Clearance from plasma of lymph chylomicrons and chylomicron remnants labelled with ^{125}I -tyramine–cellobiose

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The aims of the present study were to evaluate the metabolism of chylomicrons (CM) and of CM remnants after labelling with radioactive iodine and converting the iodinated CM into remnants *in vitro*. Lymph CM were radiolabelled with ^{125}I or sham-labelled with ^{127}I by either the ICI procedure or the tyramine–cellobiose (TC) procedure, then injected into rats. The clearance from plasma of the iodinated CM was compared with control non-iodinated lipid-labelled CM. After iodination with ICI, the plasma removal of endogenously labelled CM was significantly different from non-iodinated CM, with increased uptake of CM triacylglycerols by the liver. In contrast, the clearances from plasma and the uptake by organs of radiolabelled lipids of CM iodinated by the TC method (TC-CM) were similar to control CM. About 40 % of the label from ^{125}I -TC-CM was insoluble in 50 % propan-2-ol, indicating association with CM apolipoprotein B48. Only about 8 % of label was lipid soluble, mostly in phosphatidylethanolamine. Radioactivity from ^{125}I -TC-CM injected intravenously in rats was cleared rapidly and by 30 min only 20 % remained in plasma, whereas 48 % was recovered in the liver. After fractionation of the plasma by density-gradient ultracentrifugation, most label remained associated with d (relative density) < 1.006 lipoproteins. In intact rats label was also found associated with the low-density and high-density lipoprotein fractions of plasma. When the liver was excluded from circulation, the recovery of label in low-density- and high-density-lipoprotein fractions was greatly decreased. CM remnants were prepared *in vivo* by injecting ^{125}I -TC-CM into functionally hepatectomized donors and compared with remnants prepared *in vitro* by incubation with purified bovine milk lipoprotein lipase. Although remnants prepared *in vitro* cleared from plasma slower than remnants prepared *in vivo*, the size, lipid composition and apolipoprotein profile on gradient PAGE of the remnants were similar. We conclude that labelling of CM by the TC method avoided the ‘artefactual’ changes in metabolism seen after labelling by the ICI procedure. CM remnants when prepared *in vitro* using lipoprotein lipase were found to be similar to those prepared *in vivo* after injection into functionally hepatectomized rats.

INTRODUCTION

Cholesterol derived from diet and bile lipids and dietary triacylglycerols (TGs) are transported in CM from the intestine by way of lymph vessels into the blood stream. The CM TG is rapidly hydrolysed by the enzyme lipoprotein lipase at the endothelial surface of capillaries in adipose tissue, skeletal and cardiac muscle and other sites [1]. Before the hydrolysis of TGs is completed, the CM are released into the circulation, where they are recognized as remnant particles [2]. CM remnants (RM) are subsequently removed rapidly from the plasma, mostly by the liver. Most remnants are probably removed after binding to low-density-lipoprotein (LDL) receptors [3,4,5]. Homozygous Watanabe heritable hyperlipidaemic rabbits with defective LDL receptors show defective clearance of both LDL and RM [6,7] and accumulate retinyl esters in plasma after a meal containing vitamin A [8]. Separate receptors for binding of remnants have been postulated, but their physiological role in clearance of CM remains unproven.

Progress with the understanding of remnant metabolism has been impeded because of unsatisfactory labelling procedures. The peroxidation reactions involved in conventional radiolabelling with iodine seem likely to damage the lipids of chylomicrons. In the present work, CM obtained from mesenteric lymph were radiolabelled with iodinated tyramine–cellobiose (^{125}I -TC) [9], then converted into remnants *in vitro* by incubation with purified bovine milk lipoprotein lipase [10]. The clearances from plasma of the radiolabelled CM and their remnants were measured after injection into normal rats. Clearance was found

to be unaltered by the TC labelling procedure, in contrast with ICI.

MATERIALS AND METHODS

Animals and materials

Male Wistar rats weighing 200–250 g were obtained from the Animal Resources Center (Willetton, Western Australia, Australia) and fed on a commercial pellet diet containing approx. 5 % (w/v) fat. Intralipid [10 % (w/v) soybean oil/1.2 % (w/v) egg phospholipids/2.25 % (w/v) glycerol] was from Pharmacia. Gentamycin, GSH, BSA (essentially free of fatty acid; fraction V) and cyanuric chloride were purchased from Sigma. Iodogen [1,3,4,6-tetrachlorodiphenylglycoluril] was from Pierce Chemical, Rockford, IL, U.S.A.. Purified bovine milk lipoprotein lipase was a gift from Professor T. Olivecrona and Professor G. Bengtsson-Olivecrona of the University of Umeå, Umeå, Sweden. Radioactive [^3H]cholesterol ([7(n)- ^3H]cholesterol), [^{14}C]triolein (glycerol tri[1- ^{14}C]oleate) and Na^{125}I were purchased from Amersham International, Sydney, Australia.

Preparation of CM

Lymph CM were prepared by infusing a mixture of 10 % Intralipid and 5 % glucose (2:3, v/v) into the duodenum of rats by way of a plastic tube previously placed by surgery. The milky lymph flow was collected through a cannula placed in the mesenteric lymph duct [11] into tubes containing 2 mM-EDTA, 1.6 mM-GSH, gentamycin (0.1 mg/ml) and 1 mM-phenylmethane sulphonyl fluoride.

Abbreviations used: CM, chylomicron(s); RM, chylomicron remnants; LDL, low-density lipoproteins; VLDL, very-low-density lipoproteins; HDL, high-density lipoproteins; TC, tyramine–cellobiose; TG, triacylglycerol; d , relative density; FCR, fractional clearance rate.

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Cells were removed from the lymph by low-speed centrifugation. CM were isolated by density-gradient ultracentrifugation [12]. Lipid-labelled CM were obtained by infusing the same Intralipid/glucose mixture containing 15 μCi of [^{14}C]triolein and 40 μCi of [^3H]cholesterol. The diameter of the chylomicrons was 200 ± 20 nm (mean $\pm$ S.E.M., $n = 6$) measured with a B1-90 particle sizer (Brookhaven Instrument Corp, Ronkonkoma, NY, U.S.A.).

Iodination of CM

In experiments designed to test the effects of the iodination procedures on the metabolism of the CM, radioactive iodine was omitted and replaced with an equivalent mass of unlabelled Na^{127}I . CM labelled in the lipid moieties with [^3H]cholesterol and [^{14}C]TG were obtained from lymph-cannulated rats as described above. One portion of the lipid-labelled CM was iodinated by using the procedure as originally described by McFarlane [13], whereas the remainder was untreated and served as a control.

In another experiment, lymph CM labelled with [^3H]cholesterol and [^{14}C]TG were iodinated with unlabelled Na^{127}I by using TC as a ligand, as described by Pittman *et al.* [14]. This method allows an indirect binding of iodine to CM protein with TC as a ligand adduct. Again the remaining portion of CM was untreated and served as a control. In the experiments comparing CM remnants after preparation *in vivo* and *in vitro*, the lipid labels were omitted and radioactive ^{125}I was used to label CM with TC. The specific radioactivity of ^{125}I -TC-CM was 10–92 d.p.m./ng of protein.

Preparation of remnants

Remnants were prepared *in vivo* by injecting the CM into functionally hepatectomized rats, as described by Jeffery *et al.* [15]. CM containing 70–90 μmol of TG/kg body weight were injected into rats that were bled 30 min later. Remnants were then isolated by density-gradient ultracentrifugation of the plasma [12].

Remnants were prepared *in vitro* by using purified lipoprotein lipase as described by Chajek-Shaul *et al.* [10] with slight modification. The enzyme preparation contained 0.4 mg of enzyme protein/ml, with an activity per mg of 530 μmol of non-esterified fatty acid/min. The incubation mixture contained 30 mg of CM TG, 3 mg of rat HDL proteins and 30 μmol of BSA in Tris/HCl (0.2 M; pH 8.5), in a final volume of 12 ml. TG hydrolysis was initiated with 25 μl of lipoprotein lipase during incubation at 25 °C. At 60 min the initial turbidity had cleared and NaCl (final concn. 1 M) was added to the incubation mixture to stop the lipolysis [16]. Remnants were isolated by density-gradient ultracentrifugation of the incubation mixture [12]. Remnant diameter measured by electron microscopy was 25 ± 5 nm (mean $\pm$ S.E.M., $n = 9$). HDL was purified by sequential ultracentrifugation from rat plasma in the relative-density (d) interval 1.09–1.21 [17].

Injection studies

CM were injected into the bloodstream of conscious rats to measure the plasma disappearance and the organ distribution of the injected radioactivity. Rats were prepared with carotid and jugular cannulas as described previously [18]. At the end of the experiment, the spleen, liver and both femurs were removed. The liver was perfused with cold saline through the portal vein before removal. In the experiments using lipid-labelled CM, radioactivity was measured in lipid extracts of the minced organs. The radioactivity uptake of the bone marrow was also measured in lipid extracts from both femurs. Lipids were extracted from the organs into chloroform/methanol (2:1, v/v). Aliquots were then taken, the solvent was evaporated and radioactivity was measured

by liquid-scintillation spectrometry. Radioactivity in plasma was measured without extraction in 200 μl aliquots using ReadySolv (Beckman). For the experiments using Na^{125}I plasma, aliquots were counted directly for radioactivity in an Autogamma spectrometer (Packard; model 5360). Radioactivity in the whole spleen and 1 g pieces of weighed liver were also counted directly. Femur bone marrow was assumed to be 1/13th of the total bone marrow [19].

Characterization of CM labelled with ^{125}I -TC

Exchangeability of iodine radioactivity between CM and HDL was assessed by incubating ^{125}I -TC-CM with a 5–200-fold excess of HDL proteins for 30 min at 37 °C. The incubation mixture was separated by density-gradient ultracentrifugation [20] and the radioactivity in each fraction was measured. Whole plasma was fractionated similarly, in association with CM injection studies. Intact rats and functionally hepatectomized rats prepared by ligating the splanchnic circulation as described previously [15] were injected through the jugular cannula. Blood was sampled from the carotid cannula after 5, 10, 20 and 60 min, and plasma was separated by density-gradient ultracentrifugation as described previously [20].

Chemical analysis

Lipid phosphorus was measured as described by Bartlett [21]. Protein was determined by the Lowry procedure [22], with BSA as standard. Total cholesterol and TG mass corrected for free glycerol were determined by enzymic procedures (Boehringer Mannheim kits 390 319 and 877 557 respectively).

Protein analysis of CM and remnant apolipoproteins was by SDS/PAGE with a gradient of 5–25% polyacrylamide. The lipoprotein samples were delipidated as described by Herbert *et al.* [23] before they were loaded on to the gel. After staining the gel with Coomassie Blue R-250 (Bio-Rad) and destaining, the apolipoproteins were identified by comparison with standards of molecular mass 97400 to 14400 Da (Bio-Rad). Radioactivity in CM associated with apolipoprotein B was measured by precipitation in 50% propan-2-ol [24], after adding unlabelled carrier CM.

The lipids extracted from CM and remnants to chloroform/methanol (2:1, v/v) were separated by t.l.c. on 0.2 mm-thick layers of silica gel (DC-Alufolien Kieselgel 60; E. Merck, Darmstadt, Germany) in the solvent system light petroleum (b.p. 40–60 °C)/diethyl ether/formic acid (90:10:1, by vol.) for separation of neutral lipids. Phospholipids were then separated in the solvent system chloroform/methanol/diethyl ether/acetic acid (26:10:6:1 by volume). The bands were revealed with iodine vapour and the radioactivity was measured by autoradiography.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. Data were analysed for significant differences by analysis of variance, using SYSTAT software. Differences between group means were assessed by Tukey's procedure [23a]. Homogeneity of groups was established by an F-test of sample variance. If the F-test indicated non-homogeneity of group variances, the Kruskal-Wallis [23b] non-parametric analysis of variance was used.

RESULTS

Plasma clearance of CM iodinated by the ICI or the TC methods

Fig. 1(a) shows the plasma clearance of [^{14}C]TG from control CM and CM iodinated by the ICI (iodinated-ICI CM) or TC (iodinated-TC CM) procedures. The removal from plasma of

Fig. 1. Plasma clearances of [¹⁴C]TG (a) and [³H]cholesterol (b) after injection of control CM (●) or iodinated CM prepared either by the ICI (○) or TC (▲) procedures

Four separate preparations of lymph CM were labelled endogenously with [¹⁴C]TG (a) and [³H]cholesterol (b). A portion from each preparation was iodinated using ICI (○) or TC (▲) as described in the Materials and methods section, while the remainder was untreated and served as control CM (●). Each point is the mean of data from 14 rats, except for ●, where *n* was 17. The bars represent 1 S.E.M.

Table 1. Plasma clearance of control CM and CM iodinated by the ICI and TC procedures

Four separate preparations of lymph CM were labelled endogenously with [¹⁴C]TG and [³H]cholesterol ([³H]CHOL). A portion of each preparation was iodinated using ICI (iodinated-ICI CM) or TC (iodinated-TC CM) as described in the Materials and methods section, whereas the remainder was untreated and served as a control, then injected into normal rats. FCRs were calculated from the bi-exponential curves fitted to the data 3–30 min after injection. At 30 min after injection, organs were removed and lipids were extracted for measurement of radioactivity. Both femurs were collected and the results were expressed in terms of total body bone marrow. Results are means ± S.E.M. for the numbers of observations in parentheses. Statistical significance: ^aSignificantly different from control CM by analysis of variance (*P* < 0.05); ^bsignificantly different from control CM and iodinated-TC CM by Kruskal–Wallis procedure [23b] (*P* < 0.025); ^csignificantly different from control CM by analysis of variance and Kruskal–Wallis procedure (*P* < 0.01).

CM	Removal from plasma (FCR) (min ⁻¹)		Organ uptake of lipid radioactivities (% of injected dose)					
			Liver		Spleen		Bone marrow	
	[¹⁴ C]TG	[³ H]CHOL	[¹⁴ C]TG	[³ H]CHOL	[¹⁴ C]TG	[³ H]CHOL	[¹⁴ C]TG	[³ H]CHOL
Control	0.20 ± 0.016 (17)	0.10 ± 0.005 (17)	14.78 ± 1.08 (16)	56.58 ± 2.25 (16)	0.54 ± 0.07 (16)	1.12 ± 0.11 (16)	0.07 ± 0.01 (10)	0.23 ± 0.03 (10)
Iodinated-ICI	0.27 ± 0.038 ^c (14)	0.08 ± 0.018 ^b (14)	22.57 ± 3.00 ^a (14)	48.14 ± 2.27 (14)	1.06 ± 0.41 (14)	1.87 ± 0.55 (14)	0.09 ± 0.01 (12)	0.29 ± 0.04 (12)
Iodinated-TC	0.15 ± 0.017 ^c (14)	0.10 ± 0.009 (14)	16.60 ± 1.15 (14)	46.28 ± 5.98 (14)	0.39 ± 0.03 (14)	0.86 ± 0.13 (14)	0.10 ± 0.02 (14)	0.24 ± 0.04 (14)

Fig. 2. Plasma clearance of ¹²⁵I-TC-CM

Lymph CM unlabelled in lipids were iodinated with TC as described in the Materials and methods section. The ¹²⁵I-TC-CM were then injected into normal recipient rats. Each point is the mean of data from five rats. The bars represent 1 S.E.M.

[¹⁴C]TG in iodinated-ICI CM was faster than the control untreated CM, with less [¹⁴C]TG from iodinated-ICI CM remaining in plasma than from control CM at every time point

except at 30 min after injection. About 70 % of TG label had disappeared from the plasma 3 min after injection of the iodinated-ICI CM, compared with about 50 % from control untreated CM (Fig. 1a). The plasma removal of [¹⁴C]TG in iodinated-TC CM, however, was similar to the control untreated CM (Fig. 1a). When the data from all time points were analysed by two-way analysis of variance, the difference between iodinated-ICI CM and control untreated CM was highly significant (*P* < 0.001).

Fig. 1(b) shows the plasma-clearance curves of [³H]cholesterol from control, iodinated-ICI, and iodinated-TC CM. The plasma removal of [³H]cholesterol in the iodinated-ICI CM was slower than the control CM, with fractional clearance rate (FCR) of 0.08 ± 0.018 min⁻¹ compared with 0.10 ± 0.005 min⁻¹ in control CM (*P* < 0.025; Table 1). By 30 min, 30 % of the [³H]cholesterol label in iodinated ICI CM still remained in the plasma, compared with less than 20 % of the label from control CM. By two-way analysis of variance at all time points, the control and iodinated-ICI groups were significantly different (*P* < 0.005). By contrast, clearance from plasma of CM iodinated by TC did not differ significantly from controls.

Table 1 compares the FCR and organ uptakes (liver, spleen and bone marrow) of the control CM and CM iodinated by

Fig. 3. Radioactivity of different lipoprotein fractions after incubating ^{125}I -TC-CM with a 5-fold (■) or 200-fold (▨) excess of HDL protein for 30 min at 37 °C

The incubation mixture was centrifuged in a continuous density gradient as described in the Materials and methods section. Fractions of different densities were separated and the radioactivity in each fraction was measured.

either ICI or TC procedures. In iodinated-ICI CM, the FCR of ^{14}C TG was higher ($P < 0.01$), whereas that of ^3H cholesterol label was slower ($P < 0.025$) than control untreated CM and iodinated-TC CM. Liver uptake of ^{14}C TG from iodinated-ICI CM was significantly higher than the control CM or the iodinated-TC CM ($P < 0.05$).

Plasma clearance of ^{125}I -TC-CM and ^{125}I -TC-RM

For these studies, CM were iodinated using the TC procedure. The ^{125}I -TC-CM were injected into normal recipient rats for measurement of plasma clearance (Fig. 2). At 3 min after injection the ^{125}I label in plasma was less than 50% of that injected and this decreased to only 20% by 30 min after injection. The pattern of ^{125}I -TC-CM clearance was very similar to that of ^3H -cholesterol from control CM shown in Fig. 1(b). Liver and spleen uptakes of ^{125}I measured 30 min after injection were 47.6 ± 2.22 ($n = 6$) and 1.51 ± 0.08 ($n = 5$) of injected dose respectively. Measured 60 min after injection, 14.3 ± 1.14 ($n = 5$) of radioactivity remained in plasma, whereas 80.5 ± 7.38 ($n = 5$) was in liver.

Nenseter *et al.* [9] found with ^{125}I -TC-CM that more than 90% of the radioactivity was precipitated by 10% (w/v) trichloroacetic acid. After precipitation of apolipoprotein B in 50% propan-2-ol [24] we found 40.9 ± 2.46 ($n = 10$) to be insoluble. Chloroform/methanol extracts of the lipids from labelled CM contained 8.5 ± 2.9 ($n = 10$) of the total radioactivity, and t.l.c. showed that most of the lipid label was in phospholipids. Autoradiography of the t.l.c. plate developed in a solvent system for separating phospholipids (see the Materials and methods section), revealed that, within phospholipids, 50–60% of the label was in phosphatidylethanolamine, 20–30% in phosphatidylcholine and less than 5% in sphingomyelin.

Except for apolipoprotein B48, the proteins associated with CM are thought to be readily exchangeable. Different lipoprotein fractions were separated by density-gradient ultracentrifugation after incubating ^{125}I -TC-CM with a 5–200-fold excess of HDL proteins as described in the Materials and methods section. Fig. 3 shows that more than 80% of the radioactivity was found with CM in fraction 1, with $d < 1.006$ [25]. All other fractions up to $d = 1.21$ had low levels of radioactivity, and this pattern was not affected by a 200-fold excess of HDL protein.

Fig. 4. Radioactivity of different lipoprotein fractions in plasma after intravenous injection of ^{125}I -TC-CM in intact rats or functionally hepatectomized rats

Functionally hepatectomized rats were prepared by ligating the splanchnic circulation. Blood was sampled 5, 10 and 20 min after injection of labelled CM. Fractions of different densities were separated from the plasma and radioactivity in each fraction was measured. As indicated in Fig. 3, fraction 1 corresponds to $d = 1.006$, fraction 3 to 1.030 and fraction 6 to 1.063. In rat plasma LDL is found in fractions 3–5 and HDL in fractions 5–8. Each point is the mean of data from three rats. The bars represent 1 S.E.M.

Fig. 5. Lipolysis of ^{125}I -TC-CM by lipoprotein lipase *in vitro*

The incubation mixture included ^{125}I -TC-CM, purified bovine milk lipase, BSA and HDL at 25 °C, as described in the Materials and methods section. At time intervals up to 120 min the turbidity was measured from A_{500} (●) and the TG concentration of the incubation mixture (○) was assayed.

Fig. 6. Plasma clearances of ¹²⁵I-TC-RM prepared *in vivo* (●) and *in vitro* (○)

CM were labelled exogenously using TC (¹²⁵I-TC-CM). The ¹²⁵I-TC-CM were converted into remnants either *in vivo* or *in vitro*. RM were prepared *in vivo* (●) by injecting ¹²⁵I-TC-CM into hepatectomized rats. Preparation of *in vitro* RM (○) was by incubating ¹²⁵I-TC-CM with rat HDL, BSA and purified lipoprotein lipase at 25 °C for 60 min. The different remnant preparations were injected into normal recipient rats to obtain the plasma-clearance curves shown. Each point is the mean of data from six rats. The bars represent 1 S.E.M.

Table 2. Radioactivity in liver and spleen after injection of ¹²⁵I-TC-RM prepared *in vivo* or *in vitro*

RM labelled with ¹²⁵I-TC were prepared *in vivo* by injecting ¹²⁵I-TC-CM into liver-excluded donor rats and *in vitro* using purified bovine milk lipoprotein lipase as described in the Materials and methods section. The two remnant preparations were injected into normal rats. At 15 min after injection the organs were removed for measurement of radioactivity. Results are means ± S.E.M. for six observations in each case. Results were compared by an unpaired *t*-test. Statistical significance: ^a not significant different from *in vivo* remnants (*P* > 0.05); ^b significantly different from *in vivo* remnants (*P* > 0.01).

Organ	Radioactivity recovered (% of injected dose)	
	¹²⁵ I-TC-RM prepared <i>in vivo</i>	¹²⁵ I-TC-RM prepared <i>in vitro</i>
Liver	76.9 ± 3.25	67.5 ± 3.77 ^a
Spleen	0.62 ± 0.09	1.45 ± 0.25 ^b

Table 3. Composition of ¹²⁵I-TC-RM prepared *in vivo* or *in vitro*

Results are means ± S.E.M. for the numbers of observations given. Remnant lipids were extracted, separated on t.l.c. plates and concentrations were determined as described in the Materials and methods section. Remnant sizes were determined by a Jeol 2000 FX transmission electron microscope from selective fields as described in Fig. 5. Statistical significance: ^a significantly different from *in vivo* remnants by an unpaired *t*-test (*P* < 0.05).

Component/parameter	Composition (% , w/w)	
	RM prepared <i>in vivo</i> (n = 6)	RM prepared <i>in vitro</i> (n = 9)
TG	44.4 ± 4.16	66.2 ± 2.14 ^a
Non-esterified cholesterol	1.5 ± 0.33	3.1 ± 1.19
Cholesteryl ester	4.0 ± 0.65	2.6 ± 0.60
Protein	25.6 ± 1.77	14.6 ± 9.83 ^a
Phospholipid	24.5 ± 3.37	11.9 ± 1.49 ^a
Diameter (nm)...	25 ± 5	25 ± 5

Fig. 7. Electron-microscope appearance of ¹²⁵I-TC-RM prepared *in vivo* and *in vitro*

Remnant samples on Formvar-coated grids were negatively stained with 2% phosphotungstic acid at room temperature and viewed on a Jeol 2000 FX transmission electron microscope. Samples are shown at a magnification of 65000, and the bar indicates 120 nm. Although most appear spherical, a few particles are irregular, especially remnants prepared *in vivo*. Remnants prepared *in vivo* appear more homogeneous in size and shape than remnants prepared *in vitro*.

Fig. 4 shows the distribution of label after subfractionation of plasma in density gradients when ¹²⁵I-TC-CM were injected intravenously. In intact rats (shown in the left-hand-side panels), label was concentrated in the least dense fractions, corresponding to CM and RM, and decreased between 5 and 20 min after injection. Label was also found associated with LDL and HDL in fractions 3–8 (see Fig. 3). Similar studies in functionally hepatectomized rats with the liver excluded from circulation are shown in the right-hand-side panels. Compared with the intact rats, more label was found in the least dense fraction, whereas much less label was found in fractions 3–8.

Fig. 5 illustrates the conversion of ¹²⁵I-TC-CM into remnants *in vitro* using purified bovine milk lipase. The turbidity of the incubation mixture was measured by the absorbance at 500 nm, and TG concentration was also measured (Fig. 5). After 60 min of incubation the incubation mixture was cleared of turbidity and most TG from CM had been lipolysed. An incubation time of 60 min was used for routine remnant preparations *in vitro*.

Fig. 6 compares the plasma clearance of ¹²⁵I-TC-RM prepared *in vitro* with ¹²⁵I-TC-RM prepared *in vivo* by injection into functionally hepatectomized rats. Although the initial rates of clearance of these remnants were identical, from 3 min onwards the clearance of ¹²⁵I-TC-RM prepared *in vivo* was faster than ¹²⁵I-TC-RM prepared *in vitro*. Table 2 compares the liver and spleen uptakes 15 min after injection of remnants prepared *in vitro* and *in vivo*. Liver uptake of ¹²⁵I-TC-RM prepared *in vitro* was not significantly different from that of ¹²⁵I-TC-RM prepared *in vivo*. However, spleen uptake of ¹²⁵I-TC-RM prepared *in vitro* was significantly greater than ¹²⁵I-TC-RM prepared *in vivo* (*P* < 0.01).

Size, apolipoprotein and lipid composition of ¹²⁵I-TC-CM and ¹²⁵I-TC-RM

Size, lipid composition, and the apolipoproteins separated by SDS/PAGE were compared between ¹²⁵I-TC-RM prepared *in vivo* and *in vitro*. Fig. 7 shows the appearance of both types of remnants under the electron microscope. Most remnants appeared to be spherical and similar in size. Remnants prepared *in*

Fig. 8. Comparison of apolipoproteins from CM and their remnants by gradient SDS/5–25%-PAGE

Lipoprotein samples were delipidated and dissolved in SDS sample buffer before separation on the slab gel. The gel was stained with Coomassie Blue R250 for 2 h and destained overnight in 5% (v/v) acetic acid. Lanes 1 and 2, CM (25 and 50 µg protein respectively); lanes 3 and 4, remnants prepared *in vivo* (25 and 50 µg of protein respectively); lanes 5, 6 and 7, remnants prepared *in vitro* (20, 40 and 80 µg of protein respectively). Molecular-mass (*M*) standards in lane 8 are phosphorylase *b* (94.7 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean inhibitor (21.5 kDa) and lysozyme (14.5 kDa). Although the samples and the standards were run on the same gel, the standards were relocated to the right-hand-side for photography. Cs ≡ CI, CII, CIII

in vivo appeared to be more homogeneous in size and shape than remnants prepared *in vitro*.

Table 3 compares the lipid composition and the diameter of remnants prepared *in vivo* or *in vitro*. Remnants prepared *in vivo* appeared to have more proteins and more phospholipids and less TG than remnants *in vitro*. Free cholesterol and cholesteryl ester concentrations were not significantly different from remnants prepared *in vitro* ($P > 0.05$).

Fig. 8 compares the apolipoprotein contents of CM and of remnants prepared *in vitro* or *in vivo*. Apolipoproteins B48, AIV, E, AI and C's were seen in CM (lanes 1 and 2). Bands of molecular mass approx. 66 kDa were probably due to albumin from the lymph. Scanning by laser densitometry revealed that albumin was 6.8% of the total protein. Other CM proteins were apolipoprotein AIV (10.6%), E (14.7%), AI (28.9%), Cs (33.5%) and B48 (2.8%). Fig. 8 shows that in both types of remnant preparations there was a complete absence of apolipoprotein AIV, whereas amounts of apolipoprotein C band were similar when compared with the precursor CM in lanes 1 and 2.

More apolipoprotein B48 and apolipoprotein E were present with *in vivo* remnants than *in vitro* remnants (Fig. 8), whereas apolipoprotein AI was less with *in vivo* remnants than *in vitro* remnants. The most striking difference between the two types of remnant preparations was the heavy band of albumin at 66 kDa seen with *in vitro* remnants. The incubation mixture used to prepare the remnants *in vitro* included 30 µmol of BSA/30 mg of chylomicron TG as described in the Materials and methods section. Although most albumin was removed by the density-gradient-ultracentrifugation method, it was apparent that some albumin was retained in the top remnant fraction.

DISCUSSION

RM have been difficult to investigate because they are similar in size to small CM and to plasma very-low-density lipoproteins

(VLDL), and because of their rapid removal from plasma [28]. Understanding of remnant metabolism has been further impeded by difficulties in preparation of remnants. Commonly used radiolabelling methods employ a peroxidation reaction to introduce radioactive iodine into tyrosine residues under conditions that are known to oxidize lipoproteins. For lipoproteins such as CM that are susceptible to oxidation, such conditions might irreversibly damage the lipoprotein. For example oxidized LDL was found to bind with high affinity to macrophages [26]. Nevertheless LDL preparations have been iodinated by the ICI method [27]. CM may be more susceptible than LDL to oxidative damage because of the presence of unsaturated fatty acyl chains in the phospholipids on the CM surface [28]. Damage to CM from iodination with ICI was indicated by our data. The clearance rates for both [14 C]TG and [3 H]cholesterol in CM labelled with ICI were significantly different from non-iodinated control CM (Figs. 1a and 1b; Table 1). Increased uptake by the liver of [14 C]TG probably indicated modification of the CM and uptake by non-parenchymal liver cells.

Iodination by the TC method does not involve oxidation of the lipoprotein and the ligand adduct does not appear to alter the biological behaviour of labelled proteins, except that the label is trapped intracellularly and not degraded by lysosomal enzymes. TC labelling has been used for LDL [29], VLDL [30] and HDL apolipoprotein AI [29,31]. A recent review should be consulted for details [14].

We found that lipid-radiolabelled CM iodinated by the TC procedure retained the pattern of plasma clearance similar to that of control uniodinated CM. The uptake by the liver, spleen and bone marrow of both [3 H]cholesterol and [14 C]TG labels of TC-labelled CM were also similar to the controls (Figs. 1a and 1b, Table 1).

Nenseter *et al.* [9] previously labelled CM using the TC iodination method. They studied the intracellular localization of CM remnants after endocytosis by isolated hepatocytes, taking advantage of the intracellular trapping of the TC adduct. They found only 25% of label in the liver 60 min after injection of 125 I-TC-CM, with 60% in the blood mainly in HDL. They concluded that label in exchangeable apolipoproteins had transferred directly from CM to HDL. By contrast we found 80% of label in the liver 60 min after injection, with only 14% in the plasma. As early as 5 min after injection, a significant amount of plasma label was found in HDL-containing fractions of the plasma (Fig. 4), but most had already been taken up by the liver. Nenseter *et al.* [9] found by SDS/PAGE that CM radioactivity was distributed among apolipoproteins B48, AII and the C apolipoproteins, with minor amounts in apolipoproteins E, AI and AIV. We found a similar broad distribution of the label among the apolipoproteins by SDS/PAGE electrophoresis (results not shown). Precipitation of apolipoprotein B occurs in 50% propan-2-ol [24], and we found about 40% of label was precipitated.

Apolipoprotein B48 represents less than 5% of the total protein mass in CM and is a structural protein involved in assembly and secretion of CM by the intestinal cells. On the other hand, apolipoproteins AI, AII, AIV and C are thought to be exchangeable and therefore not a reliable marker for CM clearance studies. When we incubated labelled CM with a large excess of plasma HDL, the label was not displaced from the CM (Fig. 3). The fast disappearance of 125 I-TC-CM from plasma makes it unlikely that significant amounts of radioactivity exchanged or transferred to the plasma LDL or HDL pools, which have much slower kinetics than CM remnants. Our experiments in intact rats (Figs. 2 and 4) show that 60% of CM radioactivity was removed from the plasma in 5 min without transfer to the LDL or HDL fractions, although significant amounts of label that remained in the plasma became associated

with these fractions. Label in the LDL or HDL fractions was greatly decreased in functionally hepatectomized rats, indicating that dissociation of label may have occurred during the process of uptake of CM by the liver. Early recycling of a fraction of endocytosed residualizing label was found by Buktenica *et al.* [32]. Our findings can therefore be reconciled with those of Nenseter *et al.* [9] if some label transferred to HDL after rapid recycling when RM were endocytosed by the liver. Use of higher-molecular-mass residualizing labels may decrease recycling [33].

A strategy to eliminate the possibility of transfer of exchangeable label was to inject preformed remnant particles from which exchangeable label would be eliminated during preparation. Previous studies have used remnants isolated by density-gradient ultracentrifugation of plasma derived from liver-excluded rats, after injecting CM labelled endogenously in the lipid core [6,18]. By this method recovery is low and contamination by endogenous lipoproteins is unavoidable. Therefore remnants were also prepared *in vitro* with lipoprotein lipase [34,35]. After 60 min of incubation, more than 75% of chylomicron TG was lipolysed and the incubation mixture was no longer turbid (Fig. 5). Remnants produced *in vitro* by incubation of ^{125}I -TC-CM with purified lipoprotein lipase [10] were compared with remnants prepared *in vivo* by injecting ^{125}I -TC-CM into liver-excluded animals. Remnants from both preparations appeared spherical and had similar diameters of about 25 nm. Remnants from the *in vitro* preparation had a higher TG content and lower protein content than *in vivo* remnants, possibly a reflection of contamination of *in vivo* remnants by endogenous lipoproteins.

Plasma clearance of *in vivo* ^{125}I -TC-RM was faster than *in vitro* ^{125}I -TC-RM with a greater liver uptake. Analysis of apolipoprotein components in the two remnant preparations by SDS/PAGE revealed that ^{125}I -TC-RM prepared *in vivo* had more apolipoprotein E and apolipoprotein B48, again possibly because the remnants prepared *in vivo* were contaminated with other lipoproteins from the recipient rat plasma. A striking difference between the two remnant types was the intensity of apolipoprotein AI bands on SDS/PAGE, which was more intense for *in vitro* remnants than for *in vivo* remnants. Apolipoprotein AI is present abundantly in CM [28] and in HDL proteins [36], and has been reported to inhibit remnant binding and uptake [37]. However apolipoprotein AI associated with *in vitro* ^{125}I -TC-RM was only 8.3% of total protein, a concentration at which inhibitory effects may not be apparent. SDS/PAGE revealed that the albumin was about 25% of the total protein of *in vitro* remnants, consistent with previous work using purified lipase to prepare *in vitro* remnants [10]. The significance of albumin is unclear, but if it reflects contamination then remnant concentrations should not be expressed in terms of total protein.

Our findings show that the metabolism of CM is 'artefactually' altered by a standard method of radiolabelling with iodine, possibly because of oxidative damage to the CM lipids. CM labelled by an alternative method using TC were metabolized similarly to control unlabelled CM. The partially lipolysed remnants obtained from TC-labelled CM retained physiological metabolic behaviour whether converted into remnants *in vivo* or *in vitro*.

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