Sterol side chain length and structure affect the clearance of chylomicron-like lipid emulsions in rats and mice

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Abstract In previous work we found that sterols such as cholesterol were essential for physiological plasma clearance of lipid emulsions mimicking the structure of mammalian triglyceride-rich lipoproteins. In the present study we compared the clearances of emulsions prepared with sterols of varying alkyl chain length (straight chains, *n*–C3 to *n*–C7, or branched chains, *i*–C5 to *i*–C10) at the C-17 position. Our studies show that the length of the alkyl chain at the C-17 position of sterols markedly affects the removal of remnant particles from the plasma of rats traced by emulsion cholesteryl oleate label. An alkyl chain of 7 carbons or more was needed for normal remnant clearance. Straight and branched chains of similar length were cleared similarly, showing that the presence of a branch at the end of the alkyl chain had no effect on remnant clearance. For side chains of 7 carbons or less, substitution of sterols with an unsaturation in the alkyl chain close to the terminal carbon markedly decreased the clearance of remnants. Triolein label was used to estimate lipolysis of the injected emulsions. Lipolysis was little affected by the structure of the sterol side chain, except that lipolysis was markedly higher with emulsions containing sterols with an alkyl chain having 4 carbon atoms (*n*–C4) or with an unsaturation in the 4 carbon alkyl chain. We conclude that the length of the alkyl side chain is an important element in the essentiality of cholesterol as a regulator of metabolism of lipid emulsion models of triglyceride-rich lipoproteins.—**Martins, I. J., C. Vilchèze, B-C. Mortimer, R. Bittman, and T. G. Redgrave.** Sterol side chain length and structure affect the clearance of chylomicron-like lipid emulsions in rats and mice. *J. Lipid Res.* 1998. **39:** 302–312.

Supplementary key words sterol • alkyl chain • emulsion • cholesteryl oleate • triolein • lipolysis • clearance • rats • remnants • apoE knockout mice

The majority of dietary triglycerides and cholesterol, together with lipids from bile, are transported after their digestion and absorption from the intestine by means of the mesenteric lymph. In lymph the transport of lipids is principally in the form of triglyceride-rich particles, the lymph chylomicrons, composed mostly of lipids but also containing a number of apolipoproteins. On entry into the blood most of the triglycerides in chylomicrons are hydrolyzed by lipoprotein lipase, converting the particles to chylomicron remnants (1). The remnant particles are then removed from the plasma by receptor-mediated endocytosis, mostly into the liver.

We have previously shown that the metabolism of lymph chylomicrons can be imitated by using chylomicron-like lipid emulsions (2–4). Cholesterol is essential for metabolism of emulsions by physiological pathways (5). When cholesterol is omitted, emulsions are substrates for lipoprotein lipase but the remnants, traced by emulsion cholesteryl oleate (CO), remain circulating in the plasma for a protracted time (2, 5). We also found that for the physiological clearance of chylomicron-like emulsions, the presence of a hydroxyl group at the 3 position and an alkyl side chain at C-17 of cholesterol are essential (6).

The amphiphilic nature of cholesterol is determined by the 3β-hydroxyl group, the tetracyclic ring structure, and the isooctyl side chain at C-17. The isooctyl side chain provides important properties for interaction with phospholipids (7). A variety of side chain analogs of cholesterol have been synthesized, with the alkyl side chain ranging from being shorter or longer than cholesterol (7). The phase behavior of sterol/PC bilayers has been shown to be altered by a large degree of hy-

Abbreviations: PC, phosphatidylcholine; apoE, apolipoprotein E; CO, cholesteryl oleate; TO, triolein; BSS, balanced salt solution; FCS, fetal calf serum; MEM, minimal essential medium; PL, phospholipid; SPR, surface plasmon resonance; THP, tetrahydropyran; *u*, unsaturated. Alkyl chain lengths are designated as *n*-C3 to *n*-C7 (straight chain) or *i*-C5 to *i*-C10 (branched chain).

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drophobic mismatch between a particular sterol and the PC bilayer (7).

In PC/sterol monolayers, sterols with shorter or longer side chains (compared with isooctyl chain of cholesterol) were shown to have a much larger condensing effect when compared with 5-androsten-3β-ol (8), which lacks an alkyl side chain. Lateral domain formation was found to be present in monolayers when sterols contained a side chain of at least 5 carbons (*n* series) and at least 6 carbons (*i* series) (9–11). A small change in sterol alkyl chain length resulted in large effects on the phase behavior of PC bilayers (11, 12). Infrared studies showed that sterols with shorter alkyl side chains (<C6) induced a higher degree of conformational disorder in the hydrocarbon chains of dipalmitoyl-PC than did cholesterol. In contrast, in dipalmitoyl-PC bilayers containing sterols with long alkyl side chains (>C8), a greater degree of PL acyl chain conformational order was found when compared with cholesterol (13).

In the present experiments a series of emulsions was prepared with synthetic sterols of varying carbon chain length (*n*-C3 to *n*-C7 or *i*-C5 to *i*-C10) at the C-17 position substituted for cholesterol (cholesterol in this nomenclature *i*-C8). Emulsions contained triolein (TO), phospholipid (PL), cholesterol or other sterol, and cholesteryl oleate (CO). Radioactive labeled TO and CO were incorporated in order to monitor the plasma clearances of triacylglycerol and cholesteryl oleate uptake when emulsions were injected intravenously into rats. The effects of unsaturation in the alkyl side chain of sterols (*n* or *i* series) were also studied.

MATERIALS AND METHODS

Preparation of chylomicron-like emulsions

Chylomicron-like emulsions of the required compositions were prepared by sonication and purified by ultracentrifugation as previously described (14). TO, CO, cholesterol, and egg yolk phosphatidylcholine (PC) (all from Nu-Chek Prep, Elysian, MN), each more than 99% pure were dispensed from stock solutions in $CHCl_3$ into vials followed by [^{14}C]TO and [^{3}H] CO (Amersham, International plc, Buckinghamshire, U.K.). Solvent was removed by lyophilization. Emulsions were prepared from a lipid mixture of TO (70 mg), CO (3 mg), sterol (2 mg), and PC (25 mg). Sterols were synthesized as described previously (8), except for *n*-C4-1-*u*, which was prepared from bisnorcholenic acid in five steps by tetrahydropyran (THP) etherification of the C-3 hydroxyl group, reduction of the carboxylic group with lithium aluminum hydride, oxidation of the resulting alcohol to the aldehyde using pyridinium chlorochromate, Wittig reaction with the ylide of methyltriphenylphosphonium bromide to yield the terminal double bond, and acid hydrolysis of the THP ether. The *i*-C7-1-*u* was obtained from cholenic acid in five steps by acetylation of the C-3 hydroxyl group, activation of the carboxylic group with oxalyl chloride and cadium-catalyzed coupling reaction with methylmagnesium bromide to yield the methyl ketone. Deacetylation of the C-3 hydroxyl group followed by Wittig reaction afforded the 24-methylene derivative. 5,22-Cholestadien-3β-ol (*i*-C8-3-*u*) and 5,24,-cholestadien-3β-ol (desmosterol, *i*-C8-5-*u*) were obtained from Steraloids, Inc. (Wilton, NH).

Injection studies

For clearance studies, male rats weighing 250–350 g were fitted surgically with arterial and venous cannulas as described earlier (14). Labeled emulsions were prepared and injected to measure plasma clearance exactly as described previously (5, 14). The dose of lipid injected was 2–5 mg in an emulsion volume of 0.35–0.4 ml. Blood samples of 0.35 ml were then taken at 3, 5, 8, 12, 20, 25, and 30 min after injection of an emulsion. After separation by centrifugation, the plasma collected was measured for radioactivity by taking 150 μl and adding 5 ml of Emulsifier-Safe (Packard). Immediately after completion of the clearance study, 20 mg of sodium pentobarbitone was injected and the liver and spleen were removed for extraction of lipids (15) with chloroform–methanol 2:1 (v/v) and radioactivities in the lipid extracts were measured in 15 ml of scintillant.

Preparation of remnant-like emulsion particles

Remnant-like emulsions were prepared by sonication and purified by ultracentrifugation. The emulsions were prepared from mixtures of TO (4.5 mg), PC (2.5 mg), CO (0.5 mg), and cholesterol or *n*-C3 sterol (0.8 mg). For preparation of the fluorescently labeled particles, 0.1 mg of a fluorescent probe, cholesteryl 4, 4-difluoro-5,7-dimethyl-4-bora-3 α, 4 α-diaza-s-indacene-3-dodecanoate (CE-BODIPY), purchased from Molecular Probes (Eugene, OR), was added. After a 1-h sonication of the lipid mixture in 8.5 ml of 2.2% glycerol in water, the crude emulsion was placed at the bottom of two centrifuge tubes, and then 2.5 ml of NaCl solutions of densities 1.065, 1.040, and 1.020 g/ml were sequentially layered above. The tubes were then centrifuged in a SW 41 rotor of a Beckman L8-70M ultracentrifuge for 60 min at 30,000 rpm and 20°C. The particles that floated to the surface were removed and used for cell culture and apoE3 binding studies. The remnant-like emulsion particles were of average diameter 76 ± 6 nm (n = 30) measured by negative-stain electron microscopy.

Binding of apoE3 to remnant-like emulsions

Remnant-like emulsions prepared with either cholesterol or *n*-C3 sterol were mixed with 20 μg of human recombinant apoE3 (Pan Vera Corp., WI). The emulsion–apoE3 mixture was sonicated three times for 1 s with a 2-s interval between each sonication pulse using a Sonic and Material Inc. microtip (Danbury, CT). The emulsion–apoE3 mixtures (180 μl) were placed into airfuge tubes (ultraclear centrifuge tubes, 5 × 20 mm, Beekman). The tubes were then centrifuged at 28 psi for 20 min at 4°C in the A-100/18 rotor of a Beckman airfuge. After centrifugation, 80 μl of the supernatant was carefully removed from one side of the tubes without disturbing the emulsion pellet. The supernatant was assayed for protein content and the fraction of apoE3 bound to remnant-like emulsions was calculated.

Surface plasmon resonance

The kinetics of binding of human apoE3-remnant-like particles (containing either *i*-C8 or *n*-C3 sterols) to the ID7 monoclonal antibody purchased from the Ottawa Heart Institute Research Corporation (Ontario, Canada) was determined by surface plasmon resonance (SPR) with a BIAcore biosensor system (Pharmacia Biosensor, Uppsala, Sweden) using the CM5 sensor chip coated with carboxymethyl dextran on the gold surface (Pharmacia Biosensor, Uppsala, Sweden). The ID7 antibody has been shown to inhibit apoE-mediated binding to the LDL receptor (16). Immobilization of the ID7 antibody was carried out using the BIAcore amine coupling protocol provided with the Pharmacia coupling kit. The ID7 antibody was diluted to 50 μg/ml in the coupling buffer, 10 mm sodium acetate (PH 4.0). Twenty-μl aliquots were injected over the activated chip surface. Unreacted moieties were blocked with 1 m ethanolamine. All measurements were carried out in HEPES-buffered saline containing 10 mm HEPES, pH 7.4, 150 mm NaCl, 3.4 mm EDTA, 0.005% surfactant P-20 (Pharmacia Biosensor, Uppsala, Sweden). The analyses were performed at 25°C and the binding of the respective apoE emulsions to the antibody was measured at a flow rate of 5 μl/min. Surface regeneration was carried out with 100 mm HCl.

Association and dissociation rate constants were calculated by non-linear fitting of the sensogram data using the BIAevaluation 2 software (Pharmacia Biosensor, Uppsala, Sweden). The dissociation rate constant was derived using the equation:

$$R = R_0 e^{-k_d(t - t_0)}$$

where R is the response at time t, R_0 is the response at the start of the dissociation, and k_d is the dissociation rate constant. The association rate constant was calculated by fitting the data to the equation:

$$R = R_{eq}\left(1 - e^{-(k_a C n + k_d)(t - t_0)}\right)$$

where R is the response at time t, R_{eq} is the steady state response level, c is the molar concentration of the analyte, *n* is the steric interference factor, k_a is the association rate constant and k_d is the dissociation rate constant.

Cell culture

HepG2 cells were obtained from ATCC. The cells were cultured at 37°C in 25 ml flasks containing 5 ml of Eagle's minimal essential medium supplemented with 10% fetal calf serum (FCS) (Flow Lab., Sydney, Australia), plus 2 mm glutamine, plus antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin (Calbiochem, La Jolla, CA) under 5% CO_2/95% air incubator (Hepa Filtered, IR Incubator, Forma Scientific, Marietta, OH). The doubling time of the cells was approximately 16 h. The medium was renewed twice a week. Confluent cultures were subcultured using a split ratio of 1:10. For the binding experiments cells were plated on coverslips in 35 mm plastic culture dishes (Disposable Products, Melbourne, Australia). The cells were used when they were near confluence (70%).

Binding of remnant-like emulsions to HepG2 cells

The cells were incubated with 50 μl of fluorescently labeled remnant-like emulsions containing recombinant human apoE3 for 5 min. At the end of the incubation the cells were washed five times with balanced salt solution (BSS) (37°C) and then incubated for 1 min in fetal calf serum (FCS)-deficient minimal essential medium (MEM). The cells were again washed five times with BSS (37°C) and then fixed with 4% paraformaldehyde in 0.1 m cacodylate buffer for 10 min at 4°C. The cover slips were removed from the culture dish and mounted on slides with aquamount for examination with a confocal scanning microscope (Bio-Rad MR-1000).

Assessment of the metabolism of remnants by collection of expired $^{13}CO_2$

In these experiments, lipid mixtures containing TO (70 mg), PC (25 mg), cholesteryl [^{13}C]oleate (3 mg), and cholesterol or *n*-C sterol (2 mg) were emulsified by sonication for 1 h in 8.5 ml of 2.2% glycerol in water. Uniformly labeled [^{13}C]oleic acid was purchased from Novachem Pty. Ltd., Victoria, Australia, and cholesteryl [^{13}C]oleate was synthesized from cholesterol and [^{13}C]oleic acid as described previously (17). After sonication, the emulsion mixture was centrifuged at 3000 rpm for 10 min to remove titanium fragments and then

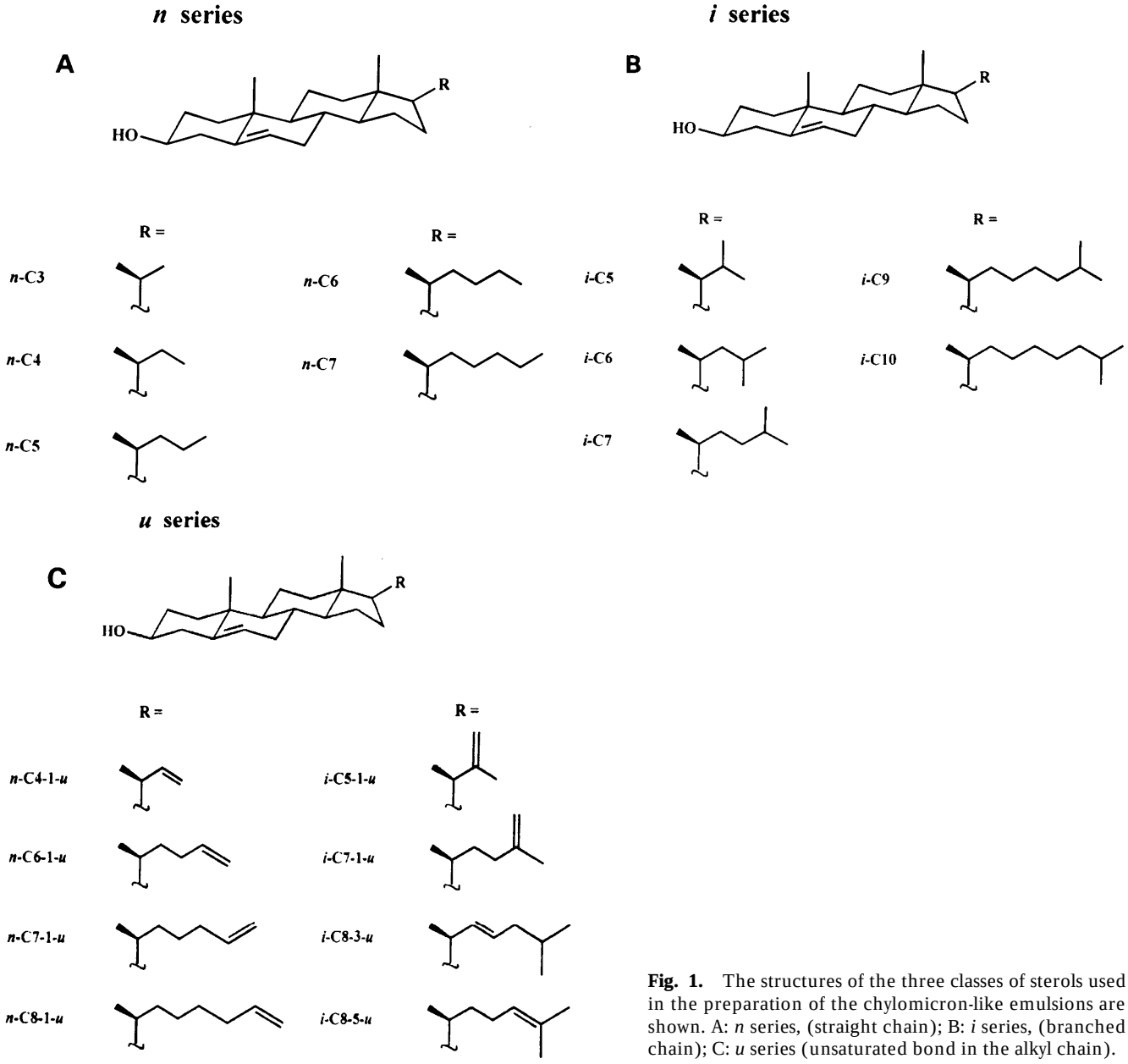

Fig. 1. The structures of the three classes of sterols used in the preparation of the chylomicron-like emulsions are shown. A: *n* series, (straight chain); B: *i* series, (branched chain); C: *u* series (unsaturated bond in the alkyl chain).

filtered through a 0.22-μm filter into sterile vessels. A volume of 100 μl of the emulsion mixture was injected via a tail vein into C57BL/6J (control) mice and apoE knockout mice. Colonies of apoE knockout mice were established from progenitor stocks obtained from the Jackson Laboratories (Bar Harbor, ME). The mice were placed in a closed chamber through which a stream of room air was passed as previously described (18). Breath samples were collected at intervals into evacuated gas sample containers (Europa Scientific Ltd, Crewe, U.K.). The enrichment of breath samples with $^{13}CO_2$ was measured by isotope ratio–mass spectrometry (ABCA, Europa Scientific, Crewe, U.K.).

Measurements of particle diameters

Chylomicron-like emulsions were analyzed by laser-light scattering using a BI-90 particle sizer (Brookhaven Instruments, Ronkonkoma, NY). Remnant emulsion particle size was determined by electron microscopy. Emulsion samples on formvar-coated grids were negatively stained with 2% phosphotungstic acid at room temperature and viewed on a Joel 2000 FX transmission electron microscope.

Chemical analysis

The lipids extracted from emulsions with chloroform–methanol 2:1 (v/v) were separated on silica gel

Table 1. Lipid composition and particle sizes of injected chylomicron-like emulsions

Sterol	Composition of Emulsion				
	TO	Sterol	CO	PC	Diameter
		%			nm
Cholesterol	82.3	1.40	4.01	12.4	144
n-C3	84.1	1.42	3.68	10.8	145
n-C4	82.5	1.62	5.35	10.6	145
n-C5	84.5	1.88	2.93	11.3	142
n-C6	82.7	1.64	3.38	12.3	143
n-C7	80.4	1.64	3.85	14.1	143
i-C5	85.1	1.66	2.64	10.6	143
i-C6	82.9	1.77	2.69	12.6	146
i-C7	86.7	1.02	2.34	10.0	145
i-C9	84.1	1.38	2.66	11.9	147
i-C10	86.9	1.23	2.40	9.7	145
n-C4-1-*u*	81.3	1.30	4.56	12.9	140
i-C5-1-*u*	75.9	1.51	5.10	17.6	143
n-C6-1-*u*	75.9	1.24	5.01	17.9	145
n-C7-1-*u*	78.8	1.90	5.45	13.8	139
i-C7-1-*u*	76.8	1.97	5.07	16.2	142
n-C8-1-*u*	80.8	1.05	4.88	13.3	142
n-C8-3-*u*	76.5	1.43	5.44	16.7	140
n-C8-5-*u*	83.4	1.02	1.90	13.7	141

Isolation by centrifugation and lipid analyses of emulsions were performed as described under Methods. Particle size was determined by laser light-scattering. TO, triolein; CO, cholesteryl oleate; PC, phosphatidylcholine.

TLC plates of 0.2 mm thickness in a solvent system consisting of petroleum ether (40–60°C)–diethyl ether–formic acid 90:10:1 (by volume). The TO, CO, and sterol bands were scraped from the plate for assay of triacylglycerol by the chromotropic acid method (19) and free and esterified cholesterol and sterols were determined by the *o*-phthaldialdehyde procedure (20). Protein assay was by the procedure of Lowry et al. (21) using crystalline bovine serum albumin as a standard. Phospholipid was measured directly on emulsion samples (22).

Statistical analysis

Statistical comparisons were made on the data of the plasma clearance (calculated from area under the curves) by analysis of variance. The *t*-test for independent means was used to compare differences of individual points.

RESULTS

Composition and size of emulsions prepared with various sterols

The structures of the various straight and branched side chain sterols used in the preparation of the emulsions are shown in **Fig. 1**.

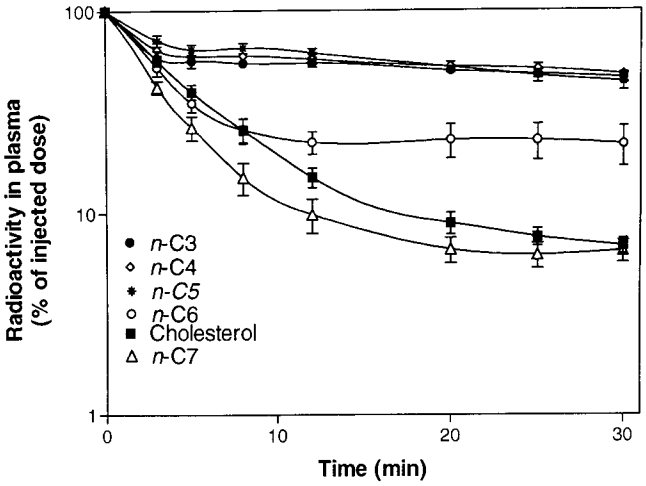

Fig. 2. The radioactivity in plasma after injection into conscious rats of chylomicron-like emulsions prepared with sterols of varying chain length (*n*-C3 to *n*-C7) at the C-17 position. Plotted are the data for [^{3}H]-CO label incorporated in the emulsions remaining in plasma at 3, 5, 8, 12, 20, 25, and 30 min post injection. Results are means ± SEM of six animals in each group.

Table 1 shows the average diameters and compositions of the various emulsions used for the clearance studies. Emulsions prepared with sterols of varying carbon chain length (*n*-C3 to *n*-C7 or *i*-C5 to *i*-C10) at the C-17 position contained approximately 84% TO and approximately 11% PC. The content of sterol was approximately 1% and the content of CO was approximately 3%. In contrast, emulsions containing sterols with a double bond in the alkyl side chain or close to the terminal carbon (*i*-C5-1-*u*, *n*-C5-1-*u* to *n*-C8-1-*u*, *i*-C7, *i*-C8-3-*u*, or *i*-C8-5-*u*) contained less TO and more CO and PC. The diameters of the emulsions prepared with the various sterols ranged between 139 and 147 nm as measured by laser light scattering.

Plasma clearance of emulsions containing the various sterols (*n, i* or *u* series)

Figure 2 compares the rates of clearance of emulsions prepared with sterols of varying carbon straight chains (*n*-C3 to *n*-C7) at the C-17 position. The clearance of the CO label (tracing remnant clearance) was markedly decreased from the plasma of rats when emulsions were prepared with sterols containing 5 carbons or fewer in the alkyl side chain. The remnant clearance was improved when emulsions were prepared with a sterol with 6 carbons; however, 7 carbons were needed for rapid clearance of remnants to produce a clearance rate similar to that of emulsions prepared with cholesterol.

Figure 3 shows the rates of clearance of emulsions prepared with sterols with branched carbon chains (*i*-C5 to *i*-C10) at the C-17 position. An alkyl side chain of

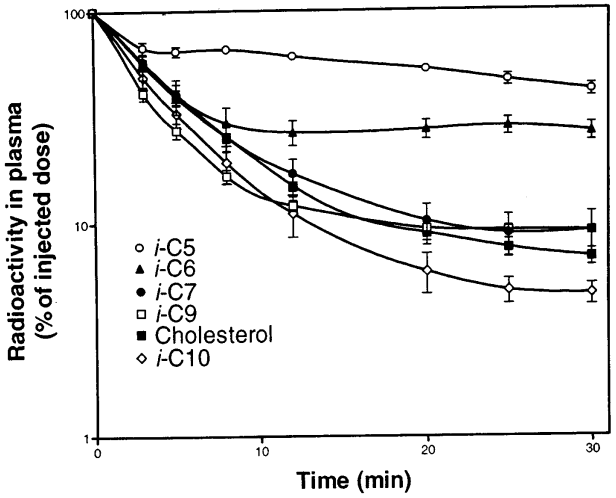

Fig. 3. The removal from plasma of cholesteryl oleate label (CO) after intravenous injections of chylomicron-like emulsions prepared with sterols of carbon chain length (*i*-C5 to *i*-C10) at the C-17 position. The results are means ± SEM of six animals in each group.

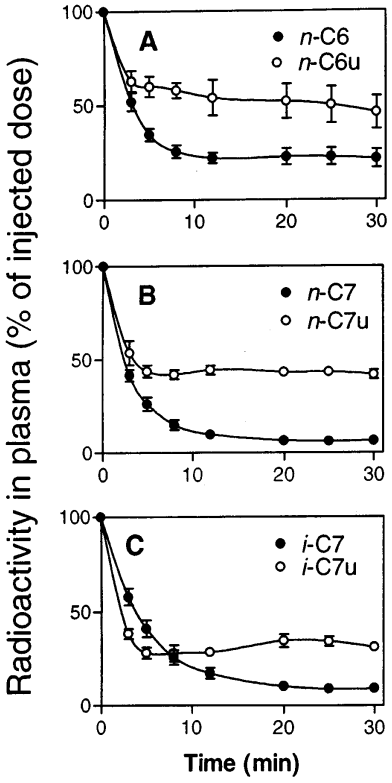

Fig. 4. Radioactivity in plasma of cholesteryl oleate (CO) label after injection into conscious rats of chylomicron-like emulsions containing sterols with or without unsaturation in the alkyl side chain close to the terminal carbon. Emulsion contained the following sterols, *n*-C6 (panel A), *n*-C7 (panel B), or *i*-C7 (panel C); *u*, ene. The results are means ± SEM of 5–6 animals in each group.

7 carbons was needed to obtain remnant clearance rates similar to emulsions prepared with cholesterol. In contrast, the clearance of remnants derived from emulsions prepared with sterols with shorter chain lengths (*i*-C5, *i*-C6) at the C-17 position was markedly slower. As shown in **Fig. 4**, the rates of clearance of emulsions containing sterols with unsaturation (*u*) in the side chain close to the terminal carbon (*n*-C6-*u*, *n*- or *i*-C7-*u*) were markedly slower compared with emulsions containing similar sterols (*n*-C6, *n*- or *i*-C7) without the unsaturation in the side chain.

The remnant clearances of emulsions prepared with various sterols are summarized in **Fig. 5**. For easier comparison of clearance rates, the data were integrated by calculating the areas under the clearance curves. This number is reciprocally related to clearance so the inverse was used for the comparisons. The upper panel (*n* series) shows that remnants derived from emulsions containing sterols with an alkyl chain of 6 carbons or less were cleared significantly slower ($P < 0.001$) than emulsions containing cholesterol or *n*-C7 sterol.

In Fig. 5 (*i* series), the clearance of remnants from emulsions containing a sterol with a 5 carbon alkyl chain was significantly slower ($P < 0.001$) than that of remnants derived from emulsions with a sterol having a 6 carbon alkyl chain. Emulsion remnants containing sterols with alkyl chain lengths between 7 and 10 carbons (*i* series) were cleared faster ($P < 0.001$) than remnants derived from emulsions with alkyl chain lengths of 6 or less carbon atoms.

When an unsaturated bond was present in the alkyl side chain of sterols, the clearance of emulsion remnants was markedly decreased ($P < 0.001$) when a sterol with an alkyl chain of 7 carbons or less was used relative to emulsions containing cholesterol, *n*-C8-*u*, *i*-C8-3-*u*, or *i*-C8-5-*u*, (Fig. 5, *u* series). In contrast, the clearance of emulsion remnants with *i*-C8-5-*u*, was faster than that of emulsions containing cholesterol, *n*-C8-*u* or *i*-C8-3-*u* ($P < 0.001$).

The lipolysis index (calculated by subtraction of the clearance of CO from clearance of TO) is shown in **Fig. 6**. The lipolysis index for emulsions prepared with most of the sterols was similar to emulsions containing cholesterol. The only marked difference was found with emulsions prepared with either *n*-C4 or *n*-C4-*u* sterol, where the lipolysis index was significantly higher ($P < 0.01$) compared with emulsions containing cholesterol or other sterols.

Figure 7 shows the liver uptake of CO and TO labels when rats were injected with emulsions prepared with cholesterol or the various sterols. When emulsions were

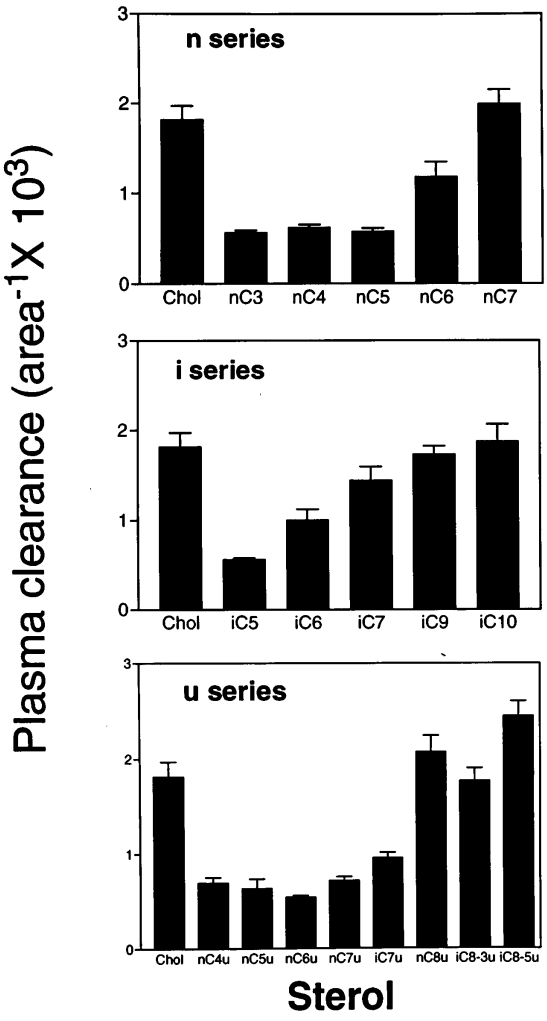

Fig. 5. The clearance of cholesteryl oleate (CO) label from the plasma of rats after intravenous injection of emulsions prepared with various sterols. The data are calculated as the area under the clearance curves. The results are means ± SEM of 5–6 animals in each group; *u*, ene.

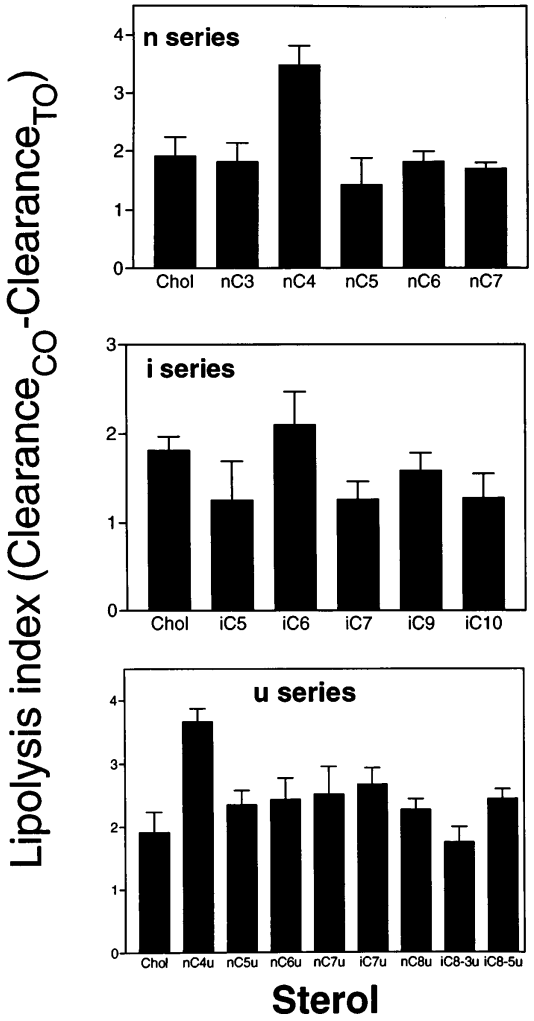

Fig. 6. The lipolysis index for the emulsions prepared with the various sterols. The lipolysis index was calculated as the difference in areas under the curves for TO and CO.

prepared with the *n* series sterols (5 carbons or less) the liver uptake of CO label was significantly decreased ($P < 0.01$) when compared with emulsions prepared with cholesterol or with sterols having 6 or 7 carbons in the alkyl chain (*n* series). The uptake of CO label was lower when emulsions were prepared with *i* series of the sterol (<7 carbons) but this difference did not reach statistical significance. When the alkyl side chain (7 carbons or less) of sterols contained an unsaturated bond (*u* series), the liver uptake was significantly reduced ($P < 0.02$). No significant differences were found in the liver uptake of the TO label between emulsions prepared with cholesterol and the other sterols.

The recovery of the CO label (calculated from the plasma and liver data) for most sterols ranged between 60 and 70%. Recovery of the CO label was greater (between 73–75%) for emulsions prepared with sterols containing either *n*-C5, *i*-C5, *n*-C6, or *i*-C8-3-*u* alkyl chains. Less CO label was recovered (approximately 55%) with sterols with either *n*-C4-*u*, *i*-C9, or *i*-C8-5-*u* alkyl chains. The recovery of the TO label for most sterols ranged between 7 and 11%, except for the *n*-C5 sterol (18%). Previous work has established that most TO is cleared by adipose tissue and muscle (1). During the course of the clearance study the TO and CO labels have been shown to be degraded and metabolized with the appearance of $^{14}CO_2$ radioactivity in the breath (18).

Measurement of remnant metabolism from CO_2 in expired breath of mice

Figure 8 shows the appearance of $^{13}CO_2$ in the expired breath of control and apoE knockout mice after

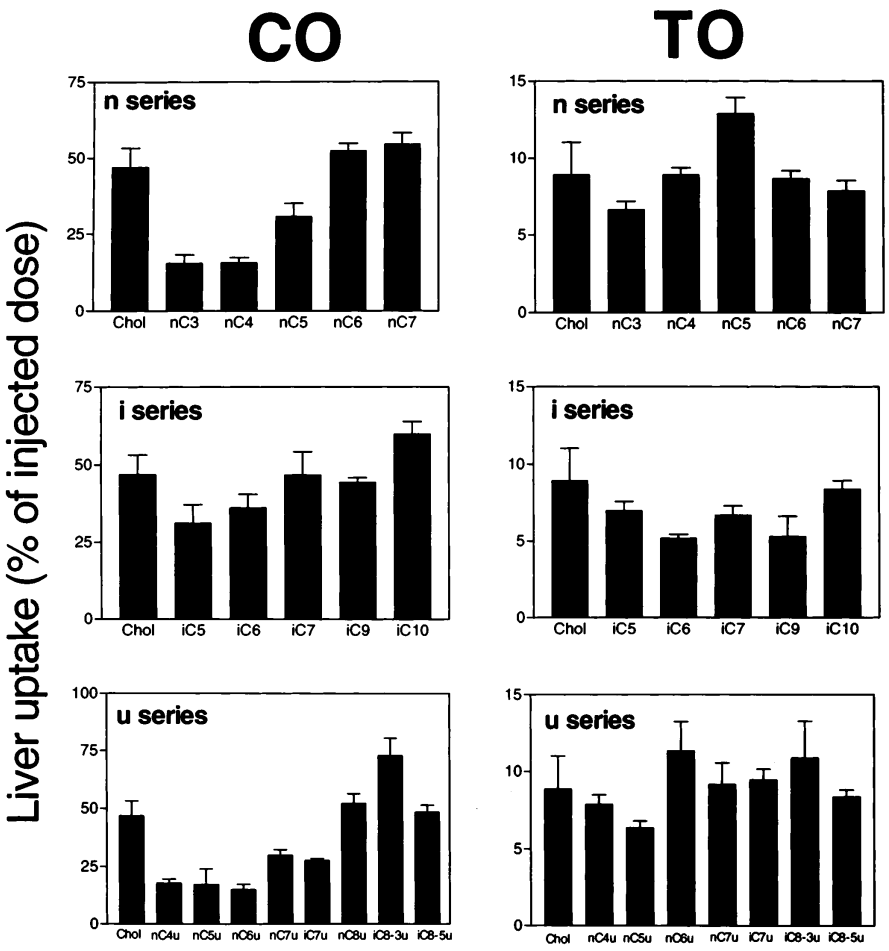

Fig. 7. The uptake of cholesteryl oleate (CO) and triolein (TO) labels in the liver after injections of chylomicron-like emulsions prepared with cholesterol or the various sterols. The results are means ± SEM of 5–6 animals in each group; *u*, ene.

injection of emulsions labeled with cholesteryl [^{13}C]oleate when prepared with either cholesterol (*i*-C8) or *n*-C3 sterol. There was no enrichment of $^{13}CO_2$ in the expired breath of apoE-deficient mice when injected with emulsions prepared with either cholesterol or *n*-C3. In contrast, in control mice the enrichment of $^{13}CO_2$ was significantly less ($P < 0.03$) at 60 and 90 min when emulsions containing *n*-C3 were compared with emulsions containing cholesterol.

Binding of apoE to fluorescently labeled remnant-like emulsions and uptake by HepG2 cells

Remnant-like emulsions containing either cholesterol or *n*-C3 sterol were each found to bind similarly approximately 30% of total recombinant human apoE3 (data not shown). When apoE-associated fluorescent remnant-like emulsions containing cholesterol were incubated with HepG2 cells, binding was avid as shown by the bright fluorescence of the cells in **Fig. 9** (panel A). In contrast, as shown in Fig. 9 (panel B), when cells were incubated with apoE-associated emulsions containing *n*-C3 sterol, there was only a low extent of cell-associated fluorescence (<10% when compared with emulsions containing cholesterol estimated by pixel intensity).

Interaction of apoE3 remnant-like emulsions with immobilized 1D7 antibody

The kinetics of binding of apoE3 remnant-like emulsions containing either *i*-C8 or *n*-C3 sterol to the ID7 antibody was monitored using SPR. Interaction of the immobilized ID7 antibody to the apoE3 emulsions was studied over a concentration range of 2.2–9 nmol/L of apoE3 associated with the remnant-like emulsions. The sensograms of the binding of apoE emulsions containing either *i*-C8 or *n*-C3 sterol to the ID7 antibody are shown in **Fig. 10**. The association and dissociation rate constants were determined from the sensograms by fit-

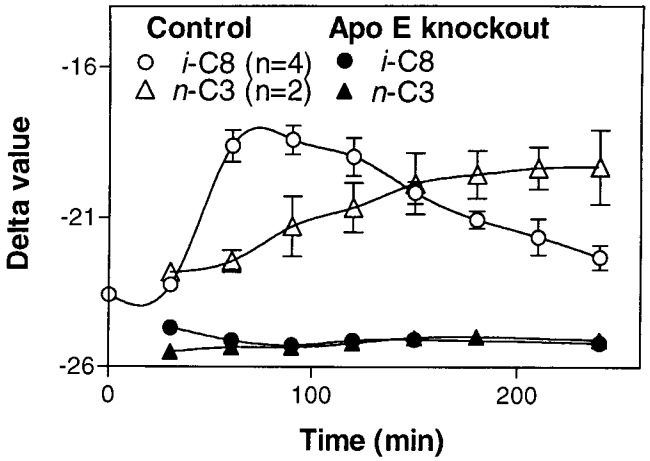

Fig. 8. The enrichment of $^{13}CO_2$ in the expired breath of control and apoE knockout mice after intravenous injection of emulsions prepared with either cholesterol (*i*-C8) or *n*-C3 sterol. Mice were injected with 100 μl of emulsion labeled with cholesteryl [^{13}C]-oleate via the tail vein and $^{13}CO_2$ was collected as described in Methods. The results are means ± SEM.

ting the data to the equations described in the Methods section. The dissociation rate constant was significantly higher ($P < 0.001$) for the apoE emulsions containing the *n*-C3 sterol ($3.19 \times 10^{-3} \pm 8.38 \times 10^{-5}$ s^{-1}) when compared with the apoE-associated emulsions containing cholesterol ($2.13 \times 10^{-3} \pm 8.36 \times 10^{-5}$ s^{-1}). The association rate constants were lower for the apoE emulsions containing *n*-C3 ($2.07 \times 10^4 \pm 0.62 \times 10^4$) but this difference was not significant when compared with emulsions containing cholesterol ($4.56 \times 10^4 \pm 1.38 \times 10^4$).

DISCUSSION

We have previously shown that for the physiological clearance of chylomicron-like emulsions, the presence of a hydroxyl group in either the α and β configuration at the 3 position and an alkyl side chain of undefined length and degree of saturation at the C-17 position of cholesterol are essential (6). To determine the importance of the structure and length of the isooctyl side chain at the C-17 position, the present clearance studies were performed with various synthetic sterols using emulsion models of lymph chylomicrons.

In the present study we have compared the plasma clearances of emulsions prepared with sterols of varying carbon chain length (*n*-C3 to *n*-C7 or *i*-C5 to *i*-C10) at the C-17 position. We found that an alkyl side chain at the C-17 position of at least 7 carbons (*n* or *i* series) was needed for normal remnant clearance from

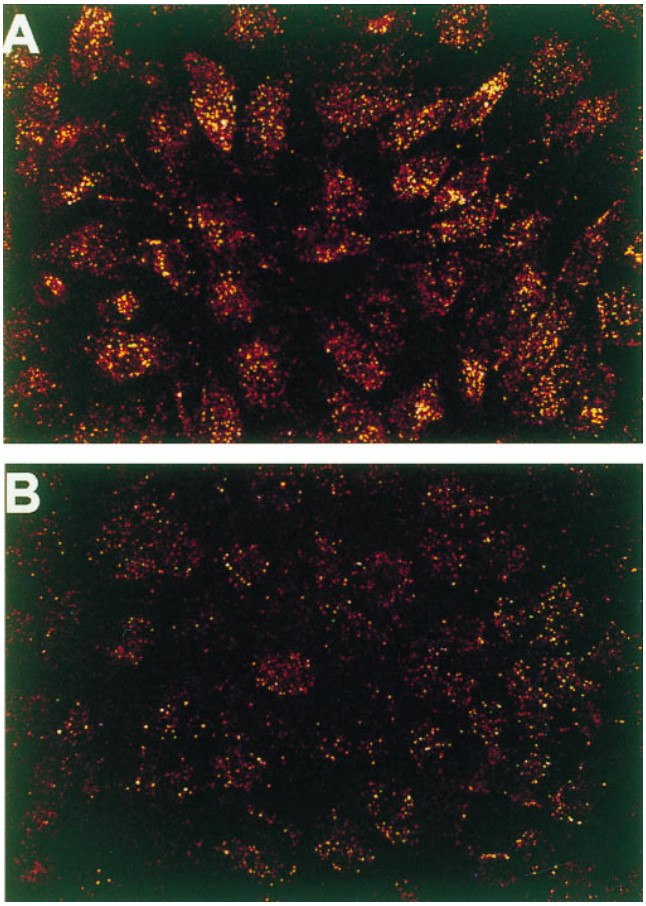

Fig. 9. The binding of fluorescent remnant-like emulsions containing either cholesterol or *n*-C3 sterol to HepG2 cells. Cells were incubated with apoE3-associated cholesteryl–BODIPY emulsions containing either cholesterol or *n*-C3 sterol for 5 min, then washed and fixed in 4% paraformaldehyde and viewed for fluorescence using a confocal microscope. The upper panel (A) shows findings when fluorescent remnant-like emulsions containing cholesterol were incubated with HepG2 cells. The lower panel (B) compares findings when similar emulsions containing *n*-C3 sterol were incubated under identical conditions.

plasma. Moreover, the metabolism of remnants as measured by $^{13}CO_2$ breath test was found to be markedly less for the short straight-chain sterol (*n*-C3) compared with the branched chain cholesterol (*i*-C8) as shown in Fig. 8.

When emulsions were prepared with sterols with unsaturation in the alkyl side chain close to the terminal carbon (*i*-C5, *i*-C7 or *n*-C6, *n*-C7) the clearance of remnants was markedly decreased. Emulsions prepared with either a sterol with unsaturation at the terminal carbon of the 8-carbon alkyl side chain (*n*-C8-1-*u*) or a double bond within the 8-carbon alkyl side chain (*i*-C8-5-*u*) were cleared rapidly from plasma similar to emulsions containing cholesterol.

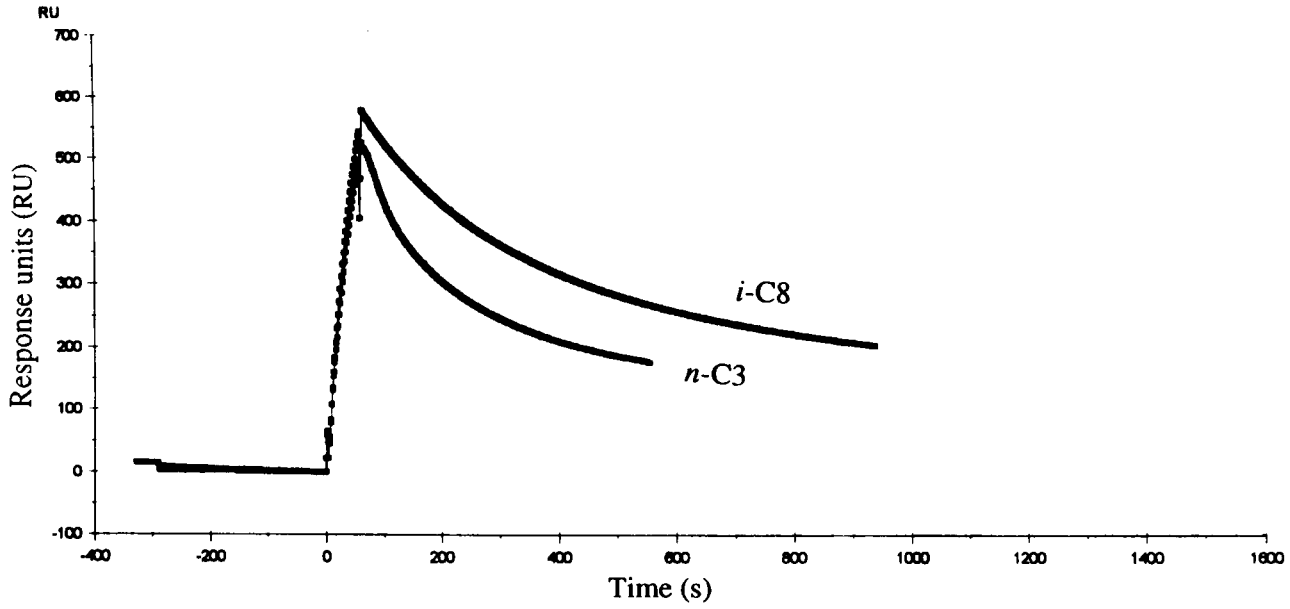

Fig. 10. The biosensor analysis of the interaction of the immobilized ID7 antibody with apoE emulsions containing either cholesterol or *n*-C3 sterol. The binding of the respective apoE emulsions to the antibody was measured at a flow rate of 5 μl/min. The concentration of apoE in emulsions was 9 nmol/L. These sensograms show that the kinetics of binding of the apoE emulsions containing cholesterol to the ID7 antibody was markedly higher when compared with the apoE emulsions containing the *n*-C3 sterol.

The lipolysis index (Fig. 6) was greater for emulsions containing sterols with an alkyl side chain with 4 carbon atoms or with an unsaturated bond at the terminal carbon (*n*-C4-1-*u*) compared with emulsions prepared with cholesterol or other sterols. However, other sterols had little effect on lipolysis consistent with previous evidence that emulsion sterols have little influence on lipolysis in vivo (5, 6). Although sterol side-chain structure did not affect the amount of apoE3 bound to emulsions, we found that in cell culture studies the binding of the *n*-C3 emulsions to HepG2 cells was markedly reduced (Fig. 9). This result suggests that the conformation of apoE or accessibility of apoE to the surface was affected in *n*-C3 emulsions, leading to altered binding to LDL receptors and decreased endocytosis. We sought evidence for a change in conformation of apoE3 by measuring the binding to the ID7 monoclonal antibody, which binds specifically to the apoE3 receptor-binding domain. Measurements by SPR of the kinetics of binding of apoE-containing emulsions to the ID7 antibody showed that the dissociation rate of the apoE emulsions containing *n*-C3 was significantly higher when compared with apoE emulsions containing cholesterol. This result provides evidence that the conformation of apoE was different in *n*-C3 emulsions, probably accounting for a higher dissociation rate from the LDL receptor leading to decreased endocytosis.

In conclusion, our studies show that the length of the alkyl chain at the C-17 position of sterols can markedly affect the removal of remnant particles from the plasma of rats and mice. Sterols with a short alkyl chain (less than 7 carbons) delay the clearance and metabolism of remnants. A branch in the alkyl chain had no effect on remnant clearance but the presence of an unsaturated bond at the terminal carbon of the alkyl chain in sterols delayed the clearance of remnants. In vitro studies show that the binding of remnants to HepG2, cells was markedly decreased with a sterol with a short alkyl chain. The reduced binding and reduced remnant clearance may be explained by the altered conformation of apoE associated with emulsions with short alkyl chains demonstrated by a higher dissociation rate for the ID7 monoclonal antibody in a BIAcore experiment.

This work was supported by grants from the National Health and Medical Research Council of Australia and Inovax Ltd. to (TGR) and US National Institutes of Health, Grant HL 16660 (RB). We thank Allan Light for the electron microscopy and Dr. Bing Ji Zeng for assistance with the cell culture studies.

Manuscript received 25 February 1997, in revised form 11 July 1997, and in revised form 7 October 1997.

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