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Action of Liproprotein Lipase on Apoprotein-Depleted Chylomicrons

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1. Rat lymph chylomicrons were exposed to soluble and to immobilized trypsin. This treatment caused no detectable changes in the chylomicron structure or lipid composition, but did result in virtually total depletion of all their tetramethylurea-soluble apoproteins. 2. The capacity of these apoprotein-depleted chylomicrons to act as substrate for lipoprotein lipase *in vitro* and *in situ* (i.e. isolated perfused rat heart) was decreased by about 90 and 75% respectively, compared with intact chylomicrons. 3. On incubation with rat plasma high-density lipoproteins, trypsin-treated chylomicrons readily acquired a full apoprotein complement. This resulted in the complete restoration of their capacity to act as substrate for lipoprotein lipase both *in vitro* and *in situ*. 4. It is suggested that with the use of trypsin-treated chylomicrons it is now possible for the first time to investigate the physiological role that individual apoproteins play in the catabolism of triacylglycerol-rich lipoproteins by lipoprotein lipase.

The initial event in the catabolism of plasma chylomicrons and VLD lipoproteins is the hydrolysis of their triacylglycerol moiety by liproprotein lipase (Robinson, 1970). Suggestions have been made that the rate of this hydrolysis may be modulated by some of the polypeptides normally present on the surface of these lipoproteins (Krauss *et al.*, 1973; Bar-On *et al.*, 1976; Schonfeld *et al.*, 1976; Rogers *et al.*, 1976). These suggestions are based on observations carried out *in vitro*, which showed that various apoproteins (apoproteins C, A and E) can affect the hydrolysis of artificial triacylglycerol emulsions by solubilized lipoprotein lipase (La Rosa *et al.*, 1970; Havel *et al.*, 1970; Brown & Baginsky, 1972; Havel *et al.*, 1973*a*; Krauss *et al.*, 1973; Miller & Smith, 1973; Bensadoun *et al.*, 1974; Östlund-Lindqvist & Iverius, 1975; Ganesan & Bass, 1975; Ekman & Nilsson-Ehle, 1975; Ganesan *et al.*, 1976; Quarfordt *et al.*, 1977). There seems to be a general agreement from these studies that apoprotein C-II has an activating effect on the hydrolysis. The role of the other aproproteins is less clear. For example, apoprotein C-I and apoprotein C-III have been variously described to have activating or inhibitory effects on the hydrolytic reaction (La Rosa *et al.*, 1970; Havel *et al.*, 1970; Ganesan *et al.*, 1971; Brown & Baginsky, 1972; Havel *et al.*, 1973*a*; Bensadoun *et al.*, 1974; Ganesan & Bass, 1975; Östlund-Lindqvist & Iverius, 1975).

Abbreviations used: VLD lipoprotein, very-low-density lipoprotein; LD lipoprotein, low-density lipoprotein; HD lipoprotein, high-density lipoprotein.

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The reasons for these differences have been ascribed to variations in the purity of the polypeptides and to the nature of enzyme preparations used (Havel *et al.*, 1973*a*). These have included purified as well as crude lipoprotein lipase preparations from post-heparin plasma (Havel *et al.*, 1973*a*; Ganesan & Bass, 1975; Ganesan *et al.*, 1976), adipose tissue (Havel *et al.*, 1973*a*; Krauss *et al.*, 1973; Ekman & Nilsson-Ehle, 1975), human and cow's milk (Havel *et al.*, 1970, 1973*a*; Miller & Smith, 1973; Östlund-Lindqvist & Iverius, 1975) and rat heart (Twu *et al.*, 1975; Chung & Scanu, 1977). In addition, differences in the substrate preparation could also account for the variations in results. Studies have shown that the hydrolytic activity of soluble lipoprotein lipase on emulsified triacylglycerol can be affected by the concentration and type of emulsifying agent used (e.g. phospholipids, Triton, gum arabic) (Chung *et al.*, 1973; Blaton *et al.*, 1974; Riley & Robinson, 1974; Heaf & Carlson, 1976). In view of these limitations, it is apparent that no assertions can yet be made about the role that the C apoproteins, as well as other apoproteins, singly or combined, may have in the hydrolysis of chylomicrons or VLD lipoprotein triacylglycerol, the physiological substrates for lipoprotein lipase.

The present investigation was initiated to examine the possibility of using chylomicrons to study the effects of C apoproteins on the catalytic action of lipoprotein lipase. The use of this substrate is complicated, however, by the fact that it already possesses these apoproteins. To avoid this problem, chylomicrons were treated with trypsin, which, under appropriate conditions, depleted the apoproteins

from the lipoprotein surface. In the present paper we describe the results of experiments that examined the ability of the apoprotein-depleted chylomicrons to act as substrate for both soluble lipoprotein lipase and for the enzyme present at its physiological site of action on the endothelial surface of capillaries.

Methods and Materials

Animals

Male Sprague–Dawley rats (180–240g) were maintained on standard laboratory chow and exposed to 12h of darkness (18:00–06:00h) each day. Unless otherwise indicated, the animals were as a routine starved for 5–8h before use in the experiments. All animals were killed while under diethyl ether anaesthesia.

Chylomicron preparation

Lymph was obtained by cannulating the mesenteric lymph duct of fed rats given 2ml of corn oil (Mazola; CPC International, Englewood Cliffs, NJ, U.S.A.) by stomach tube. The chyle was collected at room temperature and stored in an unwashed state at 4°C with 0.02% NaN_3 and 0.01% EDTA. Chylomicrons labelled in the triacylglycerol fatty acid moiety were obtained by feeding rats with 125μCi of [1-^{14}C]-palmitic acid (58mCi/mmol) (Amersham/Searle, Arlington Heights, IL, U.S.A.) dissolved in corn oil. Before their use, chylomicrons were washed by layering 40ml of Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932), pH7.4, over 20ml of the lymph in centrifuge tubes and spun in an SW 25.2 rotor at 23500 rev./min for 45min at 16°C in a Beckman model L5-75 preparative ultracentrifuge. The floating chylomicron fraction was resuspended in Krebs–Ringer bicarbonate buffer and re-centrifuged under the same conditions. The washed chylomicron preparation was used within 5h.

Trypsin treatment of chylomicrons

Twice-recrystallized bovine pancreatic trypsin (type III; Sigma Chemical Co., St. Louis, MO, U.S.A.) was solubilized in 0.05M-barbital buffer, pH8.1, and added directly to the freshly washed chylomicrons and incubated at 29°C with gentle shaking. Unless otherwise indicated, the trypsin concentration was 8μg of trypsin/mg of chylomicron triacylglycerol and the incubations proceeded for 30min. The enzymic reaction was stopped by the addition of soya-bean trypsin inhibitor (type 1-S; Sigma), also in 0.05M-barbital buffer, pH8.1, at 1.5 times the trypsin concentration. Control preparations consisted of chylomicrons incubated with pre-mixed trypsin and trypsin inhibitor. Both preparations were used without further modification as substrate for the lipoprotein lipase assays. Untreated chylomicron preparations are defined as washed chylomicrons unexposed to either trypsin or trypsin inhibitor. Untreated and control substrates did not differ in their ability to support triacylglycerol hydrolysis by lipoprotein lipase.

Immobilized trypsin was prepared essentially by the method of Cuatrecasas & Parikh (1972) by using a *N*-hydroxysuccinimide ester side chain to link the trypsin to Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Approximately 4.5mg of trypsin was bound/ml of packed Sepharose, as determined by using benzoyl-DL-arginine *p*-nitroanilide hydrochloride (Sigma) as substrate (Erlanger *et al.*, 1961). Columns (0.9cm×25cm) of immobilized trypsin–Sepharose were equilibrated with 0.05M-barbital buffer, pH8.1, and maintained at 35°C. Washed chylomicron suspensions were passed through the column by ascending flow at a rate of 0.2ml/min. The eluate contained no trypsin activity. Control chylomicrons were passed through a Sepharose 4B column of the same size at the same flow rate.

Perfusion with ^{14}C-labelled chylomicrons

All preparations of ^{14}C-labelled chylomicrons that were used for perfusion were first mixed 1:1 (v/v) with 30% (w/v) bovine serum albumin (Sigma) adjusted to pH5.0 with 1M-NaOH, and incubated at room temperature to decrease their non-esterified fatty acid content (Borensztajn & Robinson, 1970). After this, the chylomicrons were re-isolated by centrifugation as described above and added to Krebs–Ringer bicarbonate buffer, pH7.4, to a final concentration of 0.48μequivalent of triacylglycerol fatty acid/ml (Borensztajn & Robinson, 1970). Hearts were dissected out and washed for 1min by retrograde perfusion with Krebs–Ringer bicarbonate buffer, pH7.4, at 37°C in a non-recirculatory system as described by Borensztajn & Robinson (1970). This was followed by a 20min non-recirculatory perfusion with the labelled chylomicrons. The perfusates were collected into bottles containing 2ml of 10M-NaOH. The $^{14}CO_2$ produced as a result of the oxidation of the ^{14}C-labelled fatty acids was measured in 5ml samples of the perfusate acidified with 2ml of 2.5M-H_2SO_4 in a sealed flask equipped with a plastic centre well containing 0.3ml of 1M-Hyamine hydroxide (Amersham/Searle) (Borensztajn & Robinson, 1970). After standing for 4h at room temperature, the flasks were opened and the centre wells placed directly into vials containing 11ml of scintillation fluid [4g of 2,5-diphenyloxazole, 0.05g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene; Research Products International Corp., Elk Grove Village, IL, U.S.A.] and counted for radioactivity in an Isocap 300 liquid-scintillation spectrophotometer (Searle Analytic Inc., Elk Grove Village, IL, U.S.A.).

Sources of lipoprotein lipase

Lipoprotein lipase was obtained from perfused rat hearts and isolated adipocytes. Hearts were dissected out and washed for 1min with Krebs–Ringer bicarbonate buffer as described above. This was followed by a 30s perfusion with the same buffer containing 5 U.S.P. units of heparin/ml (Inolex Corp., Chicago, IL, U.S.A.) to release lipoprotein lipase from the organ. The released enzyme was used immediately after collection. Rats used for adipocyte isolation were given 1ml of 60% (w/v) glucose by stomach tube 4h before death. Both epididymal fat-pads were removed, coarsely minced and vigorously shaken at 37°C in 3ml of Krebs–Ringer bicarbonate buffer containing 3mg of glucose, 90mg of bovine serum albumin (fatty acid-poor; Pentex, Miles Laboratories, Kankakee, IL, U.S.A.) and 12mg of collagenase (Worthington Biochemical Corp., Freehold, NJ, U.S.A.). The isolated fat-cells were then washed in 4×8vol. of Krebs–Ringer bicarbonate buffer, pH7.4, containing 1.5% (w/v) fatty acid-poor albumin, the cells being reisolated each time by centrifugation for 3min at 800 rev./min. After the final wash, the cell suspension was added to 3ml of Krebs–Ringer bicarbonate buffer, pH7.4, containing 5U.S.P. units of heparin and 1.5% (w/v) albumin. This mixture was incubated for 10min at 37°C to release lipoprotein lipase. After centrifugation of this incubation mixture, the infranatant containing soluble lipoprotein lipase was removed and immediately assayed.

Assay of lipoprotein lipase activity

The standard medium for enzyme assay had a final volume of 4.5ml, containing 6.15% (w/v) albumin (Sigma), pH8.1, 0.1M-Tris/HCl buffer, pH8.1, 0.67 U.S.P. unit of heparin and various amounts of triacylglycerols (see the Results section) in the form of chylomicrons or Intralipid (Cutter Laboratories, Berkeley, CA, U.S.A.). To this mixture, 2ml of enzyme was added and incubation proceeded in a shaking water bath at 37°C for 1h. Non-esterified fatty acids released into the incubation medium were extracted and titrated as previously described (Borensztajn *et al.*, 1970). In all experiments the amount of substrate was not limiting during the period of incubation.

Incubations of chylomicrons with HD lipoproteins

HD lipoproteins (*d* 1.063–1.21) were isolated from pooled rat plasma by the method of Havel *et al.* (1955). The isolated HD lipoprotein was dialysed for 24h against 6 litres of 15mM-$(NH_4)_2CO_3$, pH8.4, containing 0.01% EDTA before further use. Chylomicrons (trypsin-treated and control) and Intralipid were added to various amounts of HD lipoproteins suspended in 15mM-$(NH_4)_2CO_3$ and preincubated for 20min at 37°C. These mixtures were then used as substrate for lipoprotein lipase assays as described above. In certain experiments the chylomicrons were reisolated from the HD lipoproteins after the preincubation. This was done by placing 0.5ml of the mixture on a column (1.5cm×35cm) of Sepharose 6B (Pharmacia) equilibrated and eluted with 0.06M-Tris/HCl buffer, pH8.2. The chylomicrons that were eluted with the void volume were used for apoprotein electrophoresis as described below.

Analytical methods

Triacylglycerols were measured by the method of Fletcher (1968). Phospholipid P was determined as described by Bartlett (1959) and a factor of 25 used to convert values for P into phospholipid quantities. Individual phospholipid classes were separated by one-dimensional t.l.c. on silica-gel HR plates (Analtech Inc., Newark, DE, U.S.A.) in the solvent system of Skipski *et al.* (1964). T.l.c. for the separation of partial glycerides was done in the solvent system light petroleum (b.p. 30–60°C)/diethyl ether/acetic acid (80:20:1, by vol.). Cholesterol was determined by the method of Rush *et al.* (1971). Free cholesterol was separated from cholesterol ester by digitonin precipitation by the procedure of Sperry & Webb (1950). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Apoprotein electrophoresis was conducted essentially as described by Kane (1973). Suspensions of untreated, trypsin-treated and HD-lipoprotein-reactivated chylomicrons were dialysed for 48h at 4°C in 12 litres of 5mM-$(NH_4)_2CO_3$, pH8.4, containing 0.01% EDTA, freeze-dried, and extracted once with diethyl ether to remove most of the lipid. The pellet obtained was dried and resuspended in 0.06M-Tris/HCl, pH8.4, to which an equivalent amount of tetramethylurea was added. The tetramethylurea-soluble proteins were electrophoresed on 7.5% polyacrylamide gels containing 8M-urea. The gels were stained with 0.3% Amido Black 10B (Sigma) overnight in acetic acid/methanol/water (7:45:48, by vol.). Destaining was conducted in acetic acid/methanol/water (7:5:88, by vol.). Gel scanning was done with a Beckman R-112 densitometer.

Microscopy

For scanning electron microscopy, control and trypsin-treated chylomicrons were added to a 2% solution (w/v) of OsO_4 in Millonig's phosphate buffer (Dawes, 1971) and left for 1h at room temperature. After centrifugation (1500 rev./min) of the fixed chylomicrons, the pellets were washed twice in Millonig's buffer, resuspended in a small volume of the same buffer, and portions placed directly on discs coated with Formvar. The samples were then dehydrated by immersion in increasing concentra-

tions of ethylene glycol (50, 70, 90 and 100%, v/v) followed by two rinses in 100% methoxyethanol. The fixed material was dried in a Bomar critical-point drying bomb (Bomar Co., Tocoma, WA, U.S.A.). The specimens were coated with gold/palladium after an undercoat of carbon and viewed in a Hitachi HFS-2 (Perkin–Elmer Corp., Silver Spring, MD, U.S.A.) scanning electron microscope fitted with a field emission tip.

Statistical significance of the difference between means was analysed by Student's *t* test.

Results

Hydrolysis of trypsin-treated chylomicrons by lipoprotein lipase

Fig. 1 illustrates the results of an experiment in which control chylomicrons and chylomicrons that had been exposed to trypsin for different times were used as substrate for heart lipoprotein lipase. Trypsin treatment caused a rapid decrease in the capacity of the enzyme to hydrolyse chylomicron triacylglycerol, with maximal decrease in hydrolysis observed within 30min. The residual hydrolysis (less than 10% of controls) was not decreased further with prolongation of exposure to trypsin (Fig. 1) or when additional trypsin at a concentration equal to that initially used was added to the mixture after 45min incubation (Fig. 1). Similar results were observed when chylomicrons were exposed to immobilized trypsin, as described in the Methods and Materials section. As in experiments with soluble trypsin, a residual hydrolysis of the treated substrate by lipoprotein lipase (3–12% of control values) was consistently present and could not be decreased further by re-exposing the lipoproteins to the Sepharose-bound trypsin.

To determine if this residual hydrolysis could still be detected when the enzyme from a source other than heart was used in the assay system, an experiment was carried out in which control and trypsin-treated chylomicrons were used as substrate for lipoprotein lipase released from isolated rat adipocytes as well as from rat heart. The results in Table 1 show that with either enzyme similar residual hydrolysis (7.9 and 9.6% of control) was observed. The results in Table 1 also show that when Intralipid was used as substrate no hydrolysis was detectable. Since triacylglycerol emulsions are readily hydrolysed by lipoprotein lipase when serum is present in the

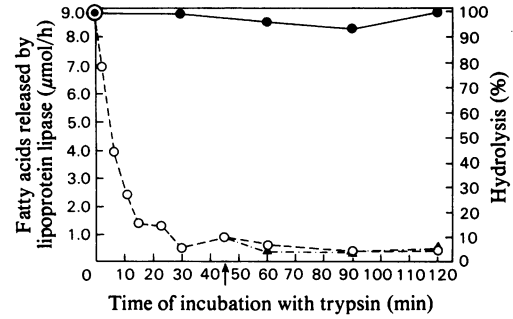
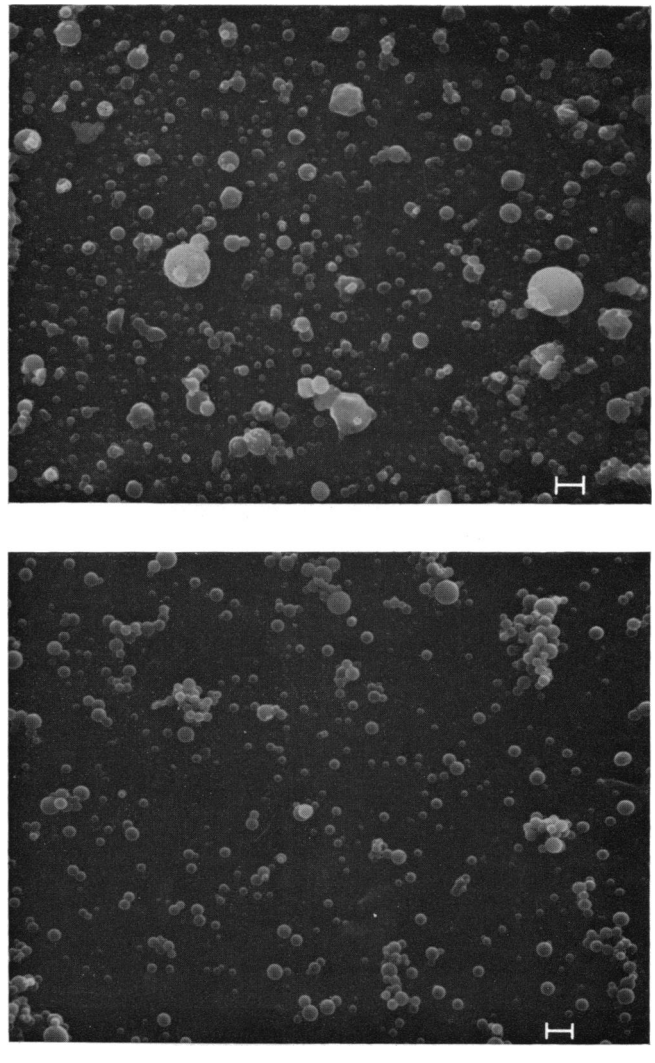


Fig. 1. *Effect of trypsin treatment on the hydrolysis of chylomicron triacylglycerol by heart lipoprotein lipase*
Chylomicrons were incubated with trypsin (8 μg/mg of chylomicron triacylglycerol) at 29°C and at the times indicated samples (30mg of chylomicron triacylglycerol) were mixed with 360 μg of trypsin inhibitor. After 45 min incubation (arrow), additional trypsin (8 μg/mg of chylomicron triacylglyerol) was added to a portion of the original mixture, and samples taken thereafter were mixed with 720 μg of trypsin inhibitor. Control chylomicrons were incubated with premixed trypsin and trypsin inhibitor. The samples of the control and trypsin-treated chylomicrons were used as substrate for heart lipoprotein lipase as described in the Methods and Materials section. Each point represents the average of determinations carried out in duplicate. ●, Control; ○, trypsin-treated; ▲, with additional trypsin.

Table 1. *Effect of trypsin treatment on the hydrolysis of chylomicron triacylglycerol by lipoprotein lipase from adipocytes and heart*
The conditions for trypsin treatment and for lipoprotein lipase assay were as described in the Methods and Materials section. The Intralipid used as substrate in the lipoprotein lipase assay was not exposed to trypsin. Each value represents the average of determinations carried out in duplicate.

	Adipocyte lipoprotein lipase		Heart lipoprotein lipase	
Substrate	(μmol of non-esterified fatty acids released/h)	(% hydrolysis)	(μmol of non-esterified fatty acids released/h)	(% hydrolysis)
Control chylomicrons	4.4	100	17.8	100
Trypsin-treated chylomicrons	0.35	7.9	1.7	9.6
Intralipid	0	—	0	—



EXPLANATION OF PLATE 1

Scanning electron micrographs of control (*a*) *and trypsin-treated* (*b*) *chylomicrons*

Preparation of the chylomicrons for viewing and their trypsin treatment with soluble trypsin were as described in the Methods and Materials section. Scale bar corresponds to 2 μm.

assay medium, the latter observation clearly demonstrates that the residual hydrolysis of trypsin-treated chylomicrons by heart or adipose-tissue enzyme was not due to contamination of the incubation medium with serum. The results in Table 1 also indicate that the non-esterified fatty acids released into the lipoprotein lipase assay media were derived from the hydrolysis of triacylglycerol. The absence of detectable non-esterified fatty acids released from Intralipid indicates that phospholipids, which constitute 12% (w/w) of this triacylglycerol emulsion, were not hydrolysed. Moreover, no phospholipase activity could be detected in the heart perfusates (T. W. Lukens & J. Borensztajn, unpublished work). Although the possible presence of mono- and diacylglycerol hydrolases in the heart perfusates and in the media used to release lipoprotein lipase from the adipocytes was not investigated, the concentration of these partial glycerides in the chylomicrons as determined by t.l.c. was too small to account for any significant release of non-esterified fatty acids.

Apoprotein electrophoresis

Fig. 2 shows the apoprotein patterns obtained by tetramethylurea/polyacrylamide-gel electrophoresis of untreated and trypsin-treated chylomicrons. The untreated chylomicrons (36.4μg of protein/mg of triacylglycerol) (gel 1) and control chylomicrons (results not shown) exhibited an overall apoprotein pattern similar to that described by Mjøs *et al.* (1975). Trypsin-treated chylomicrons (4.7μg of protein/mg of triacylglycerol) were electrophoresed on gel 2. Of importance is the complete absence of detectable protein bands, and more specifically bands corresponding to the C apoproteins. Even when the sample of trypsin-treated chylomicrons taken for electrophoresis was 10 times that used for gels 1 and 2, no C apoproteins could be detected (gel 3). The several bands present on gel 3 presumably correspond to fragments of the intact apoproteins produced by the action of trypsin.

Effect of trypsin on the lipid composition and morphology of chylomicrons

The lipid composition of control, untreated and trypsin-treated chylomicrons is shown in Table 2. No major differences in the concentrations of triacylglycerol, non-esterified and esterified cholesterol and phospholipid were found in any of the three groups. In addition, trypsin treatment had no effect on the relative amounts of individual chylomicron phospholipids.

Scanning electron micrographs of control (*a*) and trypsin-treated (*b*) chylomicrons are shown in Plate 1. It is apparent that trypsin-treated chylomicrons did not coalesce and that they retained their original spherical shape.

Effect of HD lipoprotein on trypsin-treated chylomicrons

Plasma HD lipoproteins serve as a major reservoir of C apoproteins, which under appropriate conditions can be readily transferred to other lipoproteins and to artificial triacylglycerol emulsions (Havel *et al.*,

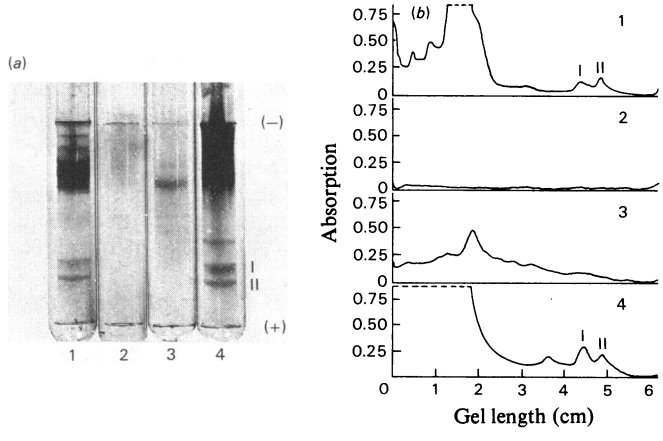


Fig. 2. *Tetramethylurea/polyacrylamide-gel electrophoresis (a) and scanning (b) of chylomicron apoproteins*
The material obtained from the delipidation of 8.5mg of chylomicrons (as determined from the triacylglycerol concentration) was applied to each gel, except for gel 3, on which the sample applied was from 85mg of chylomicrons. Chylomicrons were trypsin-treated by using Sepharose-bound trypsin as described in the Methods and Materials section. Photodensitometric scanning was conducted at 600nm. Gel 1, untreated chylomicrons; gels 2 and 3, trypsin-treated chylomicrons; gel 4, trypsin-treated chylomicrons incubated with HD lipoprotein and then reisolated. Band I corresponds to apoproteins C-III-0 and C-II; band II corresponds to apoprotein C-III-3.

Table 2. *Lipid composition of untreated, control and trypsin-treated chylomicrons*
Chylomicrons were prepared and their lipids analysed as described in the Methods and Materials section. Values are expressed as % of total lipids except the individual phospholipids, which are expressed as % of total phospholipids. The results tabulated are the average values ± S.D. obtained from the analysis of three different samples of chylomicrons.

					Phospholipid distribution (%)			
Chylomicron preparation	Triacyl-glycerol	Non-esterified cholesterol	Esterified cholesterol	Phospho-lipid	Phospha-tidyl-choline	Sphingo-myelin	Lysophos-phatidyl-choline	Phospha-tidyl-ethanol-amine
Untreated	95.3±0.2	0.33±0.06	0.17±0.05	4.9±0.4	76.3±3.2	8.1±5.4	8.5±4.1	7.2±0.8
Soluble trypsin	95.0±0.4	0.31±0.04	0.14±0.02	4.5±0.4	77.7±3.5	3.7±3.2	9.1±7.4	9.5±3.5
Control	94.6±0.5	0.35±0.07	0.15±0.02	4.9±0.5	75.0±4.5	4.8±3.2	13.1±6.7	6.9±2.7
Immobilized trypsin	94.4±0.9	0.30±0.04	0.15±0.03	5.2±0.9	73.8±11.0	7.1±1.6	11.5±5.8	7.7±1.7
Control	94.1±0.8	0.38±0.05	0.20±0.02	5.3±0.7	74.3±1.0	6.8±6.1	14.6±5.5	4.2±2.5

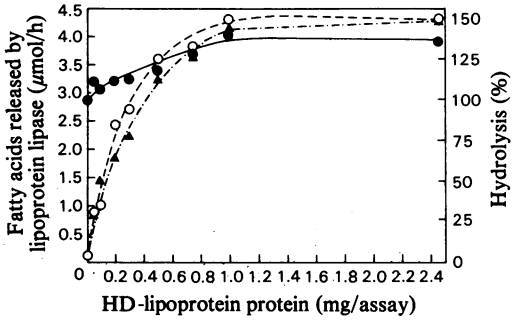


Fig. 3. *Effect of HD lipoprotein on the hydrolysis of chylomicron triacylglycerol by lipoprotein lipase*
Chylomicrons were trypsin-treated as described in the Methods and Materials section, with 20μg of trypsin/mg of chylomicron triacylglycerol. Samples of both control and trypsin-treated chylomicrons (15mg of triacylglycerol) as well as Intralipid (30mg of triacylglycerol) were added to 0.3ml of 15mM-$(NH_4)_2CO_3$ buffer, pH8.4, containing various amounts of rat HD lipoprotein. After a 20min incubation at 37°C, these mixtures were used directly as substrate in the lipoprotein lipase assay. Each point represents the average of determinations carried out in duplicate. ●, Control chylomicrons; ○, trypsin-treated chylomicrons; ▲, Intralipid.

1973*b*). Fig. 2 (gel 4) shows the electrophoretic pattern of the apoproteins from chylomicrons that had been exposed to immobilized trypsin, incubated with plasma HD lipoprotein, and reisolated as described in the Methods and Materials section. Clearly these chylomicrons have re-acquired their C-apoprotein complement lost during trypsin treatment (cf. gels 2 and 4). Incubation of trypsin-treated chylomicrons with HD lipoprotein also restored their ability to serve as substrate for lipoprotein lipase. Fig. 3 shows the results of an experiment in which control and trypsin-treated chylomicrons, as well as Intralipid, were incubated with increasing amounts of HD lipoprotein before being used as substrate for heart lipoprotein lipase. In this experiment the hydrolysis of the trypsin-treated chylomicrons in the absence of HD lipoprotein was only 5% of that observed with the control chylomicrons. Again, no hydrolysis could be detected when Intralipid was used as substrate. On addition of HD lipoprotein, the hydrolysis of the triacylglycerols of all substrates by lipoprotein lipase was readily stimulated. In the presence of about 0.3mg of HD lipoprotein, triacylglycerol hydrolysis in the trypsin-treated chylomicrons and Intralipid reached values comparable with that of control chylomicrons assayed in the absence of HD lipoprotein. Maximal stimulation (about 50% higher than control chylomicrons in the absence of HD lipoprotein) was observed when the substrates were incubated with 1mg of HD-lipoprotein protein. No further stimulation of the hydrolysis was observed when a greater HD lipoprotein concentration was used. It is noteworthy that the effects of HD lipoprotein on the trypsin-treated chylomicrons and Intralipid were similar in spite of the fact that in this experiment the concentration of trypsin-treated chylomicrons used (15mg of triacylglycerol/assay) was half that of Intralipid.

Hydrolysis of trypsin-treated chylomicrons by lipoprotein lipase in situ

The above results demonstrate that chylomicrons treated with trypsin become a poor substrate for soluble lipoprotein lipase and that their ability to be acted on by this enzyme can be completely restored after incubation with plasma HD lipoprotein. To determine if similar results could be reproduced in a system in which the enzyme is present at its physiological site of action, i.e. the luminal surface of the endothelial cells, isolated rat hearts were perfused with [^{14}C]triacylglycerol fatty acid-labelled chylo-

microns that had been exposed to trypsin or incubated with HD lipoprotein after trypsin treatment. In these experiments the rate of $^{14}CO_2$ formation as a result of the uptake and oxidation of ^{14}C-labelled chylomicron triacylglycerol fatty acid was used as an indicator of the capacity of the hearts to hydrolyse the chylomicron triacylglycerol (Borensztajn & Robinson, 1970; Rogers & Robinson, 1974). The results in Fig. 4 show that, in agreement with the observation with soluble enzyme *in vitro* (Fig. 1), the ability of trypsin-treated chylomicrons to serve as substrate for the membrane-bound enzyme was greatly decreased. At all time intervals the amounts of $^{14}CO_2$ produced by the hearts perfused with trypsin-treated chylomicrons were significantly lower ($P<0.001$) than that produced by the hearts perfused with the control chylomicrons. At the end of 20min of perfusion the total $^{14}CO_2$ formed by the hearts perfused with control and trypsin-treated chylomicrons was respectively 3.00 ± 0.43 and 0.80 ± 0.07 μequivalents of triacylglycerol fatty acid/g of heart ($P<0.001$). Perfusion of hearts with trypsin-treated chylomicrons that had been preincubated with HD lipoprotein gave results comparable with those obtained in the assay system *in vitro* (Fig. 4). The total amount of $^{14}CO_2$ formed during the 20min perfusion period was 4.2 ± 0.19 μequivalents of triacylglycerol fatty acid/g of heart, which corresponds to about a 40% increase ($P<0.001$) over the values observed with the hearts perfused with control chylomicrons.

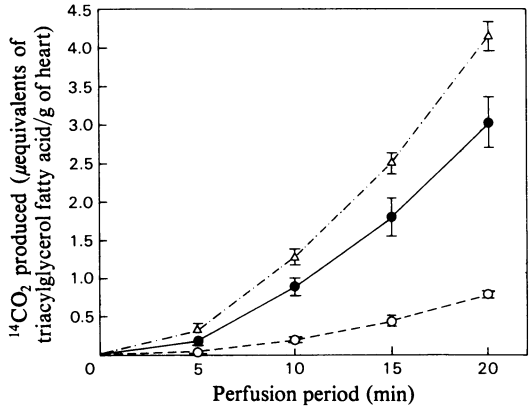


Fig. 4. *Oxidation of ^{14}C-labelled chylomicron triacylglycerol fatty acid by the perfused rat heart*

Chylomicrons were trypsin-treated as described in the legend of Fig. 3. Incubation of treated chylomicrons with HD lipoprotein (1mg of HD-lipoprotein protein/15mg of chylomicron triacylglycerol) was carried out as described in the Methods and Materials section. Hearts from 8h-starved rats (five per group) were perfused for 1min with Krebs–Ringer bicarbonate buffer and then for 20min with the same buffer containing the various ^{14}C-labelled chylomicron preparations (0.48 μequivalent of triacylglycerol fatty acids/ml). The perfusates were collected in 5min periods for the measurement of $^{14}CO_2$ as described in the Methods and Materials section. The results are expressed as means $\pm$ S.D. and have been corrected for the $^{14}CO_2$ formation that could have been derived from the oxidation of ^{14}C-labelled non-esterified fatty acids present in the chylomicron preparations (Borensztajn & Robinson, 1970). ●, Control chylomicrons; ○, trypsin-treated chylomicrons; △, incubated trypsin-treated chylomicrons.

Discussion

The results of the present investigation show that exposure of rat lymph chylomicrons to trypsin results in lipoprotein particles that are ineffective as substrate for lipoprotein lipase. Further, the results indicate that it is the loss of surface apoproteins, through their hydrolysis by trypsin, that is responsible for the inability of the chylomicron triacylglycerol to be hydrolysed by lipoprotein lipase. Trypsin treatment caused no disruption of the structure of the chylomicrons (Plate 1) or alteration in their lipid composition (Table 2). Except for the depletion of the apoproteins no other differences between control and trypsin-treated chylomicrons were detected. One possibility, apart from the apoprotein depletion, that could account for the inability of the enzyme to hydrolyse the trypsin-treated chylomicron triacylglycerol is that protein fragments resulting from the degradation of the intact apoproteins, or alternatively from the autodigestion of trypsin, might have had a direct inhibitory effect on the enzyme. This possibility is unlikely, however. Trypsin-treated chylomicrons that were reisolated by ultracentrifugation and therefore separated from most of the apoprotein fragments, and also chylomicrons that were exposed to covalently bound trypsin, could not be degraded by the enzyme.

It was not ascertained in the present study which of the apoprotein(s) lost during trypsin treatment caused the chylomicrons to become poor substrates for the enzyme. On the basis of studies carried out *in vitro* with artificial triacylglycerol emulsions (La Rosa *et al.*, 1970; Brown & Baginsky, 1972; Krauss *et al.*, 1973; Havel *et al.*, 1973*a*; Bensadoun *et al.*, 1974; Ekman & Nilsson-Ehle, 1975) it is reasonable to assume that the depletion of apoprotein C-II is a major factor in this phenomenon. As previously mentioned (see the introduction), up to the present time it has not been possible to establish the exact role that apoprotein C-II, or in fact any of the apoproteins, plays in modulating the metabolism of the physiological substrates for lipoprotein lipase, namely chylomicrons and VLD lipoproteins. With the use of trypsin-treated chylomicrons it is now

possible, however, to examine the physiological effects of individual, as well as combinations of, apoproteins on the hydrolytic action of the enzyme. Trypsin-treated chylomicrons offer definite advantages for such studies. Their lipid composition and structure is the same as that of the intact lipoproteins (Table 2 and Plate 1) so that the use of artificial substrates that require emulsifying agents such as detergents or phospholipids, which are known to affect the enzyme activity (Chung *et al.*, 1973; Blaton *et al.* 1974; Riley & Robinson, 1974; Heaf & Carlson, 1976), is entirely avoided. Further, trypsin-treated chylomicrons are capable of binding the various apoproteins, and in particular the C apoproteins, so that the rate of hydrolysis of their triacylglycerol moieties becomes indistinguishable from that of the untreated substrate (Fig. 3). Moreover, the effect of apoproteins on the degradation of chylomicrons by the enzyme can be readily tested in a physiological system (e.g. isolated perfused organs) in which the enzyme is present at its normal site of action on the endothelial surface. Thus the problem of extrapolating to the conditions *in situ* results of studies *in vitro*, which of necessity use solubilized and, in certain cases, only partially purified lipoprotein lipase preparations, can be altogether avoided.

In this study only the tetramethylurea-soluble apoproteins were monitored before and after trypsin treatment of the chylomicrons. Extensive trypsin treatment resulted in the virtually complete depletion of these apoproteins from the chylomicrons. The accessibility of these apoproteins to trypsin suggests their localization on the surface of the lipoprotein particle. This interpretation agrees with a previously proposed model of lymph chylomicron structure (Zilversmit, 1969), which places the apoproteins on the periphery of the particle along with phospholipids and free cholesterol, surrounding a large core of neutral lipid. Since a similar structure has been proposed for VLD lipoproteins, the other physiological substrate for the enzyme (Shen *et al.*, 1977), it is reasonable to assume that trypsin treatment of VLD lipoproteins would also result in the digestion of its surface apoproteins, and consequently in its inability to be catabolized by lipoprotein lipase. It should be pointed out that in the present study the fate of apoprotein B, a normal constituent of lymph chylomicron (Glickman & Kirsch, 1973) that is insoluble in tetramethylurea, was not followed. The apoprotein B of intact VLD and LD lipoproteins has been reported to be relatively resistant to tryptic digestion (Bernfeld & Kelley, 1964; Margolis & Langdon, 1966). There is no reason to believe at the present time that apoprotein B from chylomicrons is different in this regard. Indeed, the presence of essentially intact apoprotein B could in part account for the conservation of the spherical shape of the trypsin-treated chylomicrons.

When high concentrations of delipidized trypsin-treated chylomicrons were electrophoresed, small amounts of several proteins were detected (gel 3, Fig. 2). These presumably represent fragments of intact apoproteins that remain attached to the trypsin-treated particles. The possibility that these or other apoprotein fragments, too low in concentration to be detected by the electrophoretic methods used, could retain an activating effect on the enzyme was investigated. This was considered to be of importance, because a residual hydrolysis of chylomicron triacylglycerol was consistently observed even after the substrate was extensively trypsin-treated. When Intralipid, which had been incubated with HD lipoprotein and then treated with trypsin, was assayed with lipoprotein lipase, no residual triacylglycerol hydrolysis could be detected (T. W. Lukens & J. Borensztajn, unpublished work). Since Intralipid preincubated with HD lipoproteins acquires an apoprotein complement that resembles that of intact chylomicrons (Havel *et al.*, 1973*b*), a residual triacylglycerol hydrolysis would have been detected if the apoprotein fragments resulting from trypsin treatment could indeed stimulate lipoprotein lipase activity. In the light of these results, it remains to be explained why a residual triacylglycerol hydrolysis is observed with trypsin-treated chylomicrons but not with the Intralipid substrate. Although it has not been possible to completely rule out the possibility that intact apoproteins remain attached to chylomicrons after trypsin treatment, it is likely that lipoprotein lipase is capable of interacting with chylomicron triacylglycerol, but not Intralipid triacylglycerol, by a process that does not require surface apoproteins. Triacyglycerol hydrolysis by lipoprotein lipase in the complete absence of apoproteins has been described by other authors (Egelrud & Olivecrona, 1973; Bensadoun *et al.*, 1974; Ehnholm *et al.*, 1975).

The residual hydrolysis of triacylglycerol of trypsin-treated chylomicrons by the perfused rat heart, as determined by the formation of $^{14}CO_2$, was more than twice the residual hydrolysis of the same substrate when assayed *in vitro* (less than 10%). This difference is in contrast with the results obtained with trypsin-treated chylomicrons preincubated with HD lipoproteins and subsequently assayed *in vitro* and *in situ*. In both systems the chylomicron triacyglycerol was hydrolysed at a rate 40–50% greater than that for the control preparations (Figs. 3 and 4). As in isolated perfused rat hearts, lipoprotein lipase is located at its normal site of action, the results obtained with this preparation are presumably more representative of the physiological process of lipoprotein degradation. It is conceivable that the membrane-bound enzyme is more efficient in non-apoprotein-dependent triacylglycerol hydrolysis, but interacts with the substrate in a similar way to the solubilized enzyme in the

presence of intact apoproteins. However, whether the differences in the relative amounts of residual hydrolysis measured in the assay systems *in vitro* and *in situ* reflect solely differences in the enzyme–substrate interaction is unknown at present. Nevertheless, it is clear from the results of the present study that the physiological effects of apoproteins on the degradation of triacylglycerol-rich lipoproteins may now be carried out with a natural substrate of lipoprotein lipase.

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