the method described by Hamilton and Small (24). The entire proton-decoupled ^{13}C NMR spectra of egg PC vesicles prepared by cosonication with 5 mol% [1-^{13}C]TO were in agreement with the data reported by Hamilton and Small (24) (results not shown). The carbonyl regions in 5 mol% [1-^{13}C]TO and phospholipid vesicles were expanded (**Fig. 4**). The carbonyl region of the ^{13}C NMR spectra showed four carbonyl signals for the ^{13}C-enriched TO (Fig. 4); the two broader signals at higher ppm were the 'surface' phase carbonyls (Su_α and Su_β) and the two narrow signals were carbonyls in the 'oil' phase (O_α and O_β) (24). The intensity of the signals from the 'surface' carbonyls of TO increased with the SM content. The TO solubility calculated from the peak area ratio of 'surface' and 'oil' phase carbonyls by Eq. 1 was 2.7 mol% in PC vesicles without SM, and was in agreement with the reported values (24, 31, 32). **Figure 5** shows that the solubility of TO in phospholipid vesicles increases with the mol% of SM. Inhibition of lipolysis by SM, therefore, was not due to the decrease in substrate concentration at the surface layer. The invariability of the kinetic parameters by adding Chol to the emulsion surface (Table 1) also suggested that TO solubility at the surface did not play a critical role in determination of the LPL-mediated lipolysis rate.

TABLE 2. Amount of apoC-II binding to lipid emulsions at 37°C

Emulsion Type	Surface Molar Ratio	ApoC-II Amount Bound to Emulsions in Human Plasma	
		mmol/mol Surface Lipid	g/g Surface Lipid
TO-PC		13.2 ± 1.6	0.151 ± 0.019
TO-PC/SM	9/1	12.4 ± 0.6	0.143 ± 0.006
TO-PC/SM	4/1	10.9 ± 0.6	0.127 ± 0.007
TO-PC/SM	2/1	6.5 ± 0.7	0.076 ± 0.009
TO-PC/Chol	4/1	7.8 ± 0.9	0.090 ± 0.010
TO-PC/Chol	3/2	2.8 ± 0.3	0.032 ± 0.003

Data represent means ± SD of three experiments.

DISCUSSION

Inhibition of LPL-mediated lipolysis by SM in lipid emulsions

We evaluated the effects of surface lipid composition on the LPL reaction using artificial emulsions of defined lipid composition and particle size as models of plasma lipoproteins. Figure 1 clearly shows that SM is an efficient inhibitor of LPL-mediated lipolysis. SM is the second most abundant phospholipid in plasma lipoproteins. Mole percent of SM to total phospholipid is about 13% in HDL, 3% in chylomicrons, 20% in VLDL, 33% in low density lipoproteins (LDL) (12–15, 17). However, little attention has been paid to the physiological role of SM in lipoprotein metabolism. Recently, the effects of SM on lecithin:cholesterol acyltransferase (LCAT) activity in reconstituted HDL (rHDL) have been reported by several laboratories. Subbaiah and Liu (14) showed that SM inhibited LCAT reaction with rHDL and native lipoproteins. Bolin and Jonas (33) and Rye, Hime, and Barter (34) also reported that SM affects the structure and function of rHDL, and thus regulates LCAT activity. On the other hand, the effects of SM on lipolysis in vivo with artificial emulsions (35) and in mixed monolayers at the air/water interface (36, 37) have been reported. Although these studies have indicated inhibitory effects of SM on the LPL reaction, the mechanism of the effects remains to be determined. The results shown in Fig. 1 represent the first quantitative evaluation of lipolysis rates in lipid emulsions containing various amounts of SM in vitro, and are compatible with those reported by Redgrave et al. (35) in vivo.

It is considered that SM could regulate lipolysis rates by several mechanisms: inhibiting LPL binding to emulsions, inhibiting catalytic activity of LPL, inhibiting apoC-II binding to emulsions, decreasing substrate solubility at the emulsion surface, or a combination of the above. As shown in Table 1, SM decreased the apparent maximum velocity and increased the apparent

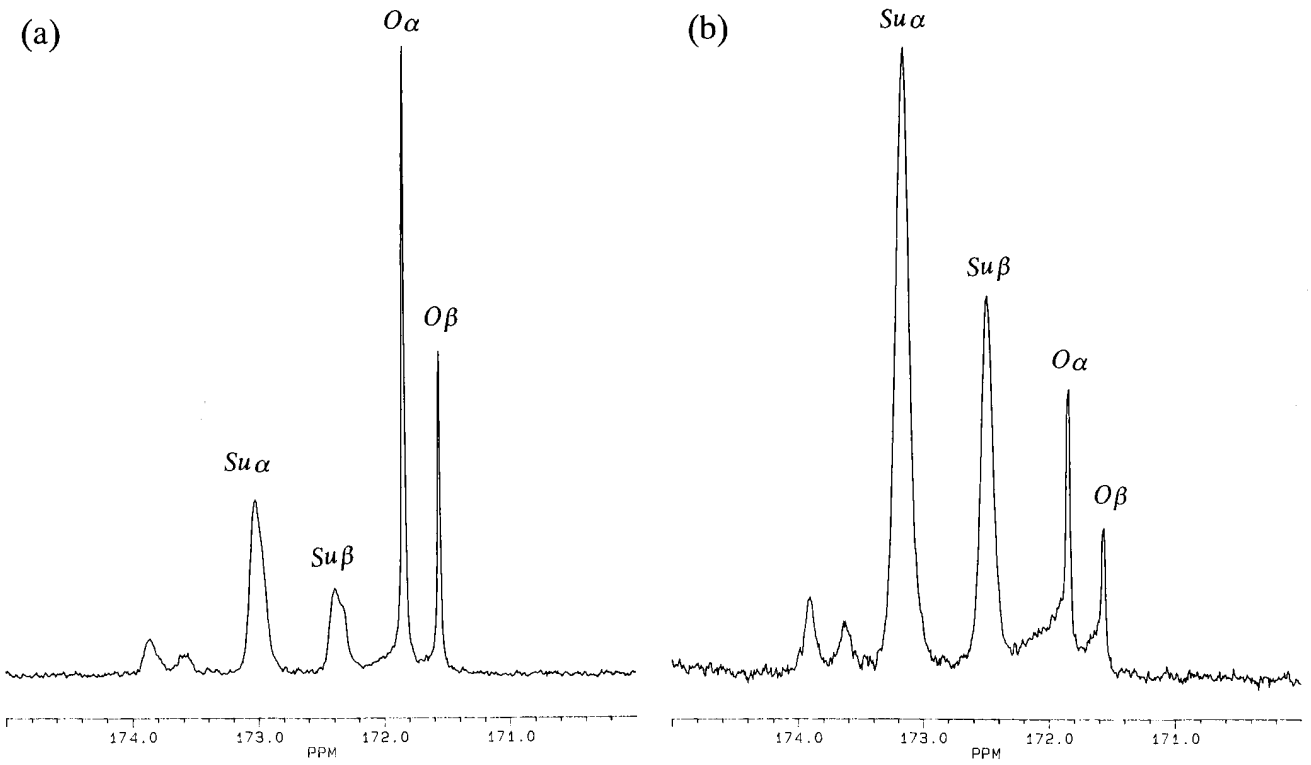

Fig. 4. Carbonyl regions of spectra recorded from PC (a) and PC/SM(2/1, molar ratio) (b) vesicles with 5 mol% [1-^{13}C]TO. Spectra were recorded under inverse-gated decoupling conditions at 37°C and given approximately the same intensity scaling. Each carbonyl peak is indicated as described in Experimental Procedures.

Michaelis-Menten constant. The results indicated that SM in the surface decreased both catalytic activity of LPL and affinity of LPL for the substrates, thereby reducing the lipolysis rates.

Unesterified cholesterol is a major surface component of plasma lipoproteins. Redgrave, Vassiliou, and Callow (38) have reported that the presence of Chol facilitated the uptake of emulsion particles by the liver. We found that both $V_{max(app)}$ and $K_{m(app)}$ for Chol-containing emulsions were almost equivalent to those for TO-PC emulsions (Table 1). Interestingly, it seems likely that the presence of Chol is a determinant of early liver uptake (38), but has little influence on LPL-mediated lipolysis.

Effects of apoC-II on the LPL reaction

It is well known that apoC-II enhances LPL activity. To elucidate the mechanism of activation by apoC-II, the effects of apoC-II on the kinetic parameters of the LPL reaction have been examined by several researchers. Jackson et al. (30) have reported that the primary effect of apoC-II on the LPL reaction was to decrease $K_{m(app)}$ with minor effects on $V_{max(app)}$ when the substrates were triacylglycerol (TG)-rich lipoproteins. Also, they have reported that the effect of apoC-II is mainly on $V_{max(app)}$ rather than on $K_{m(app)}$ with artificial TO-PC emulsions (30). Similar results have been reported for TO-PC emulsions by Posner, Wang, and McConathy (39, 40) and Cheng et al. (41). There are two possible explanations for the discrepancy between the lipoprotein and emulsion lipolysis: differences in lipid composition (i.e., TG-rich lipoproteins include other lipids such as SM and Chol, compared with TO-PC emulsions) and TG-rich lipoproteins contain a number of apolipoproteins such as apoB, apoE, apoC-III. As shown in Fig. 3, $K_{m(app)}$ was almost unchanged, while $V_{max(app)}$ increased in a saturating manner with plasma content for SM-containing emulsions. Similar results were observed with isolated human apoC-II in place of human plasma. We also observed increases in $V_{max(app)}$ and invariability of $K_{m(app)}$ after addition of plasma to lipid emulsions containing Chol (results not shown). It seems unlikely that differences in lipid composition between lipoproteins and lipid emulsions alter the mechanism of LPL activation by apoC-II. The most obvious explanation for the observed differences in the effects of activator on the kinetics parameters is that other apolipoproteins in the TG-rich lipoproteins affect these parameters.

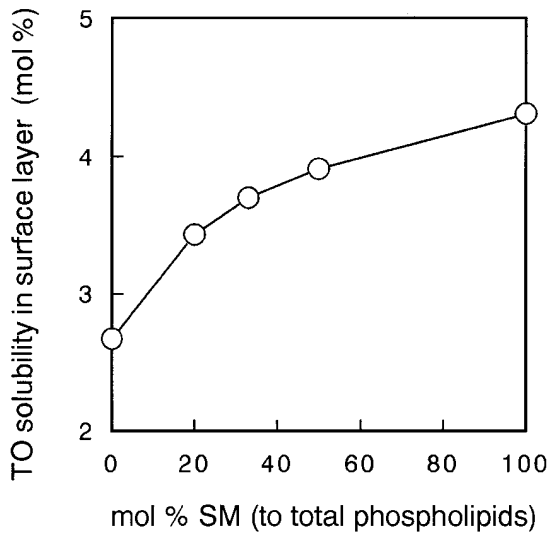

Fig. 5. Effects of SM on solubility of ^{13}C-TO in the surface layer. TO solubility in the surface layer was calculated from Eq. 1 in Experimental Procedures.

Taking into account the increase in $V_{max(app)}$ by apoC-II, it is necessary to evaluate the amounts of apoC-II binding to lipid emulsions. As shown in Table 2, both SM and Chol at the surface reduced amounts of apoC-II binding to emulsions. Replacement of PC by Chol reduced apoC-II binding but nevertheless had no effect on $V_{max(app)}$ (Tables 1 and 2), indicating that the amount of apoC-II bound to Chol-containing emulsions could activate LPL catalysis sufficiently. It is unlikely that the apoC-II binding to emulsions is a rate-limiting factor in LPL-mediated lipolysis under our experimental conditions. This is not in conflict with data suggesting that very little apoC-II is required to maximally activate LPL (30). In addition, Wang et al. (42) have reported that apoC-II concentration is normally not a limiting factor for the in vivo hydrolysis of TG-rich lipoproteins or a factor in the expression of hypertriglyceridemia. It is unlikely that SM inhibits lipolysis because of the decrease in apoC-II binding.

Effects of TO solubility in the surface layers on lipolysis.

In the kinetic model proposed by Quinn et al. (43, 44) for the interfacial enzyme, $K_{m(app)}$ and $V_{max(app)}$ are functions of the concentration of substrates at the surface layer, *S*. Neutral lipids such as triacylglycerol (TG) and cholesteryl esters are located in the cores of emulsions and have very low solubility in phospholipid layers (45, 46). Previous studies (24, 31, 32) have shown that the solubility of TO in the phospholipid surface is 2.7 mol%. Similar surface solubilities of TG have been observed for chylomicrons and VLDL (18). On the other hand, medium chain-TG (trioctanoin) is dissolved up to about 11 mol% at the emulsion surface (47). In addition, cholesterol decreases TO solubility in the surface monolayer (25). The differences in TO solubility have been presumed to affect lipolysis rate in emulsions of different lipid composition (47). As shown in Fig. 5, ^{13}C NMR measurements showed that SM increased TO solubility in phospholipid bilayers. This result indicated that the lipolysis inhibition by SM was not due to a decrease in TO solubility. The solubility of TO in PC/Chol (molar ratio, 3/2) surface layer was lower (0.7 mol%) than that in the PC surface (25), whereas the $K_{m(app)}$ and $V_{max(app)}$ values for TO-PC/Chol(3/2) emulsions were almost equivalent to those for TO-PC emulsions (Table 1). Furthermore, we found that the LPL activities of TO/cholesteryl oleate (1/1)-PC emulsions were similar to those of TO-PC emulsions (data not shown), although the incorporation of 50 mol% cholesteryl oleate reduces TO solubility by half (29). Our results indicate that TO solubility was not a rate-determining factor for the LPL reaction. In the equation reported by Quinn et al. (43, 44) $K_{m(app)}$ and $V_{max(app)}$ are independent of TO solubility, *S*, when true Michaelis-Menten constant (K_m*) $<<$ *S*. Our findings suggested that K_m* was smaller than 0.7 mol%; that is, local concentration of TO solubility in the TO-PC/Chol(3/2) emulsion surface had already achieved saturation.

The inhibition of LPL catalytic activity by SM was due neither to the decrease in apoC-II binding nor to the TO solubility in surface layers. We have shown that strong interaction between SM and TG retarded the transfer of the substrates to the catalytic pocket of LPL with mixed monolayers (37). One possible explanation for the inhibition of LPL activity by SM is this strong lipid–lipid interaction.

Roles of SM and Chol in plasma lipoproteins

In human plasma, chylomicrons containing a low mol% of SM are rapidly hydrolyzed by LPL, while the rates of hydrolysis in VLDL containing 20 mol% SM are slow (48). This difference in the susceptibility to LPL-mediated lipolysis is consistent with the present finding of the inhibition of lipolysis by SM (Table 1), suggesting that SM is one of the important factors regulating LPL activity in lipoprotein metabolism. Our results suggest that SM inhibits lipolysis both by regulating LPL affinity for substrates and by inhibiting LPL activity. On the other hand, the lipolysis of TG in chylomicrons produces Chol-enriched remnant particles (18). High Chol content in the particle surface induces a decrease in binding of apoC-II (Table 2) and TG solubility in the surface layers (25); however, these effects by Chol have no influence on LPL-mediated lipolysis (Table 1). This

is because neither amount of apoC-II binding nor TG solubility play critical roles for lipolysis rates. This enrichment of Chol content is thought to increase the ratio of apoE to apoC-II (9), and thereby promote uptake by the liver because C apolipoproteins inhibit apoE-mediated hepatic uptake (7, 8). Therefore, our results suggest that the contents of SM or Chol in the plasma lipoprotein surface play different roles in the metabolism of lipoproteins in the regulation of susceptibility to LPL and the selectivity of apolipoprotein binding.

This study was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture; by a fund from the Human Science Foundation of Japan; by a grant from Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists; and by a grant from the San-Ei Gen Foundation for Food Chemical Research. We thank Masako Akaji and Takako Nakatani for their kind help in measuring ^{13}C NMR spectroscopy.

Manuscript received 28 July 1997 and in revised form 30 September 1997.

REFERENCES

1. Havel, R. J., J. P. Kane, and M. L. Kashyap. 1973. Interchange of apolipoproteins between chylomicrons and high density lipoproteins during alimentary lipemia in man. *J. Clin. Invest.* **52:** 32–38.
2. Fielding, P. E., Y. Ishikawa, and C. J. Fielding. 1989. Apolipoprotein E mediates binding of normal very low density lipoprotein to heparin but is not required for high affinity receptor binding. *J. Biol. Chem.* **264:** 12462–12466.
3. Ishikawa, Y., C. J. Fielding, and P. E. Fielding. 1988. A change in apolipoprotein B expression is required for the binding of apolipoprotein E to very low density lipoprotein. *J. Biol. Chem.* **263:** 2744–2749.
4. Mahley, R. W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science.* **240:** 622–630.
5. van Dijk, M. C., G. J. Ziere, W. Boers, C. Linthorst, M. K. Bijsterbosch, and T. J. van Berkel. 1991. Recognition of chylomicron remnants and beta-migrating very-low-density lipoproteins by the remnant receptor of parenchymal liver cells is distinct from the liver alpha 2-macroglobulin-recognition site. *Biochem. J.* **279:** 863–870.
6. Weisgraber, K. H. 1994. Apolipoprotein E: structure–function relationships. *Adv. Protein Chem.* **45:** 249–302.
7. Weisgraber, K. H., R. W. Mahley, R. C. Kowal, J. Herz, J. L. Goldstein, and M. S. Brown. 1990. Apolipoprotein C-I modulates the interaction of apolipoprotein E with β-migrating very low density lipoproteins (β-VLDL) and inhibits binding of β-VLDL to low density lipoprotein receptor-related protein. *J. Biol. Chem.* **265:** 22453–22459.
8. Kowal, R. C., J. Herz, K. H. Weisgraber, R. W. Mahley, M. S. Brown, and J. L. Goldstein. 1990. Opposing effects of apolipoproteins E and C on lipoprotein binding to low density lipoprotein receptor-related protein. *J. Biol. Chem.* **265:** 10771–10779.
9. Saito, H., T. Minamida, I. Arimoto, T. Handa, and K. Miyajima. 1996. Physical states of surface and core lipids in lipid emulsions and apolipoprotein binding to the emulsion surface. *J. Biol. Chem.* **271:** 15515–15520.
10. Saito, H., Y. Miyako, T. Handa, and K. Miyajima. 1997. Effect of cholesterol on apolipoprotein A-I binding to lipid bilayers and emulsions. *J. Lipid. Res.* **38:** 287–294.
11. Handa, T., Y. Eguchi, and K. Miyajima. 1994. Effects of cholesterol and cholesteryl oleate on lipolysis and liver uptake of triglyceride/phosphatidylcholine emulsions in rats. *Pharm. Res.* **11:** 1283–1287.
12. Subbaiah, P. V., M. H. Davidson, M. C. Ritter, W. Buchanan, and J. D. Bagdade. 1989. Effects of dietary supplementation with marine lipid concentrate on the plasma lipoprotein composition of hypercholesterolemic patients. *Atherosclerosis.* **79:** 157–166.
13. Ben Yashar, V., and Y. Barenholz. 1991. Characterization of the core and surface of human plasma lipoproteins. A study based on the use of five fluorophores. *Chem. Phys. Lipids.* **60:** 1–14.
14. Subbaiah, P. V., and M. Liu. 1993. Role of sphingomyelin in the regulation of cholesterol esterification in the plasma lipoproteins. Inhibition of lecithin:cholesterol acyltransferase reaction. *J. Biol. Chem.* **268:** 20156–20163.
15. Gupta, A. K., and H. Rudney. 1992. Sphingomyelinase treatment of low density lipoprotein and cultured cells results in enhanced processing of LDL which can be modulated by sphingomyelin. *J. Lipid. Res.* **33:** 1741–1752.
16. Lane, J. T., P. V. Subbaiah, M. E. Otto, and J. D. Bagdade. 1991. Lipoprotein composition and HDL particle size distribution in women with non-insulin-dependent diabetes mellitus and the effects of probucol treatment. *J. Lab. Clin. Med.* **118:** 120–128.
17. Windler, E. E., S. Preyer, and H. Greten. 1986. Influence of lysophosphatidylcholine on the C-apolipoprotein content of rat and human triglyceride-rich lipoproteins during triglyceride hydrolysis. *J. Clin. Invest.* **78:** 658–665.
18. Miller, K. W., and D. M. Small. 1983. Surface-to-core and interparticle equilibrium distributions of triglyceride-rich lipoprotein lipids. *J. Biol. Chem.* **258:** 13772–13784.
19. Slotte, J. P., and E. L. Bierman. 1988. Depletion of plasma-membrane sphingomyelin rapidly alters the distribution of cholesterol between plasma membranes and intracellular cholesterol pools in cultured fibroblasts. *Biochem. J.* **250:** 653–658.
20. Slotte, J. P., G. Hedstrom, S. Rannstrom, and S. Ekman. 1989. Effects of sphingomyelin degradation on cell cholesterol oxidizability and steady-state distribution between the cell surface and the cell interior. *Biochim. Biophys. Acta.* **985:** 90–96.
21. Gupta, A. K., and H. Rudney. 1991. Plasma membrane sphingomyelin and the regulation of HMG-CoA reductase activity and cholesterol biosynthesis in cell cultures. *J. Lipid. Res.* **32:** 125–136.
22. Saito, H., K. Nishiwaki, T. Handa, S. Ito, and K. Miyajima. 1995. Comparative study of fluorescence anisotropy in surface monolayers of emulsions and bilayers of vesicles. *Langmuir.* **11:** 3742–3747.
23. Hayashi, R., S. Tajima, and A. Yamamoto. 1986. Purification and characterization of lipoprotein lipase from human postheparin plasma and its comparison with purified bovine milk lipoprotein lipase. *J. Biochem.* **100:** 319–331.
24. Hamilton, J. A., and D. M. Small. 1981. Solubilization and localization of triolein in phosphatidylcholine bilayers: a ^{13}C NMR study. *Proc. Natl. Acad. Sci. USA.* **78:** 6878–6882.

25. Spooner, P. J., and D. M. Small. 1987. Effect of free cholesterol on incorporation of triolein in phospholipid bilayers. *Biochemistry.* **26:** 5820–5825.
26. Hamilton, J. A., J. M. Vural, Y. A. Carpentier, and R. J. Deckelbaum. 1996. Incorporation of medium chain triacylglycerols into phospholipid bilayers: effect of long chain triacylglycerols, cholesterol, and cholesteryl esters. *J. Lipid. Res.* **37:** 773–782.
27. Yamamoto, I., T. Mazumi, T. Handa, and K. Miyajima. 1993. Effects of 1,2-diacylglycerol and cholesterol on the hydrolysis activity of phospholipase D in egg-yolk phosphatidylcholine bilayers. *Biochim. Biophys. Acta.* **1145:** 293–297.
28. Yamamoto, I., A. Konto, T. Handa, and K. Miyajima. 1995. Regulation of phospholipase D activity by neutral lipids in egg-yolk phosphatidylcholine small unilamellar vesicles and by calcium ion in aqueous medium. *Biochim. Biophys. Acta.* **1233:** 21–26.
29. Hamilton, J. A., K. W. Miller, and D. M. Small. 1983. Solubilization of triolein and cholesteryl oleate in egg phosphatidylcholine vesicles. *J. Biol. Chem.* **258:** 12821–12826.
30. Jackson, R. L., S. Tajima, T. Yamamura, S. Yokoyama, and A. Yamamoto. 1986. Comparison of apolipoprotein C-II-deficient triacylglycerol-rich lipoproteins and trioleoylglycerol / phosphatidylcholine-stabilized particles as substrates for lipoprotein lipase. *Biochim. Biophys. Acta.* **875:** 211–219.
31. Miller, K. W., and D. M. Small. 1982. The phase behavior of triolein, cholesterol, and lecithin emulsions. *J. Colloid Interface Sci.* **89:** 466–478.
32. Handa, T., H. Saito, and K. Miyajima. 1993. Eutectic mixed monolayers in equilibrium with phospholipid-bilayers and triolein-liquid phase. *Biophys. J.* **64:** 1760–1765.
33. Bolin, D. J., and A. Jonas. 1996. Sphingomyelin inhibits the lecithin:cholesterol acyltransferase reaction with reconstituted high density lipoproteins by decreasing enzyme binding. *J. Biol. Chem.* **271:** 19152–19158.
34. Rye, K. A., N. J. Hime, and P. J. Barter. 1996. The influence of sphingomyelin on the structure and function of reconstituted high density lipoproteins. *J. Biol. Chem.* **271:** 4243–4250.
35. Redgrave, T. G., V. Rakic, B. C. Mortimer, and J. C. Mamo. 1992. Effects of sphingomyelin and phosphatidylcholine acyl chains on the clearance of triacylglycerol-rich lipoproteins from plasma. Studies with lipid emulsions in rats. *Biochim. Biophys. Acta.* **1126:** 65–72.
36. Demel, R. A., K. Shirai, and R. L. Jackson. 1982. Lipoprotein lipase-catalyzed hydrolysis of tri[^{14}C]oleoylglycerol in a phospholipid interface. A monolayer study. *Biochim. Biophys. Acta.* **713:** 629–637.
37. Arimoto, I., M. Fujita, H. Saito, T. Handa, and K. Miyajima. 1997. Activation and inhibition of lipoprotein lipase in mixed monolayers of medium or long-chain triglycerides and phospholipids. *Colloid Polym. Sci.* **275:** 60–66.
38. Redgrave, T. G., G. G. Vassiliou, and M. J. Callow. 1987. Cholesterol is necessary for triacylglycerol-phospholipid emulsions to mimic the metabolism of lipoproteins. *Biochim. Biophys. Acta.* **921:** 154–157.
39. Posner, I., C. S. Wang, and W. J. McConathy. 1983. The comparative kinetics of soluble and heparin-Sepharose-immobilized bovine lipoprotein lipase. *Arch. Biochem. Biophys.* **226:** 306–316.
40. Posner, I., C. S. Wang, and W. J. McConathy. 1985. Kinetics of human and bovine milk lipoprotein lipase and the mechanism of enzyme activation by apolipoprotein C-II. *Comp. Biochem. Physiol. B.* **80:** 171–174.
41. Chang, Q., P. Blackett, K. W. Jackson, W. J. McConathy, and C. S. Wang. 1990. C-terminal domain of apolipoprotein C-II as both activator and competitive inhibitor of lipoprotein lipase. *Biochem. J.* **269:** 403–407.
42. Wang, C. S., W. J. McConathy, H. U. Kloer, and P. Alaupovic. 1985. Modulation of lipoprotein lipase activity by apolipoproteins. Effect of apolipoprotein C-III. *J. Clin. Invest.* **75:** 384–390.
43. Quinn, D. M., K. Shirai, R. L. Jackson, and J. A. Harmony. 1982. Lipoprotein lipase-catalyzed hydrolysis of water-soluble *p*-nitrophenyl esters. Inhibition by apolipoprotein C-II. *Biochemistry.* **21:** 6872–6879.
44. Quinn, D., K. Shirai, and R. L. Jackson. 1983. Lipoprotein lipase: mechanism of action and role in lipoprotein metabolism. *Prog. Lipid Res.* **22:** 35–78.
45. Hamilton, J. A. 1989. Interactions of triglycerides with phospholipids: incorporation into the bilayer structure and formation of emulsions. *Biochemistry.* **28:** 2514–2520.
46. Hamilton, J. A., D. T. Fujito, and C. F. Hammer. 1991. Solubilization and localization of weakly polar lipids in unsonicated egg phosphatidylcholine: A ^{13}C MAS NMR study. *Biochemistry.* **30:** 2894–2902.
47. Deckelbaum, R. J., J. A. Hamilton, A. Moser, G. Bengtsson-Olivecrona, E. Butbul, Y. A. Carpentier, A. Gutman, and T. Olivecrona. 1990. Medium-chain versus long-chain triaglycerol emulsion hydrolysis by lipoprotein lipase and hepatic lipase: implications for the mechanisms of lipase action. *Biochemistry.* **29:** 1136–1142.
48. Eisenberg, S. 1986. Plasma lipoprotein conversions. *Methods Enzymol.* **129:** 347–366.