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FILTRATION OF CHYLOMICRONS BY THE LIVER MAY INFLUENCE CHOLESTEROL METABOLISM AND ATHEROSCLEROSIS

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Summary

A fenestrated endothelial lining of sinusoids in rat liver has been shown to separate chylomicrons of different sizes following their injection into the portal vein. This sieving may have physiological importance, since during low dietary fat intake some intestinal lipoproteins are probably small enough to contact liver cells, but during high dietary fat loads most chylomicrons are too large to pass through the filter and must first be degraded to smaller remnants.

The liver plays a central role in cholesterol metabolism since it catabolises dietary cholesterol which inhibits synthesis of cholesterol to be circulated as liver-derived very low density lipoproteins (VLDL) and low density lipoproteins. The sieving of chylomicrons, remnants and other lipoproteins by the sinusoidal endothelium of the liver may thus play an important role in lipid transport, affecting the balance of various lipoprotein moieties which in turn may affect the relationship of dietary lipids to various hyperlipidaemias and atherosclerosis.

Key words: *Lipoproteins — Thoracic duct lymph — Endothelium — Space of Disse — Dietary lipids*

Introduction

Fenestrae of about 100 nm diameter have been described within endothelial extensions separating sinusoidal blood from the space of Disse and liver paren-

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chymal cells of rats [1—3]. Wisse [2] suggested that these pores functioned as filters or sieve plates. This suggestion arose from the observations of Fraser et al. [4] who had noted that most chylomicrons (the lipoproteins which transport lipids from the gut) are less than 100 nm in diameter during low dietary fat absorption, but that their diameters increase markedly during high fat absorption.

An extension of this hypothesis was proposed to explain the preferential uptake of remnant chylomicrons by the liver [5]. These circulating lipoproteins of less than 90 nm in diameter are derived from chylomicrons after partial catabolism of their triglycerides by lipases in many organs such as heart and adipose tissue. They are smaller than their parent chylomicrons, containing relatively less triglyceride and more cholesteryl esters, and are rapidly removed from the circulation by the liver [6,7].

The object of this paper is to test the theory that the endothelial pores of the liver sinus do indeed act as a barrier to the majority of relatively large chylomicrons, while only smaller lipoproteins can enter the space of Disse to contact liver cells.

Possible effects of this sieving are considered in respect to the metabolism of lipids, in particular to the relationships between dietary, synthesised and circulating cholesterol. A hypothesis is presented that the separation of chylomicrons, their remnants and other lipoproteins by the liver filter may be a factor in the production of hypercholesterolaemia and atherosclerosis.

Materials and Methods

Collection and preparation of donor lipoproteins

Young male Wistar rats, weighing 150—200 g and starved overnight, were dosed intragastrically with 40 mg powdered cholesterol (USP), dissolved in 2 ml corn oil (high fat diet) or ultrasonicated in 2 ml water (low fat diet) 5 h before thoracic duct cannulation under ether anaesthesia [4]. During the following hour, 0.5—1.0 ml of lymph was collected, pooled and defibrinated.

For perfusion of livers to be examined by electron microscopy, a supernatant of chylomicrons and intestinal VLDL of $S_f > 20$ was separated by "infinite" ultracentrifugation of the lymph through saline of density of 1.006 g/ml [4,8,9]. To quantitate the trapping of lipoproteins by the liver, lymph-lipoproteins were labelled in vivo in their cholesterol or fatty acid moieties or both. High or low fat lymph was collected as before from donor rats fed 50—150 μCi radioisotopes evaporated onto 40 mg powdered cholesterol. In order to obtain a 1—2 ml sample, lymph was pooled from three identically fed rats. The isotopes used were [1α, 2α(n)-^{3}H]cholesterol with a specific activity of 112 mCi/mg, [4-^{14}C]cholesterol, 138 μCi/mg, [9, 10(n)-^{3}H]-oleic acid, 8.5 mCi/mg and [1-^{14}C]oleic acid, 220 μCi/mg (Amersham, United Kingdom).

Intestinal lipoproteins labelled concurrently in their cholesterol and fatty acid (mainly triglyceride) moieties were obtained from donor rats fed high and low fat diets containing both ^{3}H-cholesterol and ^{14}C-oleic acid. Their thoracic duct lymph was fractionated by preparative ultracentrifugation into chylomicrons ($S_f > 400$) and VLDL (S_f 20—400). The fractions were resuspended

by repeated passage through a 23 gauge needle in isotonic saline to the same volume as the original lymph [4,8,9]. These samples were used for experiments in Table 1. However, intestinal VLDL from rats fed a high fat diet were not used for perfusion since their radioactivity was too low to give meaningful results [8].

For experiments in Tables 2 and 3, donor lymph was not fractionated but only defibrinated. In these experiments each sample of lymph had been labelled in vivo with only one of the various isotopes.

Remnant chylomicrons were prepared from a method modified from that of other workers [6,7]. Four rats were dosed intragastrically with 2 ml corn oil in which had been dissolved 40 mg of cholesterol plus concurrent markers of ^{3}H-cholesterol and ^{14}C-oleic acid. After 5 h, at the peak of fat absorption [4], the rats were anaesthetised with ether and their portal veins and hepatic arteries ligatured in order to isolate their livers from the circulation. The abdominal wall was surgically repaired and the rats were allowed to recover from anaesthesia for a further 30 min. A second anaesthetic was then given and the rats exsanguinated from their distended portal veins proximal to the ligature. Pooled serum was obtained after clotting and lipoproteins of $S_f > 20$ were separated by ultracentrifugation.

Electron microscopy of donor lipoproteins

Chylomicrons, intestinal VLDL and the chylomicron remnants were resuspended in isotonic saline to the same volume as the original pooled lymph or serum samples from which they had been derived. Portions of these reconstituted samples were fixed with osmium and placed on carbon-coated grids for electron microscopy [4,8,9] (Figs. 1 and 2). The diameters of 200 constituent spherical lipid particles were measured from electron micrographs calibrated with catalase (Calbiochem.) or with a grating of 462.9 nm periodicity (Ladd, Burlington, Vermont, U.S.A.) or with both, in a similar manner to previous calculations [4,8,9].

Liver perfusion

Portal veins of ether-anaesthetised recipient male rats with livers of 7—10 g were surgically exposed. For the perfusion experiments depicted in Table 1, 0.25 ml of reconstituted fractions containing lipoproteins of various S_f values were injected directly into the portal vein via a 23 gauge needle. This injection was standardized as much as possible, and was maintained for 3 sec with an intact portal circulation. Within 3—5 sec the thorax was opened and the inferior vena cava and aorta cut to prevent further circulation, so making this perfusion as close as possible to a single pass. The portal vein was then cannulated and the liver flushed with 20 ml isotonic saline. The rate of all perfusions was 20 ml/min.

A similar method was used for the perfusion experiments in Table 2, except that lymph for injection was from a thoroughly mixed sample of whole lymph from two groups of donor rats. One group had been fed a high fat diet and the other a low fat diet. These two samples of approximately equal proportions were mixed together immediately before perfusion, and each of a group of recipient rats was injected with 0.25 ml of the mixed sample into each portal vein.

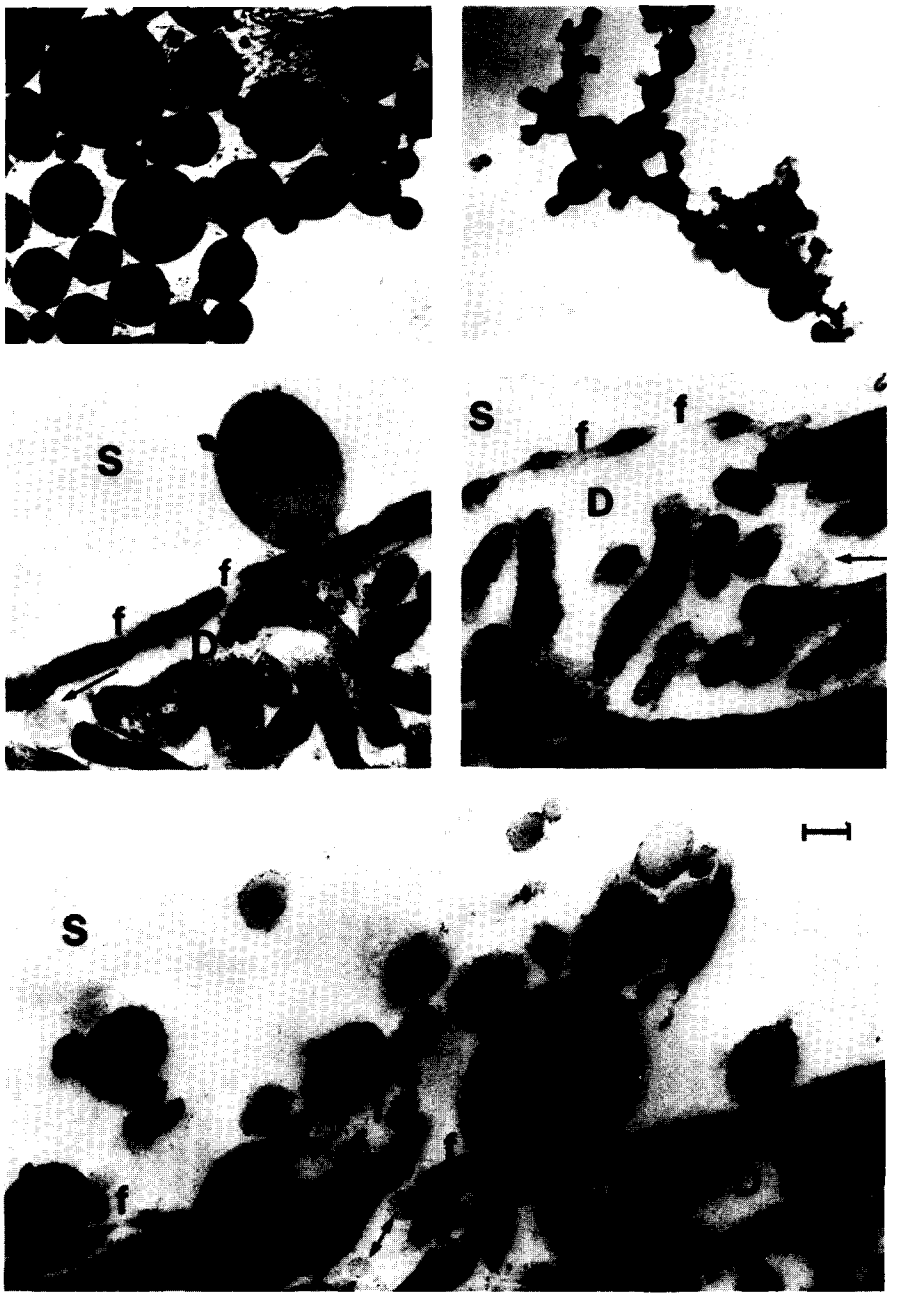

Fig. 1 shows chylomicrons and VLDL of $S_f > 20$ stained with osmium and placed on a carbon grid.

Fig. 2 shows remnant chylomicrons stained with osmium and placed on a carbon grid.

Fig. 3 shows a large chylomicron in the liver sinus and a small chylomicron amongst the microvilli of the hepatocytes in the space of Disse. The endothelial sieve separates the two.

Fig. 4 shows a small chylomicron remnant in the space of Disse.

Fig. 5 shows chylomicrons of all sizes in the liver sinus but only smaller chylomicrons in the space of Disse.

All electron micrographs are X55,300 magnification. S = liver sinus; D = space of Disse; f = fenestra; ↑ = small chylomicrons or remnants in space of Disse; bar = 100 nm.

TABLE 1

THE PERCENTAGE OF RADIOACTIVITY TRAPPED BY THE LIVER FOLLOWING A SINGLE PASS OF VARIOUS INDIVIDUAL CLASSES OF DOUBLY-LABELLED LIPOPROTEINS INJECTED INTO THE PORTAL VEIN

The lipoproteins were obtained from donor rats fed ^{3}H-cholesterol plus ^{14}C-oleic acid and their lymph or serum fractionated by preparative ultracentrifugation.

Exp.	Class of lipoprotein perfused through liver	n	% of radioactivity trapped by liver (mean ± SEM)	
			^{3}H-cholesterol	^{14}C-oleic acid
A	$S_f > 400$ (lymph chylomicrons high fat diet)	18	4.91 ± 0.41	5.15 ± 0.36
B	$S_f > 400$ (lymph chylomicrons low fat diet)	12	8.61 ± 1.39	8.73 ± 1.39
C	S_f 20—400 (lymph VLDL low fat diet)	12	11.14 ± 1.11	11.61 ± 1.00
D	$S_f > 20$ (serum remnants high fat diet)	4	22.47 ± 1.61	24.16 ± 1.89

For the experiments shown in Table 3, donor lymph was again a mixed sample from a high-fat and a low-fat fed donor group, but the method of injection into the portal vein differed from that in Table 2. The portal vein of the recipient rat was first cannulated and after the inferior vena cava and aorta had been severed, the livers were perfused with 20 ml of isotonic saline at 0°C or 37°C. Then 0.25 ml of mixed donor lymph, thoroughly mixed with 20 ml saline at the same temperature, was perfused at the same rate. The livers were finally flushed with a further 20 ml of saline at the same temperature. Each step followed the other as quickly as possible, the total procedure lasting about 5 min for each liver.

Livers to be sectioned for electron microscopy (Figs. 3—5) were similarly perfused with 20 ml isotonic saline containing a suspension of 0.5 ml lymph-lipoproteins of $S_f > 20$ (chylomicrons + VLDL) or serum remnants of $S_f > 20$. Livers were then fixed with 40 ml of cacodylate-buffered glutaraldehyde perfused into the portal vein at the same rate [2]. The final 20 ml of fixative was usually made up to contain a similar suspension of chylomicrons and VLDL or remnants as in the first saline perfusate in order to demonstrate lipid particles within blood sinuses. When plain fixative was used, only particles trapped in the space of Disse were seen, and the sinuses were flushed clear.

Electron microscopy of the liver

Immediately after perfusion-fixation, livers were removed and cut with a razor blade into 1 mm^3 blocks under buffered glutaraldehyde fixative. Blocks were washed in cacodylate buffer, post-fixed in buffered 1% osmium tetroxide for 30 min, washed in 10% acetone, stained with 2% aqueous uranyl acetate for 20 min, dehydrated through an acetone series and embedded in Spurr's resin. Sections of approximately 100 nm thickness were cut on an LKB Ultratome III or an LKB-Huxley Ultramicrotome, collected on collodion-coated 100 mesh copper grids, stained with lead citrate and examined in a JEM 100B electron

TABLE 2

THE PERCENTAGE OF RADIOACTIVITY TRAPPED BY THE LIVER FOLLOWING A SINGLE PASS OF WHOLE LYMPH CONTAINING RELATIVELY LARGE INTESTINAL LIPOPROTEINS COMPARED WITH THAT CONTAINING RELATIVELY SMALL LIPOPROTEINS

Both samples were mixed and injected into the portal vein. The differently sized lipoproteins were obtained from donor rats fed high fat (large lipoproteins) or low fat (small lipoproteins) diets labelled with ^{3}H or ^{14}C cholesterol or oleic acid.

Exp.	Lipid labelled	Lipoprotein labelled		n	% label trapped (mean ± SEM)		% small trapped / % large trapped (mean ± SEM)	Paired *t*-test *P*<
		large	small		large	small		
A	cholesterol	^{14}C	^{3}H	5	5.15 ± 0.44	6.67 ± 0.63	1.30 ± 0.07	0.02
B	cholesterol	^{3}H	^{14}C	4	4.53 ± 1.19	8.69 ± 1.62	2.37 ± 0.04	0.02
C	cholesterol	^{14}C	^{3}H	4	7.91 ± 0.91	11.88 ± 1.42	1.50 ± 0.02	0.01
D	oleic acid	^{14}C	^{3}H	4	5.87 ± 0.85	10.99 ± 1.64	1.91 ± 0.23	0.05
E	oleic acid	^{3}H	^{14}C	5	5.76 ± 0.86	11.94 ± 1.53	2.11 ± 0.11	0.01
Combined	—	—	—	22	5.81 ± 0.45	9.97 ± 0.77	1.83 ± 0.13	0.001

microscope at 80 kV. As for the chylomicrons, VLDL and remnants prepared on carbon grids, dimensions were calculated for 100 fenestrae and 100 of each type of lipid particle found in the liver sinuses and in the spaces of Disse (Figs. 3, 4 and 5).

Measurement of labelled lipids trapped by perfused livers

Recipient livers, previously perfused with labelled chylomicrons, and control livers perfused only with saline were homogenised; their lipids were extracted in chloroform : methanol (2 : 1, v/v) and washed with excess water by the method of Folch et al. [10]. Lipid extracts from control livers were used as blanks and as receptacles for known aliquots (0.01 ml) of the various perfusates from donor rats. Control livers were also used as vehicles for internal standards of the various isotopes, in order to construct efficiency and quenching correlation curves for the calculation of dpm in both ^{3}H and ^{14}C channels from external standard ratios (Packard Instruction Manual 2129). Lipid extracts from recipient livers were used to measure the amount of injected radioactivity that was trapped in the liver after perfusion of various donor samples.

Scintillation methods were as follows. Lipid extracts were evaporated in scintillation vials, to which were then added 1.2 ml water and 17 ml of a scintillant fluid comprising Triton-X-100, toluene, PPO and dimethyl-POPOP [5]. The vials were counted for 100 min at 8°C in a Packard Model 3330 liquid scintillation counter and the counts converted to dpm using the correlation curves previously constructed.

The quantity of radioactivity perfused through the liver in each group of experiments varied because of the use of different amounts and types of isotope and because of the different lipoprotein fractions perfused. The mean radioactivity per isotope per sample for perfusion was 111,000 dpm. The results were tabulated as a percentage of perfused dpm trapped by each liver.

TABLE 3

THE EFFECT OF TEMPERATURE ON THE TRAPPING OF LIPOPROTEINS BY THE LIVER

As in Table 2, large and small lipoproteins were injected into the portal vein. However, each liver had been previously flushed with saline at 0°C or 37°C and the lymph samples were mixed and suspended in saline at the same temperature. All large lipoproteins were labelled with ^{3}H and small with ^{14}C.

Exp.	Lipid labelled	0°C					37°C				
		n	% label trapped (mean ± SEM)		% small trapped / % large trapped (mean ± SEM)	paired *t*-test *P*<	n	% label trapped (mean ± SEM)		% small trapped / % large trapped (mean ± SEM)	paired *t*-test *P*<
			large	small				large	small		
A	oleic acid	4	1.30 ± 0.23	1.80 ± 0.25	1.46 ± 0.15	0.025	3	1.53 ± 0.18	2.76 ± 0.31	1.81 ± 0.04	0.020
B	oleic acid	4	1.36 ± 0.45	2.30 ± 0.77	1.65 ± 0.10	0.100	4	2.39 ± 0.68	3.41 ± 0.67	1.54 ± 0.10	0.001
C	cholesterol	4	1.35 ± 0.20	3.02 ± 0.71	2.14 ± 0.30	0.050	4	1.23 ± 0.15	2.56 ± 0.40	2.07 ± 0.15	0.050
Combined	—	12	1.33 ± 0.18	2.37 ± 0.39	1.75 ± 0.14	0.005	11	1.73 ± 0.30	2.92 ± 0.32	1.80 ± 0.10	0.001

Results

Diameters of chylomicrons and lipoproteins in perfusate

The mean diameter of chylomicrons of $S_f > 400$ from the lymph of rats fed a high fat diet was 143 nm with a 95 percentile range of 60—300 nm, while the mean from rats fed a low fat diet was 93 nm (60—160 nm). The mean diameter of intestinal VLDL of S_f 20—400 from rats fed either high or low fat diets was 54 nm (40—160 nm). The mean diameter of chylomicron remnants of $S_f > 20$ from the serum of rats fed a high fat diet was 45 nm (15—135 nm). These diameters were similar to previous measurements [4,6–9].

The fraction containing chylomicrons plus intestinal VLDL ($S_f > 20$) showed, as expected, an even wider range of diameters, varying from small intestinal VLDL to the largest of chylomicrons. The mean diameter was 129 nm with a 95 percentile range of 40–320 nm.

Diameters of fenestrae, chylomicrons and lipoproteins in the liver

The occurrence of endothelial sieve plates with pores of approximately 100 nm [2] was confirmed, and the mean diameter of the pores was 92 nm (95 percentile range 36—215 nm). These did, indeed, appear to filter chylomicrons and lipoproteins according to size (Figs. 3, 4 and 5).

Following the perfusion of chylomicrons and VLDL ($S_f > 20$) of widely varying diameters, only small particles were seen within the space of Disse (mean 61 nm, 95 percentile range 15—137 nm), whereas large chylomicrons were also present within the sinuses (mean 183 nm, 46—632) (Figs. 3 and 5). Remnant chylomicrons with a mean diameter of 46 nm (31–107) were observed to have apparently passed the endothelial sieve plate and to have come to lie within the space of Disse (Fig. 4).

Quantitation of labelled lipoproteins trapped by the liver

Table 1 shows the percentage of radioactivity of different classes of doubly labelled lipoproteins trapped in the liver after perfusion of a single class of lipoproteins. It can be seen that the lipoproteins of large diameter were not trapped to the same extent as those of smaller diameter; chylomicrons of $S_f > 400$ showing the least trapping, and chylomicron remnants the most. It can also be seen that both ^{3}H-cholesterol and ^{14}C-oleic acid were trapped to approximately the same extent within each individual class of lipoproteins. The oleic acid mainly represented triglyceride, since > 80% of these counts were shown to be in this fraction by thin layer chromatography [5].

A two-way analysis of variance was impossible due to considerable variations within each group, so the non-parametric technique of Kruskal-Wallis was employed for statistical evaluation. This test showed highly significant differences between the four groups of lipoprotein classes ($H = 48.8$, d.f. = 3, $P < 0.001$). Using procedure 1 of the method of Dunn, differences were shown between groups A and B, C and D. Differences between B and C just failed to reach significance at a 5% level. However, pairwise differences between A, B, C and D reached the 10% level. Since this test is quite conservative, a value of $P < 0.10$ can probably be considered significant [11].

Table 2 shows the percentage of radioactivity trapped by livers perfused with

a mixture of lymph from donor rats fed a high fat diet with resulting comparatively large labelled lipoproteins, and from donors fed a low fat diet with resulting comparatively small lipoproteins. It can be seen that irrespective of the isotope used, significantly more radioactivity was trapped from small lipoproteins than from large lipoproteins.

Table 3 shows that the preferential trapping of labelled lipid in small lipoproteins compared to that in large lipoproteins occurs whether livers are perfused at 0°C or 37°C. There was no significant difference in this apparent sieving effect at these different temperatures ($P > 0.995$).

In each of the 45 livers separately perfused in the experiments shown in Tables 2 and 3, a higher proportion of radioactivity was trapped from small lipoproteins than from large lipoproteins. The possibility of this being a chance occurrence is therefore 1 in 2^{45} (1 in 3.5×10^{13}).

Discussion

Our results, both from electron-microscopic observation and from quantitation of the trapping of labelled lipoproteins of different sizes, support the concept of an endothelial sieve separating sinusoidal blood from the space of Disse and the hepatocytes. The fact that differential trapping of lipoproteins occurred at 0°C as well as at 37°C (Table 3) and that radioactivity from both cholesterol and fatty acids within each lipoprotein group were equally trapped (Table 1), make it unlikely that enzymes such as hepatic lipase play an active role in this initial trapping of lipoproteins by the liver. These findings are compatible with a physical or mechanical role, such as sieving of intact lipoproteins.

The use of naturally occurring large and small lipoproteins obtained from the lymph of rats fed high and low fat diets, labelled by different isotopes and injected concurrently into the liver (Tables 2 and 3), overcame some technical difficulties experienced in experiments shown in Table 1. These included unevenness of perfusion, variations in liver weight, and possible alteration of lipoproteins by ultracentrifugation.

The liver sieve may have a physiological role in the catabolism of ingested triglycerides. During fat absorption, when most chylomicrons are large [4], it has been noted that, whereas most absorbed cholesterol is removed from the blood circulation by the liver, most triglycerides are removed by peripheral tissues [6]. During starvation or low dietary fat loads, however, the chylomicrons entering the blood circulation from thoracic duct lymph are smaller [4]. Although many are probably converted to remnants by lipases, it is possible that some do contact the liver cells direct. After contact with receptor sites on the microvilli of the hepatocytes, chylomicrons or remnants are probably then catabolised [12].

Sieving may also play a role in the metabolism of cholesterol. Cholesterol, when fed with a high dietary intake of triglycerides, is absorbed mainly in large chylomicrons. However, when fed with a low intake of triglycerides it is absorbed mainly in small chylomicrons and still smaller intestinal VLDL [8]. A recent paper [13] has shown a greater delay in the appearance in the liver of cholesterol from large chylomicrons than from small chylomicrons. The saturation and type of dietary triglycerides also alter the size and composition of

chylomicrons transporting absorbed cholesterol [8,14,15].

The first site of "recognition" by the liver of small remnant chylomicrons and "rejection" of larger parent chylomicrons is suggested by us to lie in the endothelial sieve. Felts et al. [16] have proposed an alternative or perhaps complementary explanation based on "recognition" of remnants by hepatocytes, brought about through increased lipoprotein lipase and a resulting decreased triglyceride content. We have not examined the eventual catabolism of lipoproteins by the liver but only their initial trapping.

A place for the liver sieve in the pathogenesis of atherosclerosis is suggested by us as follows.

Although the role of dietary lipids in human atherogenesis is still controversial there is no doubt of its importance in experimental animals. Increasing dietary triglycerides increases chylomicron size, and increases their relative content of diffusible free cholesterol on their surface to non-diffusible cholesteryl esters in their core [5,8]. The endothelial filter in the liver prevents large chylomicrons contacting liver cells until they have been catabolised to smaller remnants. During this process some of the diffusible free cholesterol may reach arterial walls, there to be esterified, so leading to arterial lipid deposition [17]. Eventually smaller remnants reach the liver but, being by now depleted of much dietary cholesterol, their inhibition of cholesterol synthesis [18] would probably be minimal.

On the other hand, when fed with little triglyceride, cholesterol is transported in small chylomicrons mainly as cholesteryl esters. Some of these small chylomicrons directly pass the liver filter, and those degraded to smaller remnants are unlikely to lose much of their non-diffusible cholesteryl esters. Dietary cholesterol would thus reach the liver to inhibit cholesterol synthesis and preserve cholesterol homeostasis. Cholesterol in low density lipoproteins, which is probably originally synthesised by the liver, has been shown to cause multiplication of arterial cells [19].

Thus, atherosclerosis, a disease distinguished by cholesterol deposition and proliferation of arterial cells, may partly be explained by the inability of the large chylomicrons seen after a high fat diet, or perhaps large remnants, to pass the liver sieve.

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