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Effects of Anti-Microtubular Agents and Cycloheximide on the Metabolism of Chylomicron Cholesteryl Esters by Hepatocyte Suspensions

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1. Post-heparin plasma that promoted rapid hydrolysis of about 90% of the triacylglycerol markedly stimulated the uptake or binding of chylomicron cholesteryl ester by suspended hepatocytes. The net hydrolysis of chyle cholesteryl ester after the uptake by the cells was, however, slower than *in vivo*. 2. The cholesteryl ester uptake in the presence of post-heparin plasma was larger if the cells had been preincubated for 2h. It was inhibited by the presence of colchicine, vinblastine or cycloheximide during the preincubation, and by mild trypsin treatment of the preincubated cells. 3. The results suggested that the anti-microtubular agents, but not cycloheximide, also inhibited the hydrolysis of chyle cholesteryl ester after uptake or binding to the cells. 4. The uptake of isolated chylomicron remnant particles was more efficient than that of native chyle lipoproteins. It was, however, still stimulated by heparin alone and by post-heparin plasma. The heparin-stimulated uptake was markedly decreased if cycloheximide was present during the preincubation period.

During the metabolism of chylomicron triacylglycerol by the action of lipoprotein lipase (Robinson, 1963; Havel, 1965) remnant particles which are rich in cholesteryl esters are formed (Redgrave, 1970). Chylomicron cholesteryl ester is cleared from blood, mainly by liver (Goodman, 1962; Quarfordt & Goodman, 1967; Stein *et al.*, 1969; Nilsson & Zilversmit, 1971), but several important questions about the mechanism by which chylomicron remnants are degraded have not been answered. Felts *et al.* (1975) suggested that lipoprotein lipase which is bound to the remnant particles is important for the binding of remnants to hepatocytes, but a role of other components of the particles for the binding has not been excluded. Studies by electron-microscopic radioautography suggest that the chylomicron cholesteryl esters may be hydrolysed in the hepatocyte plasma membranes before or during the penetration into the cell (Stein *et al.*, 1969). No cholesterol esterase (EC 3.1.1.13) with a distinct localization on the plasma membrane has, however, been found (Riddle *et al.*, 1975; Nilsson, 1977), whereas lysosomes (Stokke, 1972; Nilsson *et al.*, 1973), and the soluble cytoplasm as well as the microsomal fractions (Deykin & Goodman, 1962), contain high cholesterol esterase activities. These are all active *in vitro* with chylomicron remnants as substrate (Nilsson, 1977). The role of the different cholesterol esterases in chylomicron degradation is thus still unclear. Anti-microtubular agents inhibit the hydrolysis of the cholesteryl esters *in vivo* after their uptake by the liver (Nilsson, 1975*a*). A mechanism depending on intact microtubular functions, such as membrane fusion (Felts *et al.*, 1975) or lysosomal degradation after endocytosis of the whole particle, may thus be involved in remnant degradation.

The present study used suspensions of rat hepatocytes to study the degradation of chyle cholesteryl esters. Significant uptake by the cells and net hydrolysis of chyle cholesteryl esters occurred in the presence of post-heparin plasma, which promoted rapid hydrolysis of most of the added chyle triacylglycerol. In this system the effects of cycloheximide, anti-microtubular agents and mild trypsin treatment of the cells on the catabolism of chyle cholesteryl ester could be conveniently studied, to get information about the possible role of proteinase-digestible cell-surface material and of microtubules in the remnant degradation. The usefulness of the system is, however, limited by the finding that the net hydrolysis of cholesteryl ester was slow when isolated remnant particles were incubated with the cells in the absence of heparin or post-heparin plasma.

Materials and Methods

Preparation of labelled chyle

[G-^{3}H]Cholesterol, [4-^{14}C]cholesterol, [1-^{14}C]oleic acid and [9,10-^{3}H]oleic acid were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. The [^{3}H]cholesterol was purified by t.l.c. on silica gel in light petroleum (b.p.60–70°C)/diethyl ether/acetic acid (80:20:1, by vol.) and was eluted from the

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silica gel with chloroform/methanol (2:1, v/v). The radiopurity of the labelled compounds used was 96% or greater as determined by t.l.c. in the same system. Male white Sprague–Dawley rats were obtained from Anticimex AB, Stockholm, Sweden.

Thoracic-duct cannulations (Bollman *et al.*, 1948) were performed under diethyl ether anaesthesia with 0.5mm×0.8mm polyvinyl tubing. The same tubing was used to make a gastric fistula. During the first 24h the animals had free access to 2.5% (w/v) glucose, 0.5% (w/v) NaCl and 0.05% (w/v) KCl, and a constant infusion through the gastric fistula of 2ml of the same solution/h. The fat was then given through the fistula and was divided into three doses over a 6h period. Each animal received 100–400μCi of [^{3}H]-cholesterol or 50–100μCi of [^{14}C]cholesterol in 0.75ml of trioleoylglycerol. Doubly labelled chyle was prepared by also adding 10–20μCi of [^{14}C]oleic acid or 50–100μCi of [^{3}H]oleic acid to the oil. Lymph was collected for 10–12h after the first dose. Portions were analysed for the radioactivity in the different lipid classes and for the content of cholesterol and of triacylglycerol as described below. The distribution of radioactively labelled lipids between particles with $S_f > 400$ (chylomicrons) and with $S_f < 400$ was determined by ultracentrifugation (Minari & Zilversmit, 1963) in an MSE TC centrifuge and a 6×16.5ml swing-out rotor (no. 59108). It was found that 93.3–93.9% of the radioactivity of the triacylglycerol and 80.9–82.5% of that of cholesteryl esters was in chylomicrons ($S_f > 400$). The remaining part was almost entirely in particles that floated at $d = 1.006$ after centrifugation for 24h at 100000$g_{av.}$ (VLD lipoproteins*). Chylomicrons for the experiments were isolated by centrifuging chyle that had been diluted with 0.9% (w/v) NaCl at 70000$g_{av.}$ for 60min in the same rotor. They were resuspended in NaCl and were floated once more under the same conditions. The infranatant was centrifuged for 24h at 100000$g_{av.}$ in the MSE 10×10ml angle-head rotor (no. 59113) to float the remaining VLD lipoproteins. The separation of chyle lipoproteins into chylomicrons and VLD lipoproteins is an arbitrary division of particles that vary continuously in size and density (Windmueller *et al.*, 1970) and that are metabolized by the same pathways, although at different rates (Ockner *et al.*, 1969; Brunzell *et al.*, 1973). Because of these similarities, several of the experiments were therefore performed with whole chyle rather than with isolated lipoprotein fractions.

Preparation of chylomicron remnants

Post-heparin plasma was prepared from aortic blood of fed rats (weighing 300–350g) that had been injected intravenously with 250i.u. of heparin 1.5–3.0min earlier. It was used in the fresh state or after storage at −17°C for up to 3 weeks. Then 50μl (0.3–1.4mg of lipid) of doubly labelled chylomicrons VLD lipoproteins or whole chyle was mixed with 0.8ml of post-heparin plasma, and 1.8ml of 5% (w/v) bovine serum albumin dissolved in the buffer used in the cell incubation experiments (see below). The mixture was incubated for 60min at 37°C under an atmosphere of CO_2+O_2 (5:95). During the incubation, 87–94% of the radioactively labelled triacylglycerol was hydrolysed, as measured by the change in radioactivity distribution on t.l.c. The reaction was stopped by cooling to 4°C, and the mixture was chromatographed (see Fig. 3 below) on Bio-Gel A-50 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) (Rudel *et al.*, 1974). The void eluate, which contained most of the residual chylomicron triacylglycerol and cholesteryl ester, was then dialysed at 4°C against the buffer used in the cell experiments.

Cell-incubation experiments

Male white Sprague–Dawley rats weighing 200–340g were used in the fed state. Collagenase (CLS, 143–180 units/mg) was obtained from Worthington Biochemical Corp., Freehold 2, NJ, U.S.A. Hepatocytes were prepared by a collagenase procedure (Berry & Friend, 1969; Ingebretsen & Wagle, 1972) as described earlier (Nilsson *et al.*, 1974). Each preparation was checked by phase-contrast microscopy and by staining with Trypan Blue. About 88–98% of the cells excluded the stain. After a change in the laboratory routine (caused by a gain in convenience and in time) cells were prepared by the method of Seglen (1973), i.e. the liver was perfused with Ca^{2+}-free Hanks buffer (Hanks & Wallace, 1949) for 10min before the addition of 0.05% (w/v) collagenase plus 1.25mM-$CaCl_2$. The data in Table 6 (below) were obtained with cells prepared by this method.

The cells were incubated in 25ml Ehrlenmeyer flasks in a total volume of 1.5ml. The buffering medium was Hanks solution containing 25mM-$NaHCO_3$ and 19.4mM-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid] (Nilsson *et al.*, 1974). The medium contained amino acids (East *et al.*, 1973) and bovine serum albumin that had been defatted (Chen, 1967) and dialysed against the buffering medium. At low concentrations of post-heparin plasma (3.3%, v/v) rapid and extensive cell aggregation occurred without apparent cell damage, as judged by the Trypan Blue test. The cell aggregation was much less at higher concentrations of post-heparin plasma, and 26.4% (v/v) was therefore used in all experiments with post-heparin plasma that are reported (except that in Fig. 1). The incubations were performed under CO_2+O_2 (5:95). They were stopped by cooling to 4°C, and cells and media were separated by centrifugation (Sundler *et al.*, 1973). The cell pellets were resuspended and were washed once in Ca^{2+}-free Hanks solution. The supernatants

* Abbreviation: VLD lipoprotein, very-low-density lipoprotein ($d < 1.006$, $S_f = 20$–400).

and the cell pellets were analysed to determine the radioactivity in the different lipid classes as described below. Colchicine was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Vinblastine sulphate was obtained from Eli Lilly and Co., Indianapolis, IN, U.S.A. Crystalline trypsin (Trypure) was obtained from Novo Industri A/S, Bagsvaerd, Denmark. Soya-bean trypsin inhibitor (Type 1-S) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Analytical procedures

Lipids were extracted with 7–10 vol. of chloroform/methanol (1:1, v/v) (Bligh & Dyer, 1959). The lipid extract was washed and dried as described earlier (Nilsson *et al.*, 1974). The lipids were separated by t.l.c. on silica-gel G with light petroleum (b.p. 40–60°C)/diethyl ether/acetic acid (80:20:1, by vol.). The areas containing cholesteryl ester, triacylglycerol, unesterified fatty acid and cholesterol were scraped directly into counting vials and the radioactivity was determined by liquid scintillation with a dioxan-based scintillator. The radioactivity of the polar lipids was determined by counting in Instagel (Packard Instrument Co. Downers Grove, ILL, U.S.A.)/toluene (1:1, v/v) containing 4.5% (v/v) of water and 4.5% (v/v) of methanol. The radioactivity was determined in a Packard TriCarb model 3375 liquid-scintillation counter. Quenching was corrected for by aid of the automatic external standard. The percentage net hydrolysis of radioactive cholesteryl ester was calculated from the decrease in d.p.m. in cholesteryl ester and the increase in d.p.m. in unesterified cholesterol as follows:

$$\%\ \text{Net hydrolysis} = 100 - \frac{\%\ \text{Cholesterol as ester after incubation} \times 100}{\%\ \text{Cholesterol as ester before incubation}}$$

Triacylglycerols were determined by g.l.c. of the fatty acid methyl esters after isolation of the triacylglycerol by t.l.c. (Åkesson *et al.*, 1970). The typical fatty acid composition (% by weight) of chylomicron triacylglycerol was: 68.2% oleic acid; 9.4% palmitoleic acid; 8.6% palmitic acid; 7.6% linoleic acid; 5.1% myristic acid; 1.4% stearic acid. Cholesterol and cholesteryl esters were determined by the method of Zak *et al.* (1954) after the separation by t.l.c. as described above and after elution from the silica gel with chloroform/methanol (2:1, v/v). The cholesteryl ester was saponified by the method of Abell *et al.* (1952). The cholesteryl ester content of the 'chylomicron remnants' was calculated by comparing the specific radioactivity of their cholesteryl ester with that of the cholesteryl ester of the chylomicrons from which they were prepared. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Effects of post-heparin plasma

In earlier experiments (Nilsson, 1975*b*), and in the control experiments described below, the rate of cholesterol esterification in hepatocyte suspensions was low. The uptake and net hydrolysis of chyle cholesteryl ester could therefore be measured in single-isotope experiments in which simultaneous measurements of cholesterol esterification were not performed.

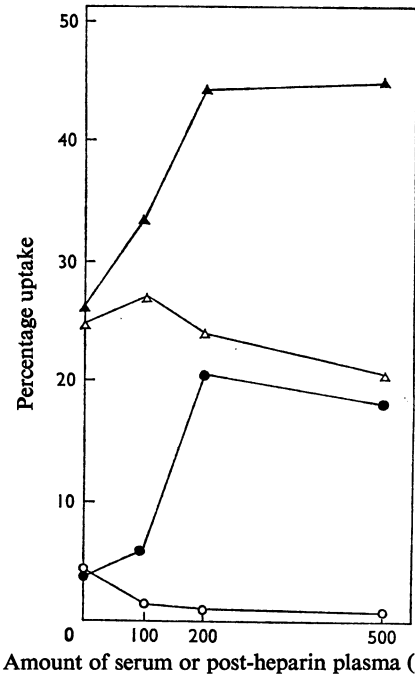

Fig. 1. *Effect of increasing concentrations of serum and post-heparin plasma on the uptake of chyle cholesterol and cholesteryl ester by hepatocytes*

An 18.0mg portion of cell protein was incubated for 60min with 25μl of chyle from a rat given [^{3}H]cholesterol. The chyle contained 0.39mg of lipids and 10μg of cholesterol; 62890d.p.m. was in cholesteryl ester and 76890d.p.m. in non-esterified cholesterol. The lower part of the Figure shows the percentage of total radioactive cholesteryl ester present in the cell pellet after incubation with increasing concentration of post-heparin plasma (●) or serum (○). The upper part of the Figure shows the percentage of total non-esterified [^{3}H]cholesterol in the cells with serum (△) and post-heparin plasma (▲) present. There was no net hydrolysis of cholesteryl ester during the incubation. In a similar experiment the cholesterol ester uptake was increased from 2.1 with zero, to 35.4 and 33.3% with 200 and 400μl of post-heparin plasma present, whereas serum decreased the uptake to 1.4–1.5%.

Table 1. *Effects of colchicine and cycloheximide on the uptake and hydrolysis of chyle cholesteryl esters in the presence of post-heparin plasma*

Cells (13.9–24.0 mg of protein) were incubated for 2 h under the conditions described in the text. Then 25 μl of chyle and 400 μl of post-heparin plasma were added. The final volume was 1.5 ml. The added chyle contained 0.39 mg of lipid and 11 μg of cholesterol. The added radioactivity was 67 100 d.p.m. of ^{3}H as cholesteryl ester and 24 690 d.p.m. as non-esterified cholesterol. The inhibitors were added either at the beginning of the cell incubation, or after 2 h together with the chyle and the post-heparin plasma as indicated. The values given are means ± S.E.M. for five experiments. The significance of differences from the controls without drugs was estimated by the *t* test (*$0.01 > P > 0.001$; †$P < 0.001$; n.s. = not significant).

Inhibitor present during incubation with chyle	Inhibitor present during preincubation	Incubation time after addition of chyle (min)	% disappearance of cholesteryl ester radioactivity from the medium	% of total cholesterol radioactivity as cholesteryl ester	% of non-esterified radioactive cholesterol in cells
None (cell-free controls)	—	0	—	68.9	—
	—	60	—	70.0 ± 0.5	—
	—	120	—	71.3 ± 0.5	—
None	None	60	50.3 ± 6.8	64.2 ± 1.3	59.3 ± 6.5
		120	65.2 ± 6.8	49.0 ± 6.4	66.6 ± 5.8
0.033 mM-Colchicine	None	60	41.0 ± 5.9 n.s.	67.3 ± 0.7*	50.9 ± 5.3
		120	48.8 ± 6.6*	62.1 ± 2.4*	60.5 ± 7.3
0.035 mM-Cycloheximide	None	60	41.0 ± 7.9 n.s.	65.1 ± 1.5 n.s.	54.1 ± 5.9
		120	48.8 ± 5.4*	56.1 ± 3.5 n.s.	62.0 ± 5.6
0.033 mM-Colchicine	0.033 mM-Colchicine	60	20.2 ± 6.4†	68.6 ± 0.3*	35.7 ± 6.4
		120	27.0 ± 7.1†	66.9 ± 1.4*	49.9 ± 8.1
0.035 mM-Cycloheximide	0.035 mM-Cycloheximide	60	16.3 ± 5.0†	69.2 ± 0.2*	31.2 ± 5.5
		120	19.4 ± 5.7†	67.6 ± 1.3*	43.1 ± 4.6

In the incubations with chyle, chylomicrons or VLD lipoproteins from animals given radioactively labelled cholesterol, the uptake or binding of radioactively labelled cholesteryl ester by the cells was less than 5% in 60–120min (Fig. 1 and Table 5). There was no significant net hydrolysis of radioactive cholesteryl ester during the incubation.

In the presence of serum the uptake of cholesteryl ester was less (Fig. 1) as found earlier for the triacylglycerol portion of chylomicrons (Felts & Berry, 1971).

During the incubation of doubly labelled ([^{3}H]oleic acid, [^{14}C]cholesterol) chyle or of chyle lipoproteins with post-heparin plasma, 87–94% of the triacylglycerol was hydrolysed within 60–120min under the incubation conditions used in the cell experiments. No net hydrolysis, but a small net formation of radioactive cholesteryl ester, occurred (Table 1). After incubation of chylomicrons most of the radioactive cholesteryl ester and of the residual triacylglycerol were still present in particles that floated at $d = 1.006$ (Fig. 2) and which migrated with the void volume on Bio-Gel A-50, i.e. they had apparent mol.wts. larger than 50000000 (Fig. 3). When intestinal VLD lipoproteins were used, much of the radioactive cholesteryl ester and the residual triacylglycerol floated at $d = 1.019$ but not at $d = 1.006$ (Fig. 2). Much of the non-esterified radioactive cholesterol had been transferred to other lipoprotein classes during the incubation (Fig. 3).

Post-heparin plasma increased the rate of disappearance of chyle cholesteryl ester from the medium. The rate of uptake of non-esterified chyle cholesterol was also increased, despite the presence of a considerable pool of unesterified plasma cholesterol in the medium that would be available for exchange reactions (Fig. 1). For significant net hydrolysis of radioactive chyle cholesteryl ester to occur, the incubations had to be continued for at least 2h. If the cells had been preincubated for 2h, however, the uptake was more efficient (Nilsson & Åkesson, 1975), and measurable cholesteryl ester hydrolysis occurred during the first hour of incubation with chyle and post-heparin plasma (Table 1). The uptake was improved during the preincubation if amino acids (East *et al.*, 1973) were present. In two experiments post-heparin plasma containing [^{3}H]cholesterol-labelled lipoproteins was used. It was found that the rate of uptake of added chylomicron [^{14}C]cholesterol ester was significantly higher than that of [^{3}H]cholesteryl ester (Table 2).

When isolated chylomicrons and VLD lipoproteins were used instead of whole chyle, the rate of uptake of the cholesteryl ester of both lipoprotein classes was increased by post-heparin plasma. The rate of uptake and the rate of net hydrolysis of cholesteryl ester was, however, larger in the experiments with chylomicrons than in those with VLD lipoproteins (Fig. 4). There was no significant difference in the extent to which the triacylglycerol of the two lipoprotein classes was hydrolysed during the incubations.

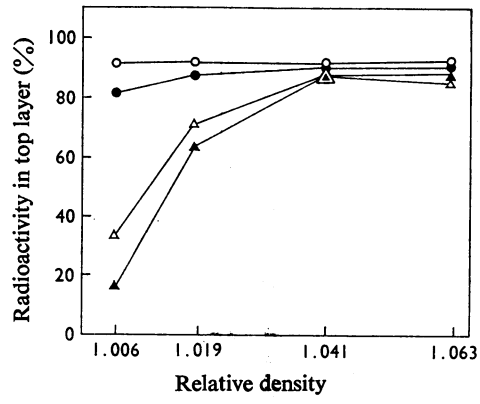

Fig. 2. *Flotation of chylomicron and intestinal VLD-lipoprotein cholesteryl ester and residual triacylglycerol after incubation with post-heparin plasma*

Chylomicrons and intestinal VLD lipoproteins from a rat given [^{3}H]oleic acid and [^{14}C]cholesterol were incubated with post-heparin plasma for 60min under the conditions described in the text. The triacylglycerol hydrolysis during the incubation was 93% in the experiment with chylomicrons and 86% in the experiment with VLD lipoprotein. After cooling to 0°C NaCl and KBr were added to the desired densities. Then the samples were centrifuged at 100000$g_{av.}$ for 24h. The Figure shows the percentage of the radioactivity remaining in chylomicron (○) and VLD-lipoprotein (△) triacylglycerol and in chylomicron (●) and VLD-lipoprotein (▲) cholesteryl ester that floated at the different densities. To each incubation was added 1.39mg (267370d.p.m. of ^{3}H) of chylomicron and 0.28mg (77570d.p.m. of ^{3}H) of VLD-lipoprotein triacylglycerol. The amounts of added chylomicron and VLD-lipoprotein cholesteryl ester were 11.7μg (78600d.p.m. of ^{14}C). The values obtained at $d = 1.006$ and at $d = 1.019$ were close to the means obtained in three experiments (S.E.M. ±1.1–2.6). Centrifugations at $d = 1.041$ and at $d = 1.063$ were performed only in the experiment shown.

Effects of metabolic inhibitors

When hepatocytes were preincubated for different periods of time, and then incubated with chyle and post-heparin plasma, the effects depended on the time at which the inhibitors were added. The inhibition of the cholesteryl ester uptake was very marked when cycloheximide or colchicine was already present during the preincubation period (Table 1). In preliminary experiments, 5mM-KCN and 20mM-NaF had the same effects. When the drugs were added

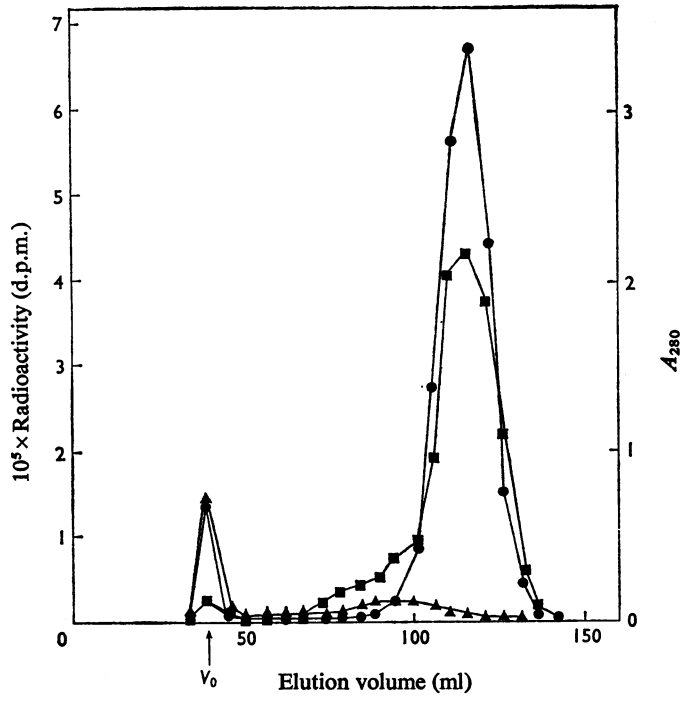

Fig. 3. *Agarose-gel chromatography of products formed during incubation of doubly labelled chylomicrons with post-heparin plasma*

Chylomicrons (1.50mg of triacylglycerol, 14μg of cholesterol, 264980d.p.m. of ^{14}C as cholesteryl ester, 179710d.p.m. of ^{14}C as non-esterified cholesterol, 1142700d.p.m. of ^{3}H, of which 87.5% was in triacylglycerol) were incubated with 0.8ml of post-heparin plasma for 60min as described in the text. It was found that 93% of the triacylglycerol was hydrolysed during the incubation. Then 2.5ml of the reaction mixture was applied to a column (1.6cm×76.5cm) of Bio-Gel A-50, and was eluted with 0.2M-potassium phosphate buffer (pH7.4) containing 0.1M-NaCl and 0.26mM-EDTA (sodium salt)/ml. Fractions of weight 5.20–5.69g were collected. ●, ^{3}H radioactivity; ▲, ^{14}C radioactivity; ■, protein. Of the ^{3}H radioactivity migrating with the void volume, 87% was in triacylglycerol, and 6.0% in non-esterified fatty acids, approx. 6% in partial glycerides and 1% in polar lipids. Of the ^{14}C radioactivity, 69% was in cholesteryl ester. In the major ^{3}H peak 0.5% of the ^{3}H radioactivity was in triacylglycerol, 83% was in non-esterified fatty acids, 12.1% in partial glycerides and 5.4% in polar lipids. Of the [^{14}C]cholesterol radioactivity in these fractions, 24.9% was in cholesteryl ester and 75.1% in non-esterified cholesterol. The Figure shows a typical elution pattern obtained with the procedure that was used as a routine for the isolation of chylomicron remnants as the void eluate. V_0 = void volume.

Table 2. *Metabolism of chyle and post-heparin-plasma cholesteryl ester in hepatocyte suspensions*

Cells (13.9–16.8mg of protein) were preincubated for 2h under the conditions described in the text. Then 25μl of chyle and 400μl of post-heparin plasma from an animal that had been injected intravenously with 10μCi of [^{3}H]cholesterol suspended in saline (Nilsson & Zilversmit, 1971) 24h earlier were added. The added post-heparin plasma contained 34260d.p.m. of ^{3}H as cholesteryl ester, 8570d.p.m. of ^{3}H as non-esterified cholesterol and 0.3mg of cholesterol. The added chyle contained 0.14mg of triacylglycerol, 4.3g of cholesterol, 1970d.p.m. of ^{14}C as cholesteryl ester, and 2095d.p.m. of ^{14}C as non-esterified cholesterol. The Table shows means±S.E.M. for two experiments.

Incubation conditions	Incubation time (min)	% disappearance of cholesteryl ester radioactivity from medium		% of total cholesterol radioactivity as ester		% of unesterified cholesterol radioactivity in cell pellet	
		^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Cell-free controls	30	—	—	81.0±0.8	50.4±1.6	—	—
	120	—	—	79.0±0.1	49.8±0.8	—	—
With cells	30	2.9±0.6	24.8±1.6	78.9±1.0	47.7±1.4	24.2±1.0	26.7±2.7
	120	3.5±0.8	45.5±4.0	81.0±0.3	41.8±1.6	46.6±3.3	57.7±4.4

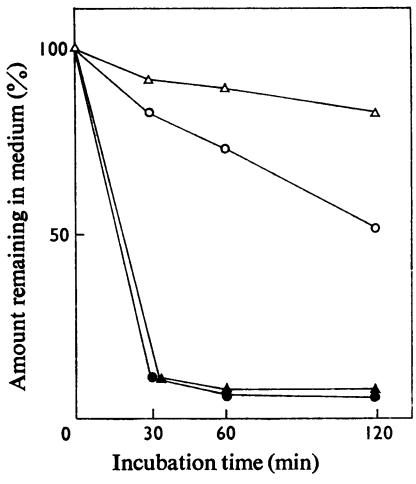

Fig. 4. *Time-course for the metabolism of doubly labelled chylomicrons and intestine VLD lipoproteins during incubation with hepatocytes and post-heparin plasma*

Hepatocytes (11.8mg of protein) were incubated for 2h under the conditions described in the text. Then 400μl of post-heparin plasma and 10μl of chylomicrons or 50μl of VLD lipoproteins was added, and the incubations were continued for the time-periods indicated. The added chylomicrons contained 0.54mg of triacylglycerol, 2.8μg of cholesterol, 6890d.p.m. of ^{14}C as cholesteryl ester, 4030d.p.m. of ^{14}C as non-esterified cholesterol and 179010d.p.m. of ^{3}H, of which 87% was in triacylglycerol. The VLD lipoproteins contained 0.34mg of triacylglycerol, 7.0μg of cholesterol, 20070d.p.m. of ^{14}C as cholesteryl ester, 8420d.p.m. of ^{14}C as non-esterified cholesterol and 111500d.p.m. of ^{3}H, of which 84% was in triacylglycerol. In a second experiment the disappearance of VLD-lipoprotein cholesteryl ester was 5.11 and 21% after 40, 80 and 120min as compared with 27.41 and 62% for the chylomicron cholesteryl ester. Symbols: $\triangle$, % of VLD-lipoprotein cholesteryl ester remaining in medium; $\circ$, % of chylomicron cholesteryl ester remaining in medium; $\blacktriangle$, % of VLD-lipoprotein triacylglycerol remaining in medium; $\bullet$, % of chylomicron triacylglycerol remaining in medium.

after 2h, at the same time as the chyle and the post-heparin plasma, the inhibition of the uptake was less efficient. Despite this, colchicine markedly inhibited the rate of net hydrolysis of radioactively labelled cholesteryl ester, whereas the inhibition of the hydrolysis by cycloheximide was variable and was related to the degree of inhibition of the cholesteryl ester uptake (Table 1 and Fig. 5).

When isolated doubly labelled ([^{14}C]cholesterol, [^{3}H]oleic acid) chylomicrons or VLD lipoproteins were used, none of the drugs was found to inhibit the hydrolysis of triacylglycerol in the cell-free incubations with post-heparin plasma. In agreement with the experiments with whole chyle, both colchicine and cycloheximide strongly inhibited the rate of uptake of chylomicron cholesteryl ester. The rate of uptake of VLD-lipoprotein cholesteryl ester was slower, and the effects of the drugs were less marked. Since 80% or more of the radioactively labelled cholesteryl ester was in particles with $S_f > 400$ in the experiments with whole chyle, these are therefore likely to show the drug effects on the metabolism of chylomicron cholesteryl ester. Vinblastine had in principle the same effects that colchicine had on the metabolism of chylomicron cholesteryl ester in hepatocyte suspensions. The inhibition of the cholesteryl ester uptake was thus most pronounced when the drug was also present during the preincubation period. When it was added after the preincubation period, the effect on the rate of uptake was less, whereas the hydrolysis of cholesteryl ester was still strongly inhibited (Table 3). None of the drugs decreased the rate of utilization of released radioactively labelled unesterified fatty acids for triacylglycerol and phospholipid synthesis by the cells. In three experiments Trypan Blue viability was examined after incubation. It was more than 80%, and was not affected by the drugs.

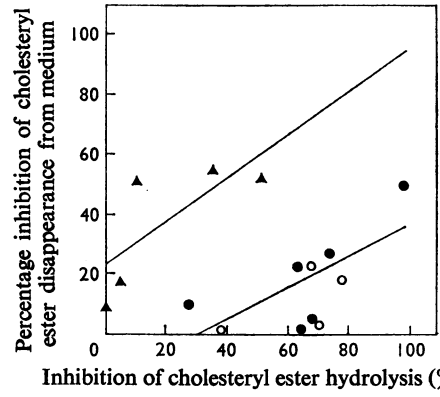

Fig. 5. *Inhibition of chyle cholesteryl ester uptake and hydrolysis with colchicine and cycloheximide*

The Figure summarizes the effects of the drugs when these were added after the 2h preincubation period. Five experiments with cycloheximide ($\blacktriangle$) and colchicine ($\bullet$) from Table 1 are included, plus another series of experiments in which only effects of colchicine were examined ($\circ$). In the latter series of experiments the incubations contained 8.8–21.6mg of cell protein, and 25μl with fat chyle containing 61080d.p.m. of ^{3}H as cholesteryl ester and 35150d.p.m. of ^{3}H as non-esterified cholesterol. As in the other series, the chyle, the drugs and 400μl of post-heparin plasma were added after a 2h preincubation period. The uptake and net hydrolysis were in the same range as in the series presented in Table 1. The Figure shows the correlation between the inhibition of the chyle cholesteryl ester disappearance from the medium and of its hydrolysis. The regression lines were obtained by calculation.

Table 3. *Effects of vinblastine on the metabolism of chylomicron cholesteryl ester in hepatocyte suspensions*
Cells (12.3–15.6mg of protein) were incubated for 2h under the conditions given in the text. Then 400μl of post-heparin plasma and 5μl of chylomicrons from a rat given [^{3}H]oleic acid and [^{14}C]cholesterol in trioleoylglycerol were added, and the incubations were continued for 2h. The vinblastine was added either from the start of the cell incubation or 2h later together with chyle and post-heparin plasma. The added chylomicrons contained 0.17mg of triacylglycerol and 2μg of cholesterol. From the beginning, 87% of the ^{3}H radioactivity was in triacylglycerol. The Table shows means±S.E.M. for two similar experiments. The triacylglycerol hydrolysis in cell-free controls was 93% and was not affected by vinblastine. The significance of the differences from the controls without vinblastine was checked by the *t* test: *$0.01>P>0.001$; †$0.2>P>0.05$; For other differences P was >0.4.

	% disappearance of radioactive cholesteryl ester from medium	% of non-esterified radioactive cholesterol in cells	% of total cholesterol radioactivity as ester
Cell-free controls	—	—	61.7±1.6
No inhibitor	65.4± 5.0	59.2±2.8	44.5±5.4
Vinblastine sulphate (50μg; added at the start of the cell incubation)	5.1± 0.5*	36.0±2.0	61.1±1.3†
Vinblastine sulphate (50μg; added after 2h)	40.5±17.5	47.9±6.2	50.1±0.1†

Table 4. *Effect of post-heparin plasma and heparin on the metabolism of cholesteryl ester of isolated remnant particles*
Cells (10.6–15.9mg of protein) were incubated for 2h before the heparin, the post-heparin plasma and the remnant particles were then added. The incubations were then continued for 2h. The remnant particles were isolated by Bio-Gel A-50 column chromatography as described in the text after 92.4–92.8% of the triacylglycerol had been hydrolysed during incubations with post-heparin plasma. The added 'remnants' contained 0.6μg of cholesterol, 3.5μg of triacylglycerol, 1680d.p.m. of ^{14}C as cholesterol, 3460d.p.m. of ^{14}C as cholesteryl ester and 1695d.p.m. of ^{3}H, of which 65% was in triacylglycerol. The values given are means±S.E.M. from four different experiments. *$P<0.01$; †$0.3>P>0.05$ for differences from control without any addition.

Additions	% disappearance of cholesteryl ester radioactivity from medium	% of total cholesterol radioactivity as cholesteryl ester	% of non-esterified cholesterol radioactivity in cell pellets
Cell-free controls	—	67.3	—
None	16.4±0.9	63.4±2.4	39.2±2.9
Heparin (12.4i.u.)	40.4±4.4*	57.4±1.3†	53.4±3.6†
26.6% post-heparin plasma	61.3±8.2*	54.2±7.2†	59.4±4.7†

Although the proportion of newly synthesized and added unesterified cholesterol that is esterified by suspended hepatocytes is small (Nilsson, 1975*b*), it might be influenced by the presence of the drugs. Experiments were therefore performed in which the net hydrolysis of chyle [^{14}C]cholesteryl ester and the esterification of added non-esterified serum [^{3}H]cholesterol were measured simultaneously. A significant uptake of [^{3}H]cholesterol occurred in these experiments, probably by exchange reactions. The proportion that had been esterified in 4h did not, however, exceed 2.4%, whether any drug was present or not. The increased rate of cholesterol esterification in the cells or in the medium may therefore not be the reason why colchicine and vinblastine appear to inhibit the decrease in radioactivity in chyle [^{14}C]cholesteryl ester. In an earlier study (Nilsson, 1975*b*), 14% of the radioactively labelled cholesterol that had been esterified by the cells in 2h had also been secreted into the medium. With the low rate of cholesterol esterification shown in Fig. 5, it is, however, unlikely that re-secretion of cholesterol that has been esterified in the cells as VLD-lipoprotein cholesterol ester will significantly affect the measurement of cholesteryl ester uptake in the present experiments.

Effects of trypsin on the uptake of chyle cholesteryl esters in the presence of post-heparin plasma

The use of >0.001% (w/v) trypsin decreased the rate of uptake of chyle cholesteryl ester when added after 2h, at the same time as double-labelled chyle and post-heparin plasma. In other experiments the trypsin was added 15min before the end of the 2h preincubation period. Post-heparin plasma, doubly labelled chyle and trypsin inhibitor were then added, and the cells were incubated for another 60min. It was

Table 5. *Effects of heparin and cycloheximide on the uptake of chylomicron and chylomicron-remnant cholesteryl ester in the presence of serum*

The cells (21.0mg of protein) were preincubated for 2h under the standard conditions with or without cycloheximide present. The remnant particles, the chylomicrons and the heparin were then added. The incubation was then continued for another 2h. The added chylomicrons contained 15540d.p.m. of ^{14}C as cholesteryl ester, 9264d.p.m. of ^{14}C as free cholesterol, 77μg of triacylglycerol, 0.73μg of cholesteryl ester and 14640d.p.m. of ^{3}H, of which 86.5% was in triacylglycerols. The added remnant particles contained 6210d.p.m. of ^{14}C as cholesteryl ester, 2690d.p.m. of ^{14}C as cholesterol, 3.1μg of triacylglycerol, 0.29μg of cholesteryl ester and 6220d.p.m. of ^{3}H, of which 81% was in triacylglycerol. In part (A) the values from two different experiments are given. In part (B) the values are means±S.E.M. from three experiments. *$0.05>P>0.02$; †$0.1>P>0.05$; ‡$0.2>P>0.1$ for differences between incubations with cycloheximide and the corresponding incubations without. §, as ‡ but for differences with and without heparin present. For other differences, P was >0.4.

Additions	% disappearance of radioactive cholesteryl ester from medium	% of total cholesterol radioactivity as ester	% of radioactive non-esterified cholesterol in cells	d.p.m. of ^{3}H as triacylglycerol / d.p.m. of ^{14}C as cholesteryl ester in the medium
(A) Chylomicrons				
Cell-free controls	—	62.5	—	8.1
None	2.4–2.7	60.9–61.6	14.4–18.2	8.1–8.2
Heparin (12.5i.u.)	9.4–19.8§	61.7–62.5	24.7–26.3	6.9–7.7§
0.035mM-Cycloheximide	1.9–2.3	61.8–61.9	18.5–22.3	7.6–7.9
Heparin (12.5i.u.)+ 0.035mM-cycloheximide	2.0–3.1‡	61.6–61.8	19.7–22.7	7.7–8.1
(B) Remnants				
Cell-free controls	—	69.6±0.9	—	0.93±0.05
None	23.2±5.0	66.3±2.2	29.5±5.7	0.90±0.06
Heparin (12.5i.u.)	35.0±8.5§	64.8±1.8	39.0±9.4	0.95±0.06
0.035mM-Cycloheximide	14.5±4.1‡	68.8±2.0	23.9±2.9	0.88±0.03
Heparin (12.5i.u.) of+ 0.035mM-cycloheximide	11.6±2.0*	68.9±1.0†	24.6±1.6	0.90±0.09

found that 0.0017% (w/v) trypsin inhibited the uptake of chyle cholesteryl ester by on average 50%, whereas trypsin inhibitor alone had no effect. Trypsin also decreased the rate of uptake of non-esterified cholesterol, but, like the inhibition with the drugs, this effect was much less pronounced than the effects on the rate of uptake of cholesteryl ester. The trypsin treatment did not decrease the rate of incorporation of labelled unesterified fatty acids into triacylglycerols and into phospholipids by the cells.

Uptake of isolated remnant particles

In nine experiments, isolated remnant particles were incubated with hepatocytes for 2h. The cholesteryl ester disappearance from the medium was 24.1±4.1% and the percentage hydrolysis of cholesteryl ester during the incubation was 3.5±1.0%. With heparin (12.5i.u.) present, the cholesteryl ester disappearance was increased to 44.0±5.1% ($0.01>P>0.001$) and the cholesteryl ester hydrolysis to 9.5±1.6% ($0.01>P>0.001$). In four of the experiments, incubations were also made with post-heparin plasma present. This was found to increase both the rate of cholesteryl ester uptake and the rate of net hydrolysis more than did heparin (Table 4). The presence of 20% (w/v) rat serum decreased the rate of cholesteryl ester disappearance from 27.7±3.3 to 20.5±4.4% ($n=4$, $0.2>P>0.1$).

If the cells had been preincubated with cycloheximide the rate of cholesteryl ester disappearance was decreased from 24.2±4.2 to 15.4±3.7% ($n=7$, $0.2>P>0.1$). This effect was more pronounced in the presence of heparin, when a decrease from 44.0±4.4 to 16.2±4.1% ($n=7$, $P<0.001$) was noticed, and the rate of net hydrolysis was decreased from 11.3±2.3 to 1.6±0.6% ($0.01>P>0.001$). The effects of heparin and of cycloheximide were also seen under the more physiological condition with 20% (w/v) serum present (Table 5).

Heparin slightly increased the rate of uptake of triacylglycerol fatty acid and of cholesteryl ester when the cells were incubated with native chyle. There was no significant change in the [^{3}H]triacylglycerol/[^{14}C]cholesteryl ester radioactivity ratio in the medium. This indicated that the increase in cellular radioactivity may be due to adhesion or to uptake of intact chyle lipoproteins rather than to the release of lipases causing lipolysis in the medium. In similar experiments with isolated remnant particles this ratio of radioactivity also remained constant. In the presence of serum, heparin stimulated the increased rate of uptake of chylomicron cholesteryl ester more markedly. In these experiments the [^{3}H]triacylglycerol/[^{14}C]cholesteryl ester radioactivity ratio of the medium decreased slightly during the incubation (Table 5).

Discussion

The present study demonstrates that, in the presence of post-heparin plasma, suspended hepatocytes take up significant amounts of added chylomicron cholesteryl ester. An extensive hydrolysis of triacylglycerol preceded the uptake of cholesteryl ester, which was shown in a separate study to occur in remnant particles containing residual triacylglycerol as well (Nilsson & Åkesson, 1975). In this respect the sequence of events is similar to that which occurs *in vivo* (Redgrave, 1970; Bergman *et al.*, 1971).

As in the perfused liver (Noel *et al.*, 1975), the rate of uptake of isolated remnant particles was more efficient than was that of native chyle lipoproteins. The conversion of chylomicrons into remnants which have a higher affinity for hepatocytes may, however, not be the only reason why post-heparin plasma increased the rate of uptake of chylomicron cholesteryl ester. Both post-heparin plasma and to a less extent heparin increased the rate of uptake also of isolated remnant particles (Table 4). The presence of unphysiological amounts of heparin and lipoprotein lipase, or of other factors in the post-heparin plasma, may thus influence the results. This might occur in several possible ways. For instance, heparin might act as a link in the binding of lipoproteins to the cell surface. It might also increase the rate of uptake of remnants by endocytosis, since it has been shown to stimulate endocytosis by other cell types (Panagiotis *et al.*, 1964; Filkins & Di Luzio, 1966). Lipoprotein lipase might bind to the remnant particles and thereby increase their affinity for hepatocytes, as suggested by Felts *et al.* (1975). Further studies are necessary to test these possibilities and to answer the question of whether glycosaminoglycans or lipoprotein lipase have a physiological role in binding remnants to hepatocytes.

Arnaud & Boyer (1976) found that 0.1 mg of heparin/ml caused an increased rate of leakage of lactate dehydrogenase (EC 1.1.1.27) from hepatocytes into the medium. The present finding that the catabolism of chylomicron cholesteryl ester was strongly inhibited by NaF, by KCN and by other drugs as discussed below, show, however, that the degradation of cholesteryl ester was an active process and was not a consequence of cellular damage caused by the post-heparin plasma. The high values for the Trypan Blue exclusion after incubation and the observation that the cells have a normal rate of biosynthesis of triacylglycerols and of phospholipids also in the presence of post-heparin plasma (Å. Nilsson & B. Åkesson, unpublished work) provide further evidence for this.

The improvement of the post-heparin plasma stimulated cholesteryl ester uptake after a 2h preincubation indicated that reversible cellular changes caused during the cell isolation procedure were rate-limiting for the metabolism of the chyle cholesteryl esters. An active protein synthesis was required for the improvement, since it was prevented by cycloheximide (Table 1) (Nilsson & Åkesson, 1975). This, and the observation that mild trypsin treatment of the preincubated cells markedly decreased the uptake of labelled cholesteryl ester, may mean that proteins necessary for the binding of the remnant particles to the cell surface are damaged during the cell isolation and in part reconstituted during incubation. The catabolism of chyle cholesteryl ester *in vivo* does not depend directly on an active turnover of plasma membrane or other proteins (Nilsson, 1975*a*). In agreement with this, there was no positive evidence for a direct effect of cycloheximide on the rate of degradation of chyle cholesteryl ester in the hepatocyte suspensions when the drug was added after the preincubation period (Table 1 and Fig. 5).

Like cycloheximide, colchicine and vinblastine strongly inhibited the increase in the post-heparin-plasma-stimulated cholesteryl ester uptake when present during the preincubation period (Tables 1 and 3). Since the anti-microtubular agents inhibit a number of secretory processes (for references, see Wilson *et al.*, 1974), it may be that they inhibit the chyle cholesteryl ester uptake because they block the transfer to the cell surface of protein or of other material that is necessary for the binding of the remnant particles.

In contrast with cycloheximide, colchicine and vinblastine also inhibited the hydrolysis of the cholesteryl ester when they were added after the preincubation, i.e. when their effect on the uptake was less marked (Table 1 and Fig. 5). This effect is comparable with that seen *in vivo*, where the same agents did not markedly affect the clearance of chyle cholesteryl ester from blood, but delayed its hydrolysis after the uptake by the liver (Nilsson, 1975*a*). Colchicine had no effect on the hydrolysis in cell suspensions of chyle cholesteryl ester that had been taken up *in vivo* (A. Nilsson, unpublished work). This indicates that colchicine has no direct and immediate effect on the hydrolytic reaction, but rather acted on a transport process by which the cholesteryl ester is made available to the hydrolytic enzyme. For instance, a fusion of the remnant particle and the plasma membrane, an interiorization of cholesteryl ester by some other mechanism or a fusion between an endocytic vesicle which contains chyle cholesteryl ester and primary lysosomes (Malawista & Bodel, 1967) might depend on intact functions of the microtubules. It is not possible to differentiate between these alternatives from the present data or from the data obtained *in vivo* (Nilsson, 1975*a*). These data do, however, indicate that in the cell suspensions hydrolysis of chylomicron-remnant cholesteryl ester does not occur during the contact between the remnant particle and the cell surface, but that the cholesterol ester has to

be interiorized or incorporated into the plasma membrane before hydrolysis. This is in agreement with the observation that a considerable portion of chylomicron cholesteryl ester that has been taken up by the liver *in vivo*, but not yet hydrolysed, can be recovered in suspended hepatocytes (Nilsson & Zilversmit, 1971).

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