Effects of Cholesterol and Cholesteryl Oleate on Lipolysis and Liver Uptake of Triglyceride/Phosphatidylcholine Emulsions in Rats

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Emulsions composed of soy bean triglyceride (TG), egg yolk phosphatidylcholine (PC), cholesterol (Chol) or cholesteryl-oleate (CO), labeled with a cholesteryl ether (^{3}H-CHE) and a triglyceride (^{14}C-TO), were injected into rats. ^{14}C-TO was removed from plasma faster than ^{3}H-CHE. The ^{14}C-labeled moiety is cleaved by digestion of the TG in the emulsion in plasma and is removed to the endothelial cells (lipolysis). In contrast, the ^{3}H-label remains stably associated and represents circulating emulsion particles. The majority (90%) of the ^{3}H-label disappearing from the plasma accumulated in the liver for all types of emulsions. On the basis of these observations, the lipolysis and the removal of emulsion particles to organs (mainly liver) were determined: 30 mole percent of cholesterol (Chol) at the TG-PC emulsion surface markedly retarded organ uptake, but the effect on lipolysis was rather small; 20 mole percent of cholesteryl oleate (CO) in the TG-PC emulsion cores delayed both organ uptake and lipolysis, and induced a rapid increase in organ uptake rate after the initial delay accompanying the gradual progress of lipolysis. Lipolysis led to the enrichment of the cores with CO. Replacement of the core TG by CO, however, induced strong suppression of the liver uptake. These results show that the lipid composition at both surface and core of emulsion particles is a crucial factor in metabolism in the rat.

KEY WORDS: emulsion; lipolysis; organ uptake; effects of cholesterol and cholesteryl ester.

INTRODUCTION

Triglyceride (TG)-phosphatidylcholine (PC) emulsions containing cholesterol (Chol) and its ester are physical models of the behavior of TG-rich lipoproteins in plasma (1–3). The metabolism of protein-free emulsions in rat is comparable to that of chylomicrons (4). Triglycerides are lipolyzed in peripheral tissue and removed from plasma, and the remnant particles formed by lipolysis are taken up predominantly by the liver (5,6). The removal of remnant particles is significantly slower in WHHL rabbits with defective LDL receptors than in normal controls, suggesting that the absence of LDL receptor function is responsible for the observed impaired remnant clearance (5). Protein-free emulsion particles are thought to combine with apo E in plasma and acquire high affinity to LDL and other apo E receptors. Metabolism of emulsion particles is influenced by the lipid composition: Cholesterol, cholesteryl esters and sphingomyelin are thought to be critical determinants in the metabolism of emulsions (5,7,8) and chylomicrons (3,4).

On the other hand, TG-PC emulsions have been employed as delivery systems for lipophilic drugs such as prostaglandins, steroid hormones and menaquinones. A drug distributed between TG cores and PC surface monolayers of emulsion particles (4b), is in dynamic equilibrium with lipoproteins and proteins in plasma (4c). The release of drugs in blood is considered to depend upon both lipolysis of TG and removal of the particles from plasma, and the control of these metabolic processes is important for proper delivery of drugs in the human body.

In the present study, we evaluated both lipolysis of TG and organ uptake of emulsion particles on the basis of the observed removal from plasma and liver-accumulation of the labeled lipids in rat. The effects of cholesterol and cholesteryl oleate on the metabolic process were estimated separately and discussed in terms of lipid-apolipoprotein interactions.

MATERIALS AND METHODS

Materials. Egg yolk phosphatidylcholine (PC) was provided by Asahi Kasei Co., the purity of which (over 99.5%) was determined by thin-layer chromatography. Soy bean triglycerides (obtained from Nakarai Tesque) were purified up to 99% by silicate (Wakogel C-200, Wako Pure Chemicals) column chromatography with chloroform as the eluent. Cholesterol (purchased from Wako Pure Chemicals) was recrystalized from ethanol. Cholesteryl oleate (CO; obtained from Sigma Chemicals) showed a single spot on thin-layer chromatography. Radiolabeled glycerol tri[1-^{14}C]oleate (^{14}C-TO) and 1,2-^{3}H-cholesteryl hexadecylether (^{3}H-CHE) were obtained from DuPont (product numbers: NEC-674 and NET-859, respectively).

Preparation of Emulsions. PC, TG, Chol, CO, and the radiolabeled lipids, ^{14}C-TO and ^{3}H-CHE, were dissolved in chloroform. After evaporation of the solvent, the mixture was dried in vacuo for 15 h, and phosphate buffered saline (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 136.9 mM NaCl, 2.7 mM KCl, pH 7.4) was then added to the dried lipid mixture to final concentrations of PC and TG of 20 mM. The suspension was briefly vortexed, degassed by nitrogen-purging, and sonicated using a UR-200P probe type sonicator (Tomy Seiko Co Ltd; power setting 100 W) for 20 min under a stream of nitrogen gas at 60°C (9). The lipid-dispersed solution was centrifuged to remove titanium dust. The overall PC/TG/Chol/CO molar ratios of emulsions investigated were 20/20/0/0 (control), 20/20/8.7/0, 20/20/0/5 and 20/0/0/10.

Quasi-elastic light scattering measurements of emulsions were performed at 25°C on a Photal LPA-3000/3100, and correlation functions were analyzed by the histogram method (10). The weight-averaged particle sizes of emulsions were 45 ± 5, 61 ± 10, 50 ± 9 and 54 ± 5 nm for overall lipid compositions of 20/20/0/0, 20/20/8.7/0, 20/20/0/5 and 20/0/0/10, respectively. The particle size evaluated agreed with that estimated by electron microscopy.

Injection of Emulsions into Rat Plasma. Double labeled emulsions were injected into the femoral vein of sodium pentobarbital-anesthetized male Wistar rats weighing 200 ± 10 g

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as a bolus of 5 μmol of emulsion PC in a volume of 250 μL. Blood samples of 200 μL were collected from the carotid vein at varying times, and immediately centrifuged to separate plasma. At 4 h after the injection of emulsions, animals were exsanguinated and the organs removed. Plasma and organ samples were incubated with alkaline solution (Soluen 350 from Pakerd) overnight to dissolve tissues, and radioactivity due to ^{14}C and ^{3}H was measured on a liquid scintillation counter (Beckman LS 5000TA). The plasma-clearances of ^{14}C and ^{3}H were simulated with a bi-exponential curve-fitting program.

Distribution of ^{14}C-radioactivity in Plasma Lipids. Lipids extracted from the plasma samples with chloroform/methanol mixtures were separated by thin-layer chromatography (Silicate Gel Plate 70 from Wako Pure Chemicals) with n-hexane/diethylether/acetic acid (55/45/1 in v/v) mixture, and ^{14}C-radioactivity of each lipid fraction was measured.

RESULTS

Clearance of Radiolabeled Lipids from Rat Plasma. PC-TG emulsions are equilibrium mixtures of emulsion particles (PC monolayers + TG cores) and liposomes (PC bilayers) (2,9,11). Complete separation of emulsion particles from the excess bilayers (liposomes) is difficult (5,12). However, the solubilities of TG and cholesteryl esters are very limited in the bilayers (2,9,13) and thus the radiolabeled lipids ^{14}C-TO and ^{3}H-CHE were exclusively incorporated into the emulsion particles, not into the liposomes.

Fig. 1 shows plasma-clearances of ^{14}C-TO and ^{3}H-CHE after injection of double labeled emulsions. The plasma concentration of radiolabeled lipid is expressed as a percentage of injected dose in 1 mL plasma. Extrapolation of the plasma concentration to time zero by use of the bi-exponential fitting curve gave an initial concentration of 13.0 ± 1.3% dose/mL. The ^{14}C-TO label was removed more quickly than the ^{3}H-CHE label. The radiolabeled lipid, ^{3}H-CHE, is metabolically stable in the plasma and is stably bound to the particles. Thus, clearance of the label from the plasma implied removal of emulsion particles to the organs. The removal of the radiolabels was retarded by incorporation of Chol into the emulsions.

The effects of cholesteryl oleate (CO) on the clearance of emulsions are shown in Figs. 2a and 2b. 20% CO in the emulsion cores brought about a delay in the removal of ^{3}H-CHE (i.e. emulsion particles) and a depression of the removal of ^{14}C-TO from plasma. Removal was enhanced after an initial delay of 40–50 min. Replacement of TG in the emulsion cores by CO (i.e. overall PC/TG/Chol/CO = 20/0/0/10) caused strong suppression of particle-clearance as shown in Fig. 2b.

Lipolysis and Liver Uptake of Emulsion Lipids. Radiolabeled TO was most rapidly removed from the plasma when control emulsions (overall PC/TG/Chol/CO = 20/20/0/0) were injected. Emulsion TG is hydrolyzed by lipoprotein lipases (LPL) on endothelial cells (5,6). The radiolabeled free fatty acids (FFA) produced are taken up by the endothelial cells, where they are incorporated into phospholipids, glycerides and other lipids, and re-secreted into the plasma (14,15). Lipid fractions were separated from the plasma after

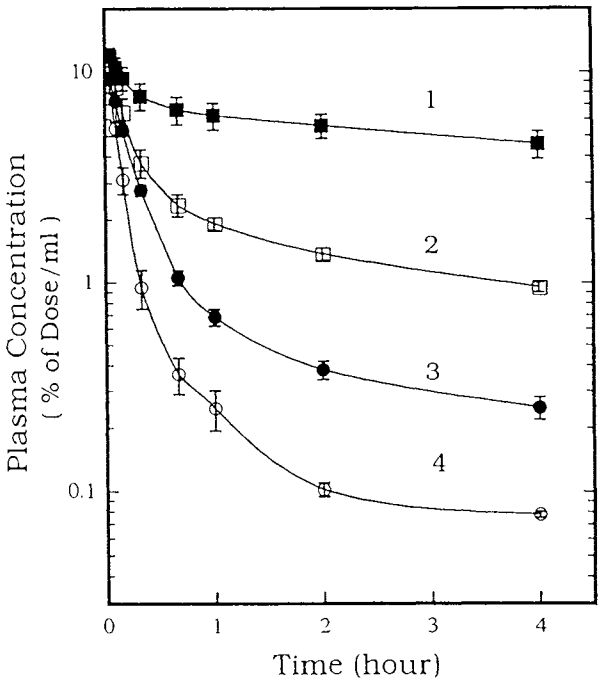

Fig. 1: Plasma-clearance of ^{14}C-TO and ^{3}H-CHE incorporated into emulsions. Plasma concentration of radiolabel is expressed as a percentage of total injected radioactivity in 1 mL plasma. The initial concentration was evaluated as 13.0 ± 1.3% dose/mL (see text). Clearance of ^{3}H-CHE (1) and ^{14}C-TO (2) of the Chol-containing emulsions (overall: PC/TG/Chol/CO = 20/20/8.7/0, surface: PC/Chol = 20/8.3, core: Chol/TG = 0.4/20), (n = 3). Clearance of ^{3}H-CHE (3) and ^{14}C-TO (4) of the control emulsions (overall: PC/TG/Chol/CO = 20/20/0/0 molar ratio, surface: PC, core: TG), (n = 3).

the injection of emulsions, and the distribution of ^{14}C was determined as shown in Table 1. More than 90% of the radioactivity was observed in the TG fraction, while low activities were found in the FFA and diglyceride fractions. Radioactivity in the phospholipid fraction was negligible. The results indicated the emulsion ^{14}C-TO as the major source of the ^{14}C-radioactivity in plasma in the initial 60 min after injection of emulsions.

Rats were sacrificed 4 h after the injection, and the organ-distribution of ^{3}H-CHE was determined. The majority of radioactivity was observed in plasma and liver (Fig. 3). For the control and the CO-containing emulsions, 90% of the injected label was detected in liver. Half of the ^{3}H-label in the Chol-containing emulsions (Fig. 1, line 1) and about three quarters of the label in the CO-core (Fig. 2b, line 1) emulsions were cleared from the plasma at 4 h after injection, and the majority (about 90%) of the label cleared was accumulated in the liver. These results showed the exclusive liver uptake of emulsions. TG-rich lipoproteins, chylomicrons, are similarly taken up by liver cells, predominantly by parenchymal cells (14), after lipolysis of TG (16–18).

Emulsion TG was removed from plasma by two processes: the lipolysis of TG at the endothelial surface of capillaries and uptake by the organs (mainly liver)of the emulsion particles containing residual TG. The overall removal of ^{14}C-TO can be represented as

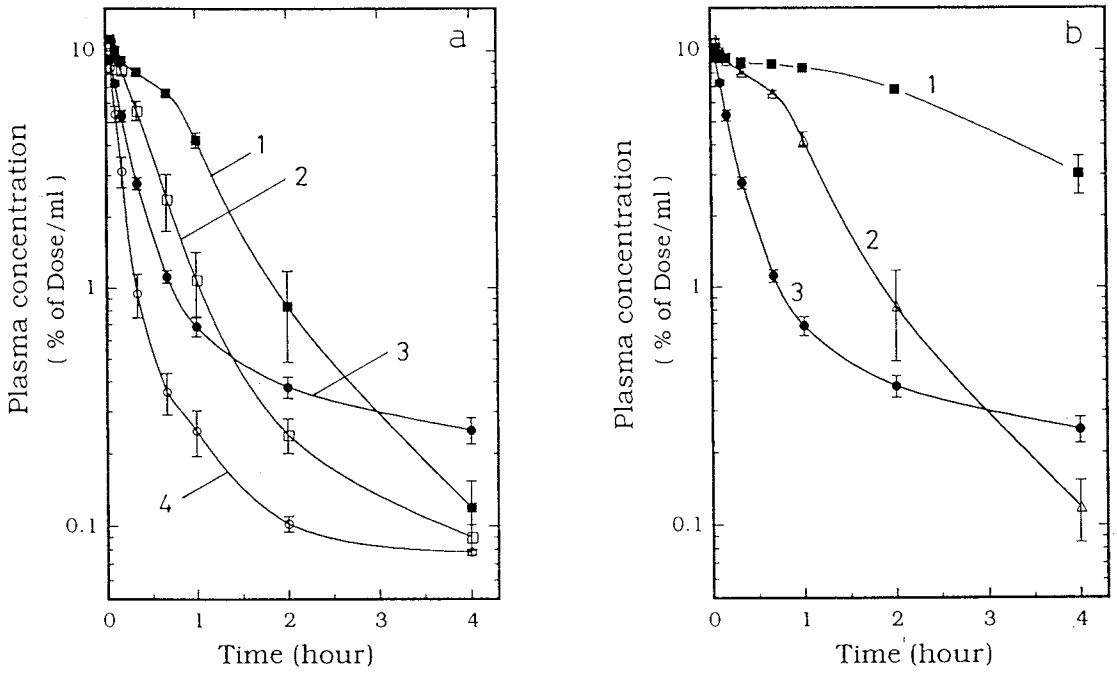

Fig. 2: Effects of CO on plasma-clearance of emulsions. a: Clearance of ^{3}H-CHE (1) and ^{14}C-TO (2) of the CO-containing emulsions (overall: PC/TG/Chol/CO = 20/20/0/5, surface: PC, core: TG/CO = 20/5) (n = 3), and clearance of ^{3}H-CHE (3) and ^{14}C-TO (4) of the control emulsions (n = 3). b: Clearance of ^{3}H-CHE. 1, The core TG of emulsions was replaced by CO (overall: PC/TG/Chol/CO = 20/0/0/10, surface: PC, core: CO), (n = 3); 2, CO-containing emulsions (n = 3); 3, The control emulsions (n = 3).

$$[^{14}C-TO]_0 - [^{14}C-TO]_t = \text{lipolysis} + \int_0^t ([^{14}C-TO]_t/[^{3}H-CHE]_t) \times (-d[^{3}H-CHE]/dt)dt \quad (1)$$

Where, $[^{14}C\text{-}TO]_0$ and $[^{14}C\text{-}TO]_t$ are the plasma concentrations of ^{14}C-TO immediately after emulsion injection and at time t, respectively. The second term on the right hand side of eq. 1 is the amount of TG removed through the endocytosis of emulsion particles by organ cells. The value of $d[^{3}H\text{-}CHE]/dt$ is obtained by differentiating the simulated curves for plasma clearance (Figs. 1 and 2a). The value, $[^{14}C\text{-}TO]_t/[^{3}H\text{-}CHE]_t$ was not constant and was obtained from the experimental removal curves. Lipolysis evaluated by eq. 1 is shown in Fig. 4. Addition of Chol to the emulsion particles did not significantly affect the cumulative digestion of TG, while CO in the emulsion cores caused retardation of TG lipolysis.

TABLE 1. Distribution of ^{14}C-Radioactivity in Plasma Lipids after Injection of Labeled Emulsions[a] (n = 3)

time (min)	radioactivity in plasma lipid fractions (percent)			
	PL[b]	DG[c]	FFA[d]	TG[e]
6	0.2 ± 0.1	1.9 ± 0.3	3.2 ± 0.2	94.6 ± 2.7
15	0.3 ± 0.1	1.3 ± 1.0	3.7 ± 1.0	94.7 ± 4.6
30	0.4 ± 0.1	2.2 ± 0.3	4.0 ± 0.5	93.4 ± 1.8
60	0.8 ± 0.2	2.4 ± 0.1	5.0 ± 1.0	91.8 ± 2.6

a) Injection of control emulsions (overall composition: PC/TG/Chol/CO = 20/20/0/0), showing the most rapid TG-clearance.
b) Phospholipids. c) Diglycerides. d) Free fatty acids. e) Triglycerides.

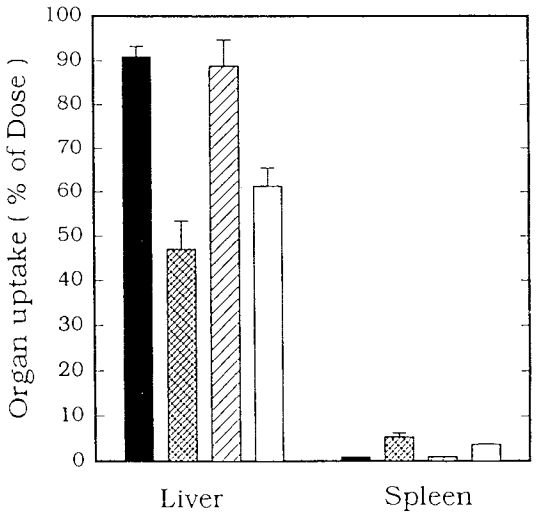

Fig. 3: Distribution of ^{3}H-CHE radiolabel (marker of emulsion particles) in rat organs at 4h after the emulsion injection. The majority of activity was observed in plasma, liver and spleen. About 50 and 30% of the label remained in plasma of rats injected with the Chol- and CO-containing emulsions, respectively. From left to right, control, Chol-containing, CO-containing, and CO-core emulsions.

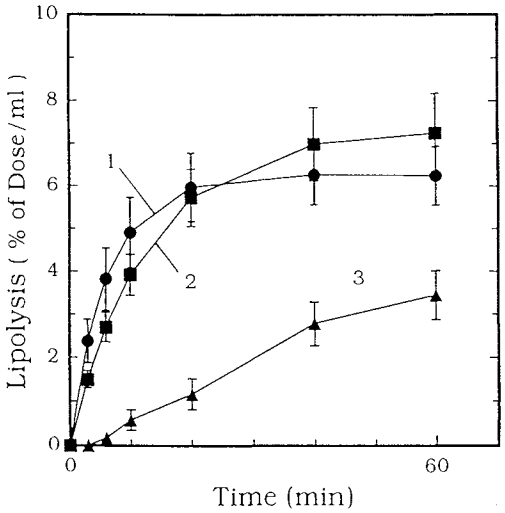

Fig. 4: Lipolysis of emulsional TG. 1, Control emulsions; 2, Chol-containing emulsions; 3, CO-containing emulsions.

DISCUSSION

Emulsion Particles and Bilayers. The digestion of TG by LPL (lipolysis) reduces size of emulsion cores, and the redundant surface monolayers of PC change into bilayers, fusing with some elements of high density lipoproteins (HDL) (19). Levels of 80 and 90% removal from plasma of PC of TG-PC emulsions are attained at 1 and 2 h, respectively, after the injection of emulsions into rats (19). PC removal is more rapid for emulsions of smaller diameter (in press; 1993 Human Science Foundation Report-3, Japan). Therefore, the majority of TG remaining in plasma is indicated to be in the emulsion cores rather than in the PC bilayers even at 1 h after injection (Table 1). On the other hand, 90% of ^{14}C-TO and ^{3}H-CHE were cleared from rat plasma at 20 and 40 min after the injection of the control emulsions, respectively (Fig. 1).

Effects of Lipid Composition on Emulsion Catabolism. Miller and Small (1,2) have shown that Chol in emulsions is localized in the bilayers, while CO partitions to the cores. We evaluated the partitioning of Chol and CO into the emulsion monolayers and into the liposome bilayers as similar on the basis of interfacial tension measurements on PC-Chol mixed monolayers at the TG/saline interface (data not shown). The monolayer/core partition coefficient of Chol was about 20 in the mole fraction base, comparable to the value of 22 obtained for partitioning into the bilayers (1). Calculation of Chol distribution indicated that 95% of that added was solubilized in the emulsion PC monolayers and the coexisting liposome PC bilayers in the present study.

The behaviors of emulsions determined in the present study are summarized in Fig. 5: 1) The TG-PC (control) emulsions underwent quick lipolysis and rapid liver uptake in rats (Figs. 1 and 4); 2) Chol added to the emulsion surface monolayers retarded removal of the lipid particles from plasma to liver cells (Fig. 1), but the effect on the lipolysis was small (Fig. 4); 3) CO in the emulsion cores delayed both

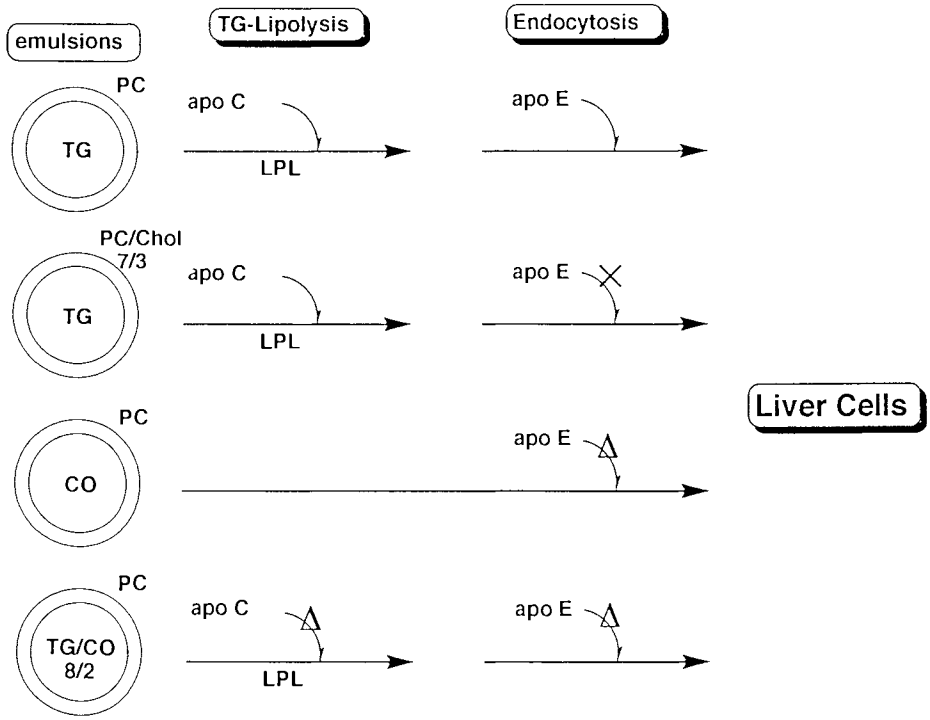

Fig. 5: Lipolysis and liver uptake (endocytosis by liver cells) in rat of emulsions with different lipid compositions. Chol in the surface monolayers led to retardation of liver uptake but little effect was observed on lipolysis. CO in the TG cores induced an initial delay of liver uptake and depression of lipolysis. Recovery of liver uptake accompanied the gradual progress of lipolysis. Replacement of the core TG by CO strongly suppressed liver uptake. (see text).

liver uptake in the initial stage (see Fig. 2) and lipolysis of TG (Fig. 4), and liver uptake was accelerated after this initial delay of 40–50 min (Fig. 2); 4) Replacement of the core TG by CO strongly depressed the uptake of lipid particles by liver cells (Figs. 2 and 4).

The activation of lipoprotein lipases (LPL) involves apolipoproteins and lipids. Apolipoprotein Cs, especially apo C-2, modify interactions between LPL and TG-rich lipoproteins such that catalytic rate for TG is increased manyfold (20). The suppression of lipolysis induced by addition of 20% CO to the TG emulsion cores (Fig. 4, line 3) implies a reduction in the binding of apo C to the emulsion surface.

Apolipoprotein E (apo E) is a plasma protein that serves as a specific ligand for LDL receptors in liver and other organs (21). Oswald and Quarfordt (22) have shown that apo E significantly increases the uptake of emulsions by rat hepatocytes in culture. Chol at the emulsion surface led to the suppression of liver uptake of the emulsion particles. Derksen and Small (23) showed a sharp decrease in the apo E binding capacity of emulsions by saturation with Chol. 30% Chol in the PC monolayers at the emulsion surface may lead to the reduction in binding of apolipoprotein E.

The liver uptake rate of the CO-containing emulsion particles recovered after an initial delay of 40–50 min (Fig. 2). The lipolysis of TG proceeded gradually during the delay period (Fig. 4) and caused a relative elevation of the CO content in emulsion cores. On the other hand, complete replacement of the core TG by CO resulted in a pronounced retardation in the plasma-removal of the lipid particles (Fig. 2b). These observations are indicative of a complicated relationship between the organ (liver) uptake and the CO enrichment in emulsions.

The effects of Chol and CO on emulsion metabolism were different, and, in one case, opposite. The manner of interaction between emulsion particles of different lipid compositions and apolipoproteins are predicted to play important roles in metabolism.

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