Biochem. J. (1979) **178**, 455–466
Printed in Great Britain

The Metabolic Conversion of Very-Low-Density Lipoprotein into Low-Density Lipoprotein by the Extrahepatic Tissues of the Rat

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(*Received* 24 *July* 1978)

1. The work reported was designed to provide quantitative information about the capacity of the extrahepatic tissues of the rat to degrade injected VLD lipoproteins (very-low-density lipoproteins, $d<1.006$) to LD lipoproteins (low-density lipoproteins, d 1.006–1.063) and to study the fate of the different VLD-lipoprotein apoproteins during the degradative process. 2. Rat liver VLD lipoproteins, radioactively labelled in their protein moieties, were produced by the perfusion of the organ and were either injected into the circulation of the supradiaphragmatic rats or incubated in rat plasma at 37°C. At a time (75min) when approx. 90% of the triacylglycerol of the VLD lipoproteins had been hydrolysed, the supradiaphragmatic rats were bled and VLD lipoproteins, LD lipoproteins and HD lipoproteins (high-density lipoproteins, d 1.063–1.21) were separated from their plasma and from the plasma incubated *in vitro*. The apoproteins of each of the lipoprotein classes were resolved by gel-filtration chromatography into three main fractions, designated peaks I, II and III. 3. Incubation of the liver VLD lipoproteins in plasma *in vitro* led to the transfer of about 30% of the total protein radioactivity to the HD lipoproteins. This transfer mainly involved the peak-II (arginine-rich and/or apo A-I) and peak-III (apo C) proteins. There was also a small transfer of radioactivity (about 5% of the total) to the LD lipoproteins. 4. Injection of the liver VLD lipoproteins into the circulation of the supradiaphragmatic rat resulted in the transfer of about 15% of the total VLD-lipoprotein radioactivity to the LD lipoproteins. This transfer involved mainly the peak-I (apo B) proteins and accounted for about 20% of the total apo B protein radioactivity of the injected VLD lipoproteins. When the endogenous plasma VLD lipoprotein was taken into account the transfer of apo B protein was about 35%. 5. The transfer of peak-II protein radioactivity from the VLD to the HD lipoproteins was greater in the plasma of the supradiaphragmatic rat than in the incubated plasma, suggesting that there was a net transfer of peak-II apoproteins during the VLD lipoprotein degradation. The transfer of peak-III protein radioactivity was not greater in the plasma of the supradiaphragmatic rat, but there was a loss of this radioactivity from the circulation.

The primary event in the metabolism of plasma chylomicrons and VLD lipoproteins is the hydrolysis of their triacylglycerol, a core constituent (Shen *et al.*, 1977), through the action of the extrahepatic enzyme lipoprotein lipase (Robinson, 1970). Studies utilizing supradiaphragmatic and hepatectomized animal preparations have shown that the extrahepatic degradation of these lipoproteins results in the formation of triacylglycerol-depleted particles that, in the rat, have been termed remnants. By comparison with the parent lipoproteins, these are enriched in cholesteryl esters (Redgrave, 1970) and in the protein species known as apo B (Mjøs *et al.*, 1975). Such remnants have generally been considered to be the end products of the extrahepatic degradation (Eisenberg, 1976) that are then taken up from the plasma as intact complexes by the liver and further metabolized (Redgrave, 1970; Eisenberg & Rachmilewitz, 1973; Faergeman & Havel, 1975; Noel *et al.*, 1975; Floren & Nilsson, 1977; Cooper, 1977).

Still unsettled, however, is the question of the site of production of the plasma LD lipoproteins. These are known to be formed through the metabolism of chylomicrons and VLD lipoproteins, but, although it has been suggested that the LD lipoproteins could be released from the liver after remnant degradation in the organ (Felts *et al.*, 1975), this view has not been supported by any experimental evidence. Our recent work with the supradiaphragmatic rat (Suri *et al.*,

Abbreviations used: VLD lipoproteins, very-low-density lipoproteins ($d<1.006$); LD lipoproteins, low-density lipoproteins (d 1.006–1.063); HD lipoproteins, high-density lipoproteins (d 1.063–1.21); SDS, sodium dodecyl sulphate.

1978) has led us to question the view that remnants are the true end products of extrahepatic metabolism. Instead, we believe that the remnants can be better represented as intermediates in an on-going degradation of the plasma VLD lipoproteins, and we have suggested that this might well continue as far as LD lipoproteins without the intervention of the liver. In the present study, we confirm the ability of the extrahepatic tissues of the rat to metabolize VLD lipoproteins to LD lipoproteins and provide a quantitative measure of this process in the supradiaphragmatic rat. We also examine the fate of the different VLD lipoprotein apoproteins in this preparation. For these purposes we have used VLD lipoproteins labelled in their protein moiety and prepared by perfusion of the rat liver. We believe that such preparations are more suitable for the studies than plasma VLD lipoprotein fractions whose apoproteins have been labelled with ^{125}I (see, e.g., Eisenberg & Rachmilewitz, 1973; Glangeaud *et al.*, 1977). These last will normally contain complexes of both hepatic and intestinal origin and some of them may already be partially degraded. Furthermore, the iodination of lipoproteins causes very different degrees of labelling of the distinct apoprotein species (Fidge & Poulis, 1974) and results in considerable incorporation of radioactivity into the lipid moiety. Both these factors can complicate the interpretation of the results obtained (Shepherd *et al.*, 1976; Dory *et al.*, 1978).

On their release from the liver into the plasma, some of the VLD-lipoprotein apoproteins exchange with those of other lipoprotein classes (Eisenberg & Levy, 1975; Lewis, 1977), and this results in a net acquisition of apoprotein by the VLD lipoproteins (Osborne & Brewer, 1977). To try to distinguish such movements of apoproteins from the transfers between lipoprotein classes that occur during the degradation of VLD lipoproteins in the supradiaphragmatic rat, we have used rat plasma incubated with radioactively labelled liver VLD lipoproteins as a control system.

Materials and Methods

Materials

Fluorescamine {4-phenylspiro[furan-2(3),1'-phthalan]-3,3'-dione} was purchased from Roche Products, London W.1, U.K., and NCS was supplied by Hopkin and Williams, Chadwell Heath, Essex, U.K. The L-[4,5-^3H]leucine (40–58Ci/mol) and ^{125}I-labelled human albumin (2.5μCi/mg of albumin) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Sephadex G-150 and Blue Dextran were products of Pharmacia (G.B.) Ltd., London W.5, U.K. Oleic acid, penicillin, Triton X-114, clinical-grade dextran (average mol.wt. 264000) and proteins for use as molecular-weight markers were all purchased from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. 2,5-Diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Sources of all other materials used were as specified by Suri *et al.* (1978).

Animals

Male Wistar albino rats (A. Tuck and Son, Rayleigh, Essex, U.K.) maintained on Oxoid pasteurized 41B diet (Herbert Styles, Bewdley, Worcs., U.K.) were used throughout the study.

Livers for perfusion were obtained from animals weighing between 320 and 350g. The maintenance diet of the liver donors was replaced by a fructose-rich diet (Suri *et al.*, 1978) 3 days before the perfusion experiments in order to increase the output of VLD-lipoprotein triacylglycerol and protein (Petersburg *et al.*, 1975).

For the experiments with supradiaphragmatic rats, the animals were starved overnight and weighed 340–380g.

Liver perfusion

This was carried out in an isothermal cabinet at 37°C by a modification of the method of Hems *et al.* (1966), as described by Petersburg *et al.* (1975). The perfusion medium consisted of Krebs–Ringer bicarbonate buffer, pH7.4 (Krebs & Henseleit, 1932), containing washed rat erythrocytes (20%, v/v), bovine serum albumin (2.5%, w/v), glucose (300mg/100ml), penicillin (11×10^5 units/100ml) and an amino acid mixture (Tolman *et al.*, 1973) from which leucine had been omitted.

The rat erythrocytes were washed three times with 0.15M-NaCl solution and then with the Krebs–Ringer bicarbonate buffer. A stock serum albumin (25%, w/v) solution at pH7.4 was dialysed against several changes of 0.15M-NaCl solution before being stored frozen.

Each liver was perfused with 85ml of the perfusion medium, which was continuously recirculated. When the flow rate of perfusion medium through the liver had stabilized to approx. 15ml/min, 3ml of an oleic acid–albumin complex (28mM with respect to oleic acid and with a molar ratio of oleic acid to albumin of 38) was added to the perfusion medium, and the same complex was infused throughout the remainder of the perfusion period at a rate of 3ml/h. The complex was prepared by the method of Van Harken *et al.* (1969). L-[4,5-^3H]Leucine (0.4mCi) was injected into the portal-vein cannula after the addition of the oleic acid–albumin complex, and the perfusion was continued for 4h thereafter. At the end of this period, erythrocytes were removed from the perfusion medium by centrifugation at 1500*g* for 20min.

Isolation of lipoproteins

VLD lipoproteins were isolated from the perfusion medium by centrifugation of 7.5ml samples for 22h in a Beckman model L2-65B ultracentrifuge at 106500*g* ($r_{av.}$ 5.9cm) at 5°C in a Beckman Ti 50 rotor. At the end of the centrifugation, the top 1.5ml of fluid from each tube was removed with a Pasteur pipette and its density was raised to 1.04 by the addition of KBr/NaCl solution of *d* 1.346. Samples (2.8ml) were pipetted into polycarbonate tubes (1.6cm×7.5cm) and overlayered by 2.5ml and 3.0ml of KBr/NaCl solutions with respective densities of 1.027 and 1.01. After further centrifugation under the same conditions for 3h, the VLD lipoproteins in the top 1.5ml of the salt gradient were again removed and dialysed against three changes of 200vol. of 0.9% NaCl solution containing L-leucine (20mg/litre) to remove unincorporated [^{3}H]leucine (Petersburg *et al.*, 1975).

VLD lipoproteins isolated as described above were then concentrated to a volume of approx. 10ml by dry dialysis against dextran. For each supradiaphragmatic-rat experiment, VLD lipoproteins synthesized by the perfusion of four livers were required.

Various lipoprotein classes were also isolated from plasma. For these separations, a modification of the procedure of Havel *et al.* (1955) was used and VLD lipoproteins (*d*<1.006), LD lipoproteins (*d* 1.006–1.063) and HD lipoproteins (*d* 1.063–1.21) were isolated.

For the separation of VLD lipoproteins, 5ml samples of plasma were overlayered with 2.5ml of an NaCl solution of *d* 1.006 and the tubes were centrifuged for 22h under the conditions described above. The VLD lipoproteins were layered in the top 2ml of the fluid in each tube, and this was collected by means of a Pasteur pipette. To ensure quantitative recovery of the VLD lipoproteins, the infranatant solution was again overlayered with NaCl solution of *d* 1.006 and the centrifugation and recovery of VLD lipoproteins was repeated. The two VLD lipoprotein samples were combined.

The density of the infranatant solution was then adjusted to 1.063 by the addition of a KBr/NaCl solution of *d* 1.346. Samples (5ml) of this solution were overlayered with 3ml of a KBr/NaCl solution of *d* 1.063, and centrifugation was carried out as for the isolation of VLD lipoproteins. The top 1ml fraction was removed from each tube as above, and this constituted the LD lipoprotein class.

Finally, the density of the infranatant solution was adjusted to 1.21 with salt solution of *d* 1.346 and, after overlayering 5ml samples with 3ml of a KBr/NaCl solution of *d* 1.21, centrifugation was carried out at 122250*g* for 24h. The top 1ml fraction of each tube, which contained the HD lipoproteins, was removed and washed once by resuspension in a KBr/NaCl solution of *d* 1.21.

All the lipoprotein samples were dialysed exhaustively against 0.15M-NaCl solution containing 1.3mM-EDTA, pH8.6.

VLD-lipoprotein injection and incubation

Supradiaphragmatic rats were prepared by the method of Bezman-Tarcher & Robinson (1965) and injected with liver VLD lipoproteins (0.3ml) via the left jugular vein. The rats were bled after 75min via the abdominal aorta into tubes containing EDTA (final concn. 2.7mM), and the plasma obtained from 14–16 animals was pooled and stored at 4°C.

The same preparation of liver VLD lipoproteins was also incubated with plasma, obtained from control groups of starved rats, for 75min at 37°C *in vitro*. The concentration of liver VLD lipoprotein in the plasma in these incubations was the same as that calculated to be attained initially in the plasma of the injected supradiaphragmatic rat preparations. Bezman-Tarcher & Robinson (1965) showed that the mean plasma volume of the supradiaphragmatic rat was 28% of that of the whole animal (40.4ml/kg body wt.; Wang, 1959). We have confirmed this in studies with ^{125}I-labelled albumin and showed further that there is no change in the plasma volume of the supradiaphragmatic rat over a 75min period. The amount of VLD lipoprotein to be added to the plasma was calculated from this information, due allowance being made for the volume of VLD lipoprotein injected.

The lipoproteins of the incubated and the supradiaphragmatic plasma were isolated as described above. None of the plasma was stored for longer than 8h before the isolation was started.

Delipidation and gel chromatography

All the isolated lipoproteins were delipidated by the method of Scanu & Edelstein (1971). Protein recoveries after delipidation were generally greater than 90%. The apoproteins were dissolved in 1ml of 0.19M-Na_2HPO_4/0.01M-NaH_2PO_4 buffer, pH8.0, containing 0.1M-SDS and samples were chromatographed at room temperature (21°C) on Sephadex G-150 columns (2cm×70cm) equilibrated with 0.2M-sodium phosphate buffer, pH8.0, containing 2mM-SDS and 50mg of merthiolate/litre. The flow rate was 15ml/h per cm^2, and 2.2ml fractions were collected. Molecular-weight markers used for the calibration of the column were Blue Dextran, bovine serum albumin and cytochrome *c*.

Polyacrylamide-gel electrophoresis

This was carried out by urea/polyacrylamide-gel electrophoresis as previously described (Suri *et al.*, 1978), except that tetramethylurea was omitted from the samples, since delipidated apoproteins were

loaded on the gels. The apoprotein solutions were first dialysed for 24h against several changes of 0.15M-NaCl solution containing 6M-urea. The dialysis was carried out in acetylated tubing (Vanaman *et al.*, 1968) to avoid loss of low-molecular-weight proteins into the diffusate.

Measurement of radioactivity

Radioactivity was measured in a Beckman LS230 liquid-scintillation counter. The lipoprotein samples (20μl) were incubated with NCS (60μl) in capped vials for 45min at 25°C. The contents were then mixed with 2μl of acetic acid and 10ml of a toluene/methoxyethanol (3:1, v/v) scintillant containing 6.6g of 2,5-diphenyloxazole and 200mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene per litre and stored in the dark for 24h before radioactivity counting. Samples (0.5ml) of the apoproteins from the gel-filtration-column fractions were mixed with 1ml of water and 10ml of a xylene/Triton X-114 (3:1, v/v) scintillant containing 6g of 2,5-diphenyloxazole/litre.

Preparation of antisera to apoprotein B

LD lipoproteins (*d* 1.020–1.050) were isolated from the plasma of starved rats as described above and washed twice by resuspension and centrifugation in a KBr/NaCl solution of *d* 1.050. After delipidation, the apoproteins were separated by gel filtration, and the void-volume peak was recovered as apo B. Portions of the peak material were emulsified with equal volumes of Freund's complete adjuvant and injected subcutaneously into rabbits (50μg/kg body wt.) by using multiple injection sites. The rabbits were given booster injections at 2-week intervals for 6 weeks, and 2 weeks after the last injection blood was obtained from the ear vein. From the blood, antiserum was prepared as described by Sargent & George (1975). On double diffusion (Ouchterlony, 1949), the antiserum produced a single precipitin line against the apoproteins of VLD and LD lipoproteins and no precipitin line against the apoproteins of HD lipoprotein.

Analytical methods

Triacylglycerol was measured by the method of Fletcher (1968). The protein content of lipoproteins and their apoproteins was measured by the method of Lowry *et al.* (1951), or by a modification of the fluorimetric procedure of Udenfriend *et al.* (1972). The latter was used for the determination of the low concentrations of protein in column fractions. Up to 300μl of the column fractions, containing 1–10μg of protein, was used and sufficient 0.2M-sodium borate buffer (0.2M-boric acid adjusted to pH8.5 with NaOH) was added to give a final volume of 1.85ml. The tubes were then held on a Vortex mixer, and 150μl of a solution of fluorescamine in acetone (30mg/100ml) was added. Bovine serum albumin was used as a standard. The fluorescence was measured on an Amicon–Bowman SPF125 spectrofluorimeter which was set at an excitation wavelength of 390nm and an emission wavelength of 485nm.

Analysis of data

The results of three experiments are given in the text as the means±S.E.M. of the values obtained.

Results and Discussion

Three separate preparations of liver VLD lipoproteins, labelled in their apoprotein components with [^{3}H]leucine, were used in three separate experiments in the study. In each experiment, the VLD lipoproteins were injected into the bloodstream of groups of starved supradiaphragmatic rats. Samples of the same preparations were also incubated with starved-rat plasma *in vitro* at 37°C at the initial concentrations attained in the injected animals (see the Materials and Methods section). The initial concentration of the endogenous plasma triacylglycerol was 46± 9mg/100ml and the respective calculated initial concentrations of injected VLD-lipoprotein triacylglycerol and protein were 63±20 and 3.9±1.5mg/100ml of plasma. In two of the experiments, the injected VLD lipoprotein had a triacylglycerol/protein ratio of about 15. In the other it was about 25,

Table 1. *Distribution of radioactivity among plasma fractions after injection or incubation of ^{3}H-labelled VLD lipoproteins*
Preparations of ^{3}H-labelled liver VLD lipoproteins were either injected into the circulation of supradiaphragmatic rats or incubated in rat plasma at 37°C at the concentrations attained initially in the injected animals (see the Materials and Methods section). After 75min, the supradiaphragmatic rats were exsanguinated, and lipoprotein fractions were separated from both the plasma of the supradiaphragmatic animals and the incubated plasma. In three separate experiments the mean recoveries (±S.E.M.) of the injected radioactivity in the separated fractions were 101±1 and 81±4% respectively for the incubations *in vitro* and the injections *in vivo*.

System	Percentage of recovered radioactivity in			
	VLD lipoproteins	LD lipoproteins	HD lipoproteins	Proteins of $d>1.21$
Incubated plasma	56±5	5±1	30±4	9±2
Injected supradiaphragmatic rat	43±6	15±1	34±5	7±2

reflecting in this case a greater degree of loading with triacylglycerol during the liver perfusion. The radioactivity associated with liver VLD lipoprotein injected/100ml of plasma was $17.6\times10^6\pm5.5\times10^6$ d.p.m.

After 75min the supradiaphragmatic rats were exsanguinated. At this time the plasma triacylgycerol concentration had fallen by approx. 90%. This is consistent with our previous results and reflects the action of lipoprotein lipase on both the endogenous and injected triacylglycerol (Bezman-Tarcher *et al.*, 1965; Suri *et al.*, 1978).

Distribution of liver VLD-lipoprotein radioactivity among plasma lipoproteins

The distribution of VLD-lipoprotein radioactivity among the different lipoproteins in the incubated plasma and in the plasma of the supradiaphragmatic rat is shown in Table 1. In accordance with previous findings (Eisenberg & Rachmilewitz, 1973, 1975), there is a marked loss of VLD-lipoprotein radioactivity both *in vitro* and *in vivo* and, in both situations, this is accounted for mainly by the appearance of radioactivity in the HD lipoproteins. Although a small percentage of the injected radioactivity is found in the LD lipoproteins after the incubations *in vitro*, a substantially higher percentage is present in the supradiaphragmatic-rat plasma. This is consistent with our previous evidence for LD-lipoprotein formation from VLD lipoprotein in this preparation (Suri *et al.*, 1978). The radioactivity in the $d>1.21$ infranatant fraction can be accounted for by a combination of contamination of this fraction by HD lipoproteins and by pressure dissocation of apoproteins during the ultracentrifugation procedures (Koga *et al.*, 1969; Mahley & Holcombe, 1977; Fainaru *et al.*, 1977).

Redistribution of liver VLD-lipoprotein apoproteins and apoprotein radioactivity among plasma lipoproteins in vitro and in vivo

The results in Table 1 provide no information about possible redistributions of individual VLD-lipoprotein apoproteins and of apoprotein radioactivity that may occur in the circulation of the supradiaphragmatic rat or during the incubations with rat plasma *in vitro*. To provide this, each of the isolated lipoproteins was delipidated and their apoproteins were fractionated by gel-filtration chromatography. The protein and radioactivity contents of the peak fractions were then determined.

VLD-lipoprotein apoproteins. Fig. 1 shows typical elution profiles of the apoproteins from VLD lipoproteins of the liver perfusate, of the incubated plasma and of the plasma of the supradiaphragmatic animals.

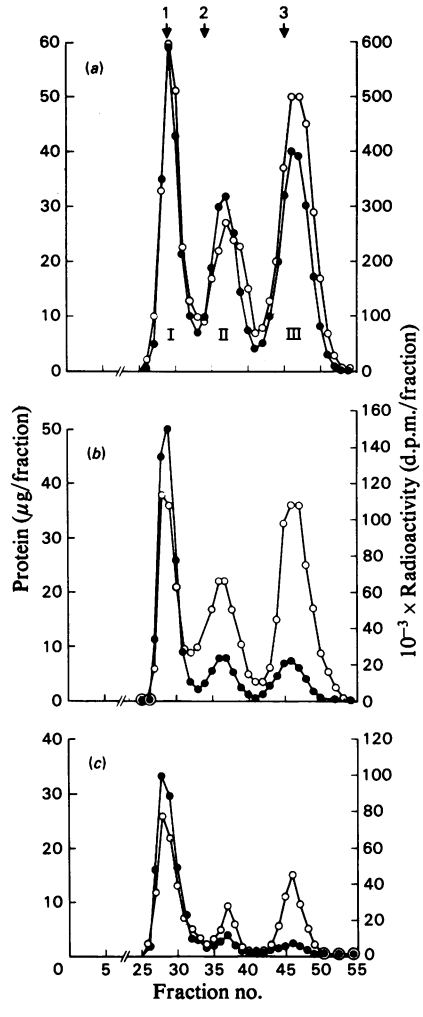

Fig. 1. *Gel-filtration chromatography of VLD-lipoprotein apoproteins*

The VLD-lipoprotein apoproteins were prepared and fractionated as described in the Materials and Methods section. The sources of ^{3}H-labelled VLD lipoproteins were: (*a*) liver perfusate; (*b*) rat plasma incubated with liver VLD lipoproteins; (*c*) plasma from supradiaphragmatic rats injected with liver VLD lipoproteins. The amounts of apoprotein applied to the columns were: (*a*) 0.69mg; (*b*) 0.43mg; (*c*) 0.17mg. The protein (○) and radioactivity (●) contents under peaks of each eluted sample (designated I, II and III; see *a*) were determined as described in the Materials and Methods section, the percentage recoveries (mean ±S.E.M.) of protein and radioactivity in three experiments of the kind shown being respectively: (*a*) 98±3 and 90±4; (*b*) 82±1 and 87±2; (*c*) 86±2 and 92±6. The eluted positions of molecular-weight markers labelled 1, 2 and 3 and representing Blue Dextran, bovine serum albumin and cytochrome *c* respectively are shown on (*a*).

The elution profiles of the apoproteins of liver VLD lipoproteins (Fig. 1*a*) and of the incubated rat plasma VLD lipoproteins (Fig. 1*b*) are similar to those previously reported (Bersot *et al.*, 1970). Three well-defined fractions are separated. The apoprotein in the void-volume peak (peak I) corresponds to the apo B of VLD lipoprotein, being excluded from the running gel on electrophoresis (Bersot *et al.*, 1970). Peak II produced two bands in the upper region of the running gel on electrophoresis, a pattern similar to that previously described (Windmueller *et al.*, 1973). By comparison with the electrophoretic band patterns of VLD-lipoprotein apoproteins obtained by other workers (see, e.g., Hamilton *et al.*, 1976), the slower of the two bands was considered to be the arginine-rich protein (or apo E) and the faster band to be apo A-I. However, the identification of these bands remains tentative, since both these apoproteins can exhibit multiple bands with slightly different mobilities (Edelstein *et al.*, 1972; Weisgraber *et al.*, 1977). The third peak (peak III) gave three well-defined fast-migrating bands on electrophoresis, representing the apo C proteins of the VLD lipoproteins (Swaney *et al.*, 1977). A faint slower-moving apoprotein band was also observed that corresponded in its migration distance to apo A-II (Hamilton *et al.*, 1976). The electrophoretic band patterns of supradiaphragmatic-rat VLD-lipoprotein apoproteins were very similar to those of liver and incubated plasma, except that the peak-II apoprotein showed only one band and the peak-III apoproteins showed no band corresponding in migration distance to apo A-II.

Peak I contained $33\pm1\%$ of the total protein and $42\pm4\%$ of the total radioactivity of the liver VLD lipoproteins. Peak II contained $25\pm2\%$ of the protein and $20\pm5\%$ of the radioactivity, and for peak III the corresponding values were $42\pm2\%$ and $38\pm2\%$.

After the incubation of the liver VLD lipoproteins with rat plasma *in vitro*, the distribution of the total protein among peaks I, II and III, respectively $28\pm2\%$, $25\pm1\%$ and $47\pm3\%$, was not markedly changed. Much more significant were the changes in the distribution of radioactivity among the VLD-lipoprotein apoproteins during the incubations *in vitro*. The radioactivity associated with peak III fell markedly to $16\pm1\%$ of the total and there was a smaller fall to $13\pm3\%$ in that associated with peak II. Evidently the overall loss of VLD-lipoprotein radioactivity (Table 1) is largely due to these falls, which lead to a corresponding rise in the radioactivity associated with peak I to $73\pm3\%$ of the total.

After their reisolation from the circulation of the supradiaphragmatic rat, the VLD lipoproteins showed additional changes with respect to the distribution of both the apoprotein protein and the apoprotein radioactivity (Fig. 1*c*). The percentage of the total protein in peak I rose to $51\pm2\%$, whereas that associated with peaks II and III fell to $17\pm1\%$ and $31\pm1\%$ respectively. So far as the radioactivity distribution was concerned, there was a further concentration in peak I beyond that already evident during the incubations *in vitro*. Thus this peak contained $85\pm4\%$ of the total, whereas that associated with peaks II and III declined further to $8\pm2\%$ and $8\pm1\%$ respectively. The significance of these changes will be considered later in the paper.

LD- and HD-lipoprotein apoproteins. Fig. 2 shows typical elution profiles of the delipidated LD lipoproteins separated from the incubated plasma (Fig. 2*a*) and from the plasma of the supradiaphragmatic rats (Fig. 2*b*). Figs. 3(*a*) and 3(*b*) provide the corresponding information for the HD lipoproteins.

The LD-lipoprotein apoprotein elution profiles were similar in the incubated plasma and in the plasma of the supradiaphragmatic rats. The electrophoretic band patterns of the isolated apoproteins were also similar and the bands in their migration distance corresponded to those from VLD lipoproteins. As expected (Koga *et al.*, 1971), the predominant peak was peak I, corresponding to the apo B and accounting for $61\pm3\%$ and $62\pm2\%$ of the total protein in Figs. 2(*a*) and 2(*b*) respectively. Peaks II and III contained respectively $14\pm2\%$ and $25\pm2\%$ of the total protein in the incubated plasma (Fig. 2*a*) and respectively $15\pm2\%$ and $23\pm1\%$ of the total protein in the plasma from the supradiaphragmatic rats (Fig. 2*b*).

The presence of about 40% of the total protein in peaks II and III is unlikely to be accounted for by contamination with VLD and HD lipoproteins. Although apo B is often considered to be characteristic of the LD-lipoprotein class, recent studies have shown that the *d* 1.006–1.063 lipoprotein fraction from rat plasma contains additional apoproteins, including the arginine-rich, apo A-I and the apo C group (Mahley & Holcombe, 1977; Weisgraber *et al.*, 1977). Moreover, these have been shown to be associated with a distinct lipoprotein, designated HD lipoprotein$_1$ (Weisgraber *et al.*, 1977).

Very little of the VLD-lipoprotein radioactivity appeared in the LD lipoproteins in the incubated plasma, as previously noted (Table 1), and the distribution among peaks I, II and III was respectively $52\pm4\%$, $14\pm4\%$ and $34\pm7\%$. The radioactivity in this lipoprotein could be accounted for by a small contamination of the fraction and/or by a limited exchange of the apoproteins of peaks II and III.

In the plasma of the supradiaphragmatic rat, the amount of radioactivity present in the LD lipoproteins is increased (Table 1). It is clear (cf. Figs. 2*b* and 2*a*) that almost all of this increase is due to peak-I apoprotein radioactivity, which rises to $76\pm2\%$ of the total, whereas that in peaks II and III declines to respectively $9\pm2\%$ and $15\pm3\%$.

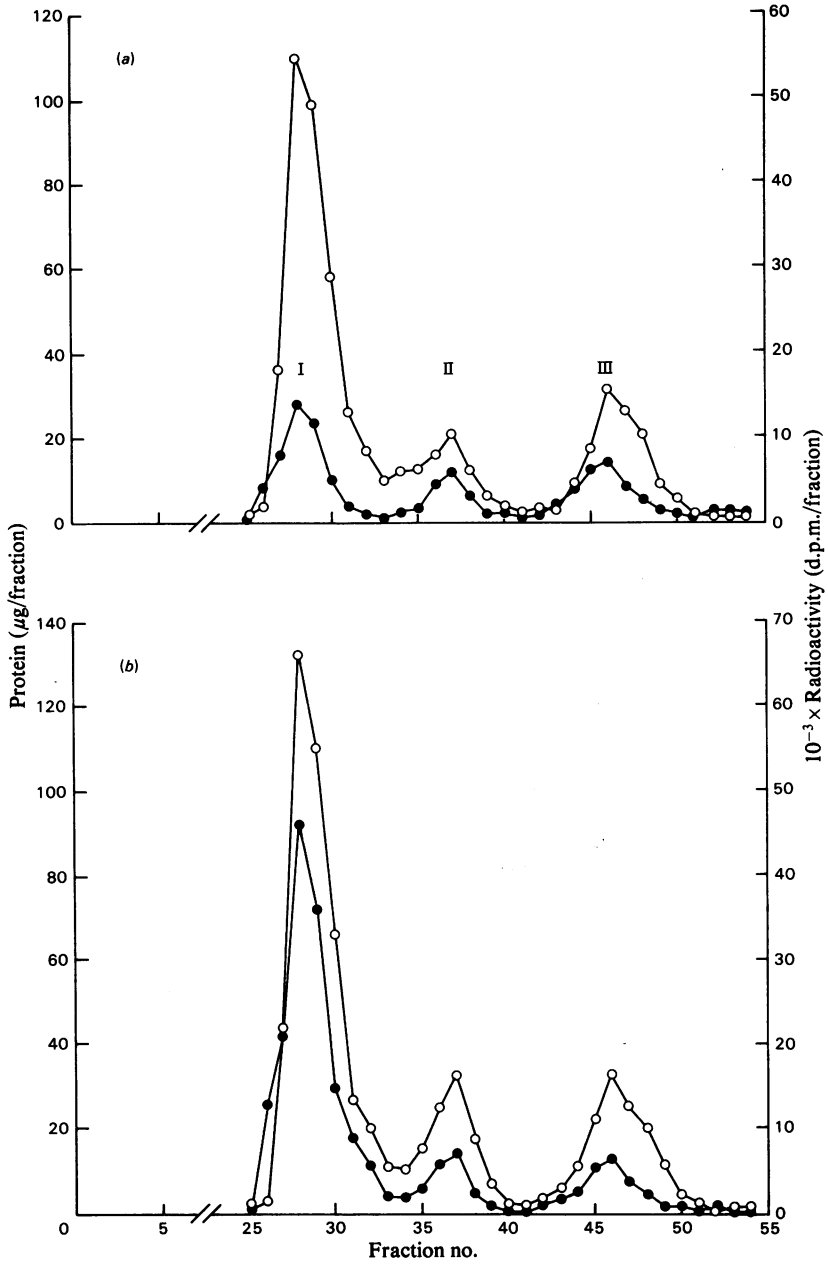

Fig. 2. *Gel-filtration chromatography of LD-lipoprotein apoproteins*

The LD-lipoprotein apoproteins were prepared and fractionated, as described in the Materials and Methods section, from: (*a*) rat plasma incubated with ^{3}H-labelled liver VLD lipoproteins; (*b*) plasma from supradiaphragmatic rats injected with ^{3}H-labelled liver VLD lipoproteins. The amounts of apoprotein applied to the columns were: (*a*) 0.60mg; (*b*) 0.68mg. The protein (○) and radioactivity (●) contents under peaks of each eluted fraction (designated I, II and III; see *a*) were determined as described in the Materials and Methods section, the percentage recoveries (means±S.E.M.) of protein and radioactivity in three experiments of the kind shown being respectively: (*a*) 85±5 and 100±5; (*b*) 88±2 and 91±2.

Gel filtration resolved the delipidated HD lipoprotein into two well-defined peaks that have been designated II and III (Fig. 3) and which correspond to the HS-2 and HS-3 peaks of Bersot *et al.* (1970). No distinct void-volume peak, corresponding to the HS-1 peak of Bersot *et al.* (1970), was observed. Since this is believed to be due to aggregation of the peak-II (HS-2) apoproteins (Swaney *et al.*, 1977), presumably such aggregation was avoided under the conditions of delipidation and solubilization of the apoproteins used in our study. The protein in the void-volume fractions produced no precipitin line when examined by Ouchterlony double diffusion against antisera to apo B. Therefore, for clarity in presentation of the data, we have considered the small amount of protein and radioactivity in the void-volume fractions as part of peak II.

The peak-II apoproteins were separated into two slow-moving bands on polyacrylamide-gel electrophoresis. The first of these corresponds to the arginine-rich protein in its mobility (Koga *et al.*, 1971) and the second to the major apoprotein of the HD lipoprotein, apo A-1 (Koga *et al.*, 1971; Fainaru *et al.*, 1976). The apoproteins of peak III separated into four major bands, and it can be assumed that the three fast-migrating bands represent the apo C proteins, and the slowest-migrating represents apo A-II (Herbert *et al.*, 1974).

Most of the total apoprotein of the HD lipoprotein was represented by peak II, both in the fraction isolated from the incubated plasma ($81\pm2\%$) and in that isolated from the plasma of the supradiaphragmatic rats ($78\pm2\%$). However, most of the radioactivity was associated with peak III in both situations. This contained $71\pm4\%$ of the total in the incubated plasma and $61\pm3\%$ in the supradiaphragmatic-rat plasma.

Quantitative transfer of VLD-lipoprotein radioactivity

The information contained in gel-filtration profiles of the kind shown in Figs. 1, 2 and 3 can be used to provide quantitative information about the redistribution of radioactivity that occurs when liver VLD lipoproteins are incubated in rat plasma *in vitro* or injected into the circulation of the supradiaphragmatic rat *in vivo*. However, in carrying out such an analysis it is necessary to appreciate the existence of two distinct complicating factors.

One concerns the appearance, both *in vitro* and *in vivo*, of a small proportion of the liver VLD-lipoprotein radioactivity in the $d>1.21$ infranatant fraction (Table 1). Though the amount is similar in the incubated plasma and in the plasma of the supradiaphragmatic rat, it is not possible to assume either that it occurs proportionally for the apoproteins of each of the peaks I, II and III, or that the involvement

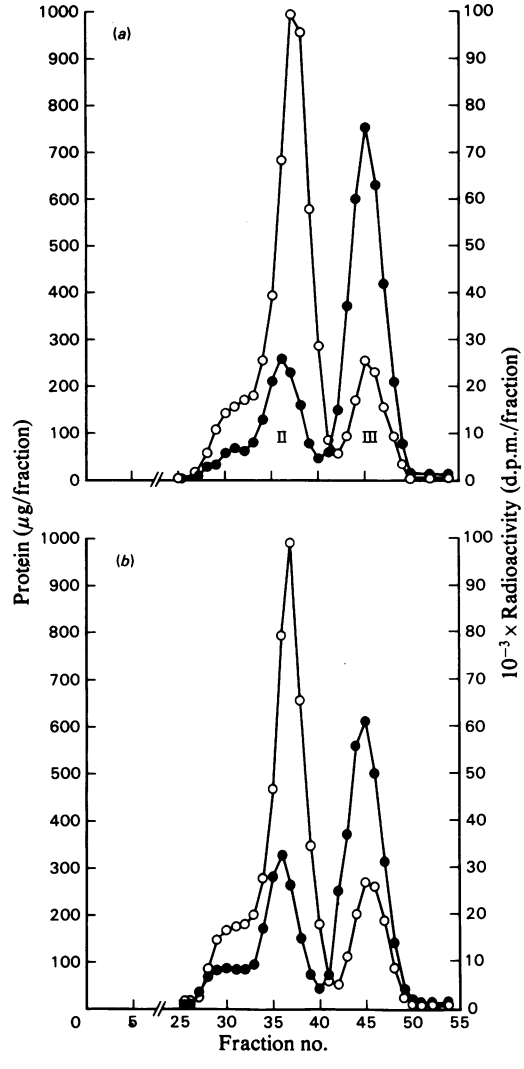

Fig. 3. *Gel-filtration chromatography of HD-lipoprotein apoproteins*

The HD-lipoprotein apoproteins were prepared and fractionated, as described in the Materials and Methods section, from: (*a*) rat plasma incubated with ^{3}H-labelled liver VLD lipoproteins; (*b*) plasma from supradiaphragmatic rats injected with ^{3}H-labelled liver VLD lipoproteins. The amounts of apoprotein applied to the columns were: (*a*) 6.2mg; (*b*) 5.9mg. The protein (○) and radioactivity (●) contents under peaks of each eluted sample (designated II and III; see *a*) were determined as described in the Materials and Methods section, the percentage recoveries (means±S.E.M.) of protein and radioactivity in three experiments of the kind shown being respectively: (*a*) 80 ± 4 and 87 ± 4; (*b*) 82 ± 5 and 88 ± 7.

of the different apoproteins is the same in the two situations.

The second complicating factor arises from the fact that, although the added VLD-lipoprotein radioactivity was recovered quantitatively from the incubated plasma, the recovery from the plasma of the supradiaphragmatic rat was only 81% (see legend to Table 1). This difference could be due either to a greater loss during the fractionation procedures in the latter situation, though this seems unlikely, or to loss of apoproteins from the plasma during VLD-lipoprotein degradation in the supradiaphragmatic animal (Suri *et al.*, 1978). In either case, it is again not possible to assume that it will occur proportionally from the apoproteins of each of the peaks I, II and III.

Because of the foregoing considerations, we have not felt justified in making any allowances for either the radioactivity in the $d > 1.21$ infranatant fraction or for the differences in the recoveries of radioactivity. However, from a knowledge of the distribution of radioactivity between peaks I, II and III in the liver lipoproteins initially, we have been able to calculate for each peak the percentage of its radioactivity that appears in the same peak in the gel-filtration profiles of the delipidated VLD, LD and HD lipoproteins in each experiment, and we have done this for both the studies *in vivo* and *in vitro* (Table 2). Because of the complicating factors already considered, these percentages do not total 100% and the extent of the deficit varies from one peak to another and for the situations *in vitro* and *in vivo*. We do not believe that these considerations limit the significance of the findings for the conclusions we wish to draw.

Studies in vitro. Incubation of the liver VLD lipoproteins with rat plasma *in vitro* clearly results mainly in the loss of radioactivity from peaks II and III, and the main recipient is the HD lipoprotein (Table 2). It has been known for some time (Eisenberg *et al.*, 1972; Eisenberg & Rachmilewitz, 1975) that the incubation of ^{125}I-labelled VLD lipoproteins with rat plasma *in vitro* leads to the movement of radioactivity from the apo C proteins of peak III to the HD lipoproteins. However, similar evidence has not been available hitherto for the transfer of peak-II apoprotein radioactivity (either arginine-rich protein or apo A-I) to HD lipoproteins.

Supradiaphragmatic-rat studies. The substantial loss of radioactivity from peak I of the VLD lipoproteins that occurs in the supradiaphragmatic rat is accounted for in large part by the gain in the peak-I radioactivity of the LD lipoproteins. Since the peak-I radioactivity is due to apo B, this provides strong confirmatory evidence for the formation of the LD lipoprotein from VLD lipoprotein in the preparation (Suri *et al.*, 1978). Moreover, since the recovery of the peak-I radioactivity is 91%, it is clear that little or no apo B leaves the circulation during the VLD-lipoprotein degradation.

There is an additional loss of VLD-lipoprotein radioactivity from peaks II and III in the circulation of the supradiaphragmatic rat beyond that which occurs during the incubations *in vitro*. Most of that additionally lost from peak II can be accounted for by the further rise in the radioactivity of the same peak in the HD lipoprotein. This would be consistent with a net transfer of peak-II apoprotein to the HD lipoproteins during the metabolism of the VLD lipoproteins in the supradiaphragmatic rat. Other workers (Weisgraber *et al.*, 1977; Kushwaha & Hazzard, 1978) have reported similar findings in intact animals.

Table 2. *Percentage of the radioactivity in peaks I, II and III of ^{3}H-labelled liver VLD-lipoprotein apoproteins present in each of the equivalent peak fractions of the lipoprotein apoproteins separated from plasma after the injection or incubation of liver VLD lipoproteins*

Preparations of ^{3}H-labelled liver VLD lipoproteins were either injected into the circulation of supradiaphragmatic rats or incubated in rat plasma at 37°C at the concentrations attained initially in the injected animals (see the Materials and Methods section). Lipoprotein fractions were separated from the plasma of the supradiaphragmatic animals and the incubated plasma after 75min. The apoproteins of the liver VLD lipoproteins and of the two groups of plasma lipoproteins were separated by gel filtration into three main peaks (peaks I, II and III) as described in the Materials and Methods section. From the elution profiles the percentage of the total radioactivity in each peak in the liver VLD lipoprotein that was accounted for by that present in each of the corresponding peaks of the plasma VLD, LD and HD lipoproteins was calculated (see the text and legends to Figs. 1–3). The values represent means (±S.E.M.) of three experiments.

		Percentage distribution of peak radioactivity of liver VLD-lipoprotein apoproteins in					
		Incubated rat plasma			Supradiaphragmatic-rat plasma		
Lipoprotein fraction	Peak ...	I	II	III	I	II	III
VLD		94±3	36±5	23±1	68±6	13±2	8±2
LD		5±2	3±1	4±1	23±4	6±3	5±1
HD		—	47±6	57±7	—	61±8	45±7
	Sum ...	99	86	84	91	80	58

However, in their studies no control incubations of VLD lipoproteins with plasma *in vitro* were carried out, and the observed changes could not therefore be ascribed definitely to the degradation of the VLD lipoprotein.

The additional loss of peak-III radioactivity from the VLD lipoproteins cannot be accounted for by a further rise in the peak-III radioactivity in the LD or HD lipoproteins; nor was there any increase in the radioactivity in the $d > 1.21$ infranatant fraction of the plasma of the supradiaphragmatic rat. It appears therefore that peak-III radioactivity, presumably apo C-protein radioactivity, is lost from the circulation. The report by Dory *et al.* (1978) that, when labelled VLD lipoproteins are perfused through the rat heart, their degradation is accompanied by the uptake of over 40% of the radioactivity by the heart tissue, would be consistent with this.

Net transfer of VLD-lipoprotein apoproteins during metabolism in the supradiaphragmatic rat

From the radioactivity data alone it is not possible to distinguish movements of apoproteins between lipoprotein classes that are due to exchange processes from those involving net movements of apoproteins from one class to another. In order to do this, direct measurements of the protein of peaks I, II and III were made and these were used to calculate the amounts present in the incubated plasma and in the plasma of the supradiaphragmatic rats. Such a comparison also takes account of the existence in the plasma of endogenous, as well as of injected, liver VLD lipoproteins.

The concentrations of the VLD-lipoprotein apoproteins of peaks I, II and III were all lower in the plasma of the supradiaphragmatic animals than in the incubated plasma (Table 3). This is as expected. However, the percentage decrease was much less for peak I (46%) than for peaks II and III (76% and 80% respectively). This is entirely consistent with the radioactivity data, and it can again be concluded that the residual VLD lipoprotein in the supradiaphragmatic animal is enriched in apo B.

The peak-I apoprotein that is lost from the VLD lipoproteins is largely accounted for in the LD-lipoprotein fraction. Both the direct protein estimations and the radioactivity data therefore support the view that apo B is transferred to the LD lipoproteins during the extrahepatic degradation of the VLD lipoproteins.

The data in Table 2 show that the greater loss of radioactivity from peak II of the VLD lipoprotein that occurred in the plasma of the supradiaphragmatic rat was largely accounted for by the greater gain of radioactivity in the HD lipoproteins. This was consistent with a net transfer of the peak-II apoproteins during the VLD-lipoprotein degradation. The data in Table 3 do not provide any evidence for such net transfer. However, the expected increase in the peak-II protein mass of the HD lipoprotein would be very small in relation to the total, and could well have remained undetected.

It has been concluded from the data in Table 2 that peak-III radioactivity, presumably associated with apo C proteins, was lost from the circulation during VLD-lipoprotein degradation (see above). The data in Table 3 do not, however, show any overall decrease in the total circulating peak-III proteins in the supradiaphragmatic preparation. Again this discrepancy may be accounted for by the size of the circulating peak-III protein pool, which is such that small movements of injected radioactive apoproteins out of the plasma could remain undetected. The small rise in the amount of peak-III protein in the HD lipoprotein of the supradiaphragmatic rat is not significant. Nevertheless, it was observed in all the experiments and could signify that a net movement of apo C

Table 3. *Mass of lipoprotein apoproteins in gel-filtration fractions derived from the plasma of supradiaphragmatic rats injected with VLD lipoproteins and from rat plasma incubated with VLD lipoproteins*

Preparations of liver VLD lipoporteins were either injected into the circulation of supradiaphragmatic rats or incubated in rat plasma as described in the legend to Table 2. VLD-, LD- and HD-lipoprotein fractions were separated from the plasma of the supradiaphragmatic animals, and the incubated plasma after 75min and the apoproteins of the lipoproteins were separated into three main peaks (I, II and III) as described in the Materials and Methods section. From determinations of the protein under peaks I, II and III of the different lipoprotein fractions, the plasma concentrations of the apoproteins in each peak were calculated. The values represent means (±S.E.M.) for three experiments.

	Plasma lipoprotein apoprotein concentration (mg/100ml) in					
Peak ...	I		II		III	
Lipoprotein fraction	Incubated rat plasma	Supradiaphragmatic-rat plasma	Incubated rat plasma	Supradiaphragmatic-rat plasma	Incubated rat plasma	Supradiaphragmatic-rat plasma
VLD	2.4±0.1	1.3±0.2	2.1±0.1	0.5±0.1	4.0±0.5	0.8±0.1
LD	4.0±0.6	5.0±0.5	0.9±0.1	1.2±0.1	1.6±0.2	1.8±0.1
HD	—	—	72.7±2.0	70.4±4.0	16.3±2.0	18.6±1.1

proteins to the HD lipoproteins is also associated with VLD-lipoprotein degradation.

Significance of LD-lipoprotein formation from VLD lipoprotein by the extrahepatic tissues

The results of the present study extend our previous work (Suri *et al.*, 1978) showing that the degradation of VLD lipoproteins by the supradiaphragmatic rat can proceed as far as the formation of complexes with the density characteristics of LD lipoproteins. In the rat, the LD-lipoprotein class displays considerable heterogeneity with respect to the apoprotein species present (see above). Nevertheless, under conditions in which most of the VLD-lipoprotein triacylglycerol is hydrolysed in the supradiaphragmatic animal, approx. 20% of the apo B of the injected VLD-lipoprotein radioactivity is transferred to the LD-lipoprotein class within 75 min (Table 2). When the endogenous VLD lipoprotein is also taken into account (Table 3), it appears that the transfer may be as high as 35%.

This evidence for a substantial capacity of the extrahepatic tissues to degrade VLD lipoproteins as far as LD lipoproteins is supported by work showing that the perfused rat heart has a similar capacity (Dory *et al.*, 1978). Such degradation will be accompanied in the circulation of the supradiaphragmatic rat by the concomitant movement of VLD-lipoprotein cholesterol and phospholipid to other plasma lipoprotein classes and may also require the action of plasma phosphatidylcholine–cholesteryl acyltransferase to bring about appropriate changes in the cholesterol/cholesteryl ester ratio of the lipoproteins (Lewis, 1977). In addition, as the present study indicates, it seems likely to involve transfers of arginine-rich apo C and perhaps other apoproteins from the VLD lipoproteins to other lipoprotein classes, as well as the loss of some apoprotein (particularly apo C) from the circulation.

There is nevertheless a considerable body of evidence to show that partial-degradation products of VLD-lipoprotein metabolism are rapidly taken up by the liver in the intact rat (see the introduction). It appears therefore that there may be two pathways for the degradation of VLD lipoproteins (and probably also of chylomicrons) that normally operate in concert in the intact animal (see also Faergeman *et al.*, 1975). Both would involve the removal of triacylglycerol by lipoprotein lipase action in the extrahepatic tissues. However, whereas the one studied here, when taken to its conclusion, would lead to the formation of lipoproteins with the density characteristics of LD lipoproteins, the other would involve the removal or modification of triacylglycerol-depleted VLD lipoprotein by the liver. This last process seems likely to predominate in the intact rat, since the half-life of VLD-lipoprotein apo B in the plasma is very short in this species (Eisenberg & Rachmilewitz, 1973; Fidge & Poulis, 1975; Faergeman *et al.*, 1975) and the plasma LD-lipoprotein concentration is extremely low. In man, the half-life of VLD-lipoprotein apo B is considerably longer (Eisenberg *et al.*, 1973; Berman *et al.*, 1978) and there appears to be little or no removal from the circulation by the liver (Sigurdsson *et al.*, 1975). Moreover, the plasma LD-lipoprotein concentration is much higher. The conversion of VLD lipoprotein into LD lipoprotein in the human may, therefore, be brought about solely by the metabolic activities of the extrahepatic tissues.

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