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HIGH PERFUSION PRESSURE DAMAGES THE SIEVING ABILITY OF SINUSOIDAL ENDOTHELIUM IN RAT LIVERS

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Summary.—Fenestrated endothelium lining liver sinusoids forms an ultrastructural sieve between blood and hepatocytes which at physiological perfusion pressures has previously been shown to shield hepatocytes from large triglyceride-rich chylomicrons. In the study reported in this paper, enlargement of endothelial fenestrae at high perfusion pressures has been confirmed and a concurrent increase in trapping of large chylomicrons by the liver noted. These findings suggest the importance of employing physiological perfusion pressures in studies designed to examine hepatic lipoprotein metabolism and also suggest a possible mechanism in the pathogenesis of fatty change seen in the "nutmeg" liver of chronic venous congestion.

SIEVE PLATES formed by extensions of endothelial cells which separate sinusoidal blood from the space of Disse and hepatocytes of rat livers contain multiple fenestrae or pores about 100 nm in diameter which have been suggested to sieve lipoproteins of different sizes (Wisse, 1970). This suggestion was prompted by the findings of Fraser, Cliff and Courtice (1968), who had noted that most chylomicrons (the triglyceride-rich lipoproteins which transport lipids of intestinal origin) are less than 100 nm in diameter during low dietary fat absorption, but that their diameters increase markedly during high fat absorption.

The shielding of the space of Disse and hepatocytes from large chylomicrons, but the passage of smaller chylomicrons through the sinusoidal endothelial filter, has been confirmed in the *in vivo* liver in normal rats, both by electron microscopy and by the trapping of isotopes after injection into the portal vein of labelled chylomicrons of differing sizes (Fraser, Bosanquet and Day, 1978; Naito and Wisse, 1978). This sieving, however, was not noted by other workers perfusing the rat liver with chylomicrons of varying sizes (Sherrill and Dietschy, 1978). A possible explanation of the different results in the sieving of chylomicrons may lie in the findings of Nopanitaya *et al.* (1976), who noted enlargement of pores in hepatic sinusoids after venous outflow had been obstructed for several hours. They suggested either pressure or hypoxia as a cause of this enlargement.

The object of this present study is to determine whether increased pressure of perfusion of portal veins acutely enlarges the size of pores within the sinusoidal endothelium, so altering its ability to sieve chylomicrons.

MATERIALS AND METHODS

The effect of increased portal perfusion pressures on the diameters of pores in the fenestrated hepatic sinusoidal endothelium and their ability to sieve chylomicrons of different sizes was compared with those perfused at physiological pressures. The diameters of pores were measured by electron microscopy, and variations in the trapping within the livers of

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large and small chylomicrons, differentially labelled in their triglyceride moieties and concurrently perfused through the livers, were compared by liquid scintillation. Methods used were similar to those previously described for livers perfused at physiological pressures (Fraser *et al.*, 1978).

Experimental animals.—All rats in the experiments were 16 weeks old female Wistars weighing 280–310 g, allowed free access to water and commercial rat pellets.

All operative procedures were performed under open ether anaesthesia.

Perfusion techniques.—For single pass *in situ* perfusions the portal vein was cannulated and isotonic saline infused at a constant rate of 10 ml/min, which is about half the physiological rate of flow of 4 ml/g of liver/min (Ryan *et al.*, 1978). For livers to be perfused at low pressures, the inferior vena cava was bisected close to the diaphragm immediately before perfusion, while for those to be perfused at higher pressures the vena cava was first clamped until 10 ml or 20 ml saline had been infused, after which the vena cava was cut.

For continuous re-cycling perfusion, livers were perfused *in situ* by the method of Hems *et al.* (1966) for 10 min. The perfusate consisted of Krebs–Ringer buffer, pH 7·4, containing Ficoll-70, 30 g/l (Pharmacia, Sweden), glucose 5·6 mmol/l and sodium bicarbonate 25 mmol/l. The perfusate was gassed with oxygen: carbon dioxide, 95: 5. The pressure of perfusion at the portal vein was 15 mmHg. All perfusates were at 37°.

Pressure recordings.—Portal pressure was measured *via* a 25-gauge needle inserted into the portal vein. This needle was attached to a saline-filled Portex manometer tube which led to a Bell and Howell 4-327 Pressure Transducer. Pressures were recorded on a Devices 8 Channel Recorder. Pressures were measured in 6 normal rats, 6 rats cannulated and infused with saline at 10 ml/min with the inferior vena cava previously bisected, 6 rats infused with 10 ml saline before bisection and 6 rats infused with 20 ml saline before bisection.

Electron microscopy.—Following the previously mentioned perfusions, those livers for electron micrography were perfused with 40 ml cacodylate-buffered gluteraldehyde, also at the rate of 10 ml/min. For transmission electron microscopy immediately after perfusion-fixation livers were cut with a razor blade into $1mm^3$ 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 (UA) for 20 min, dehydrated through an alcohol series and embedded in Spurr's resin. Sections approximately 100 nm thick were cut on an LKB Ultratome III, collected on collodion-coated 100 mesh copper grids, stained with lead citrate and examined in a JEM 100B electron microscope at 80 kV.

Transmission electron micrographs of fenestrated sinusoidal endothelium at known magnification, calibrated against a grating of periodicity 462·9 nm (Ladd, Burlington, Vermont, U.S.A.), were compared with the livers of rats perfused at different pressures. The diameters of a large number of pores were measured from each rat and the results shown in Table I and Fig. 1.

For scanning electron microscopy blocks approximately 5 mm × 5 mm × 2 mm in size taken from perfusion-fixed livers were post-fixed and dehydrated as for transmission electron microscopy, omitting the UA staining step. Blocks were transferred (under alcohol) to a Polaron E3000 critical point drying apparatus and dried in carbon dioxide. Following sputter coating with gold in a Polaron E5100 unit, specimens were viewed in an ISI40 SEM operated at 15 kV in the secondary electron mode.

Preparation of large and small chylomicrons.—The preparation of large and small intestinal lipoproteins depends on previously reported findings that their size depends on an animal's dietary fat load. Triglycerides from the gut are transported *via* thoracic-duct lymph to the blood stream mainly in large intestinal chylomicrons with diameters greater than 100 nm (large chylomicrons) when fed with a high fat diet, but in smaller lipoproteins (small chylomicrons and intestinal very low density lipoproteins) when fed with a low fat diet (Fraser *et al.*, 1968; Fraser and Courtice, 1969).

To obtain labelled large chylomicrons, rats were fed a high fat diet containing ^3H-labelled lipid. 150 μCi of ^3H-oleic acid (9, 10n-^3H-oleic acid, sp. act. 8·5 mCi/μg, Amersham, U.K.) were evaporated on 20 mg powdered cholesterol (USP) which was then dissolved in 2 ml corn oil. Three donor rats, starved overnight, were each dosed intra-oesophagically with this high-fat diet 3 h before cannulation of their thoracic duct. Lymph was collected for 1 h and approximately 3 ml was pooled. The resulting pooled lymph contains mainly large chylomicrons (mean diameter 143 nm with a mid-95 percentile range of 60–300 nm) with more than 80% of their ^3H-oleic acid incorporated in their triglyceride moiety (Fraser *et al.*, 1978).

To obtain labelled small intestinal lipoproteins, rats were fed a low-fat diet containing C^{14}-labelled lipid. 80 μCi of ^{14}C-oleic acid (1-^{14}C-oleic acid, sp. act. 8·5 mCi/μg, Amersham, U.K.) were evaporated on 20 mg powdered cholesterol which was then homogenized in 2 ml of water by ultrasound. Three donor rats, starved overnight, were dosed with this low-fat diet and approximately 1 ml of pooled thoracic-

duct lymph collected as before. The resulting lymph contains mainly small chylomicrons (mean diameter 93 nm with a mid-95 percentile range of 60–100 nm) and intestinal very-low-density lipoproteins (54 nm, 40–160 nm) with more than 80% of their ^{14}C-oleic acid now incorporated in their triglyceride moiety (Fraser *et al.*, 1978).

Perfusion of labelled chylomicrons.—After defibrination and immediately before perfusion, the pooled whole-lymph samples from donor rats of high- and low-fat diets were thoroughly mixed. 0·1 ml of the mixed lymph was thoroughly mixed in 20 ml isotonic saline which was then perfused at a rate of 10 ml/min through various livers now with bisection of the inferior vena cava, but which had been previously subjected to various pressure of perfusions as already described. After perfusion with labelled chylomicrons, the livers were flushed with 40 ml of isotonic saline also at 10 ml/min (Fraser *et al.*, 1978).

Measurement of labelled triglycerides trapped by perfused livers.—The proportion of radioactivity from the large chylomicrons (^{3}H) to small chylomicrons (^{14}C) was compared in the mixed lymph perfused through livers with that trapped in livers previously subjected to varying pressures of perfusion. This was achieved by liquid scintillation of lipid extracts from livers and perfusate by concurrently counting ^{3}H and ^{14}C and converting counts to dpm after corrections for quenching (Fraser *et al.*, 1978).

RESULTS

The mean portal pressure recorded in normal rats was 8 mmHg (range 4–12 mmHg), and in rats perfused following bisection of the inferior vena cava was 7 (5–12) mmHg. In those perfused with the inferior vena cava clamped, portal pressures peaked at 20 (15–23) mmHg after perfusion with 10 ml of isotonic saline and at 34 (20–51) mmHg after perfusion with 20 ml of isotonic saline. As stated in the METHODS section, for continuous recycling perfusion the head of pressure was 15 mmHg.

Table I and Fig. 1 show the diameter of pores, as measured by transmission electron microscopy, increasing from a median of 100 nm in those perfused at a pressure of 7 mmHg to 240 nm at one of 34 mmHg. In the 10-min continuous recycling perfusion experiments at 15 mmHg, the median diameter of pores was intermediate at 160 nm. The 3 distributions were shown to be significantly different ($P < 0·005$) using a 2-sided k-sample Smirnov test. This test required equal sample series, so all were randomly reduced to 116 observations.

TABLE I.—*A comparison of the distribution of diameters of pores within hepatic sinusoidal endothelium, as measured by transmission electron microscopy, from rat livers perfused at different pressures. Those at 15 mmHg pressure were continuously perfused* in vitro *for 10 min, while those at 7 and 34 mmHg were subjected to single-pass perfusion*

Pressure of perfusion (mmHg)	n		Diameter of pores (nm)		
	Rats	Pores	Median	Mean	Mid 95% range
7	5	186	100	117	40–260
15	2	164	160	193	60–600
34	3	116	240	386	80–1300

Fig. 2 shows typical scanning electron micrographs of hepatic sinusoids perfused at pressures of 7 mmHg and 34 mmHg. From this and from Fig. 1 it is apparent that many large fenestrae are seen after high pressures of perfusion, so increasing the exposure of the space of Disse and microvilli of the hepatocytes to the sinusoidal lumen.

Table II shows the variations in trapping by the livers of radioactivity from perfused labelled large and small chylomicrons. Whereas the ratio of radioactivity in large to small chylomicrons was 25·6 in the perfusate, the median of the ratios of those trapped in livers perfused at 7 mmHg was only 8·1. At perfusion pressures of 20 mmHg the median of the ratios increased to 14·4, while at 34 mmHg the median had increased to 22·9. These results are highly significant ($P < 0·001$ by Kruskal–Wallis test on the data giving a χ^2 value of 45·6 with 2 degrees of freedom) and indicate a decreased sieving ability of sinusoidal endothelium when subjected to high pressures of perfusion.

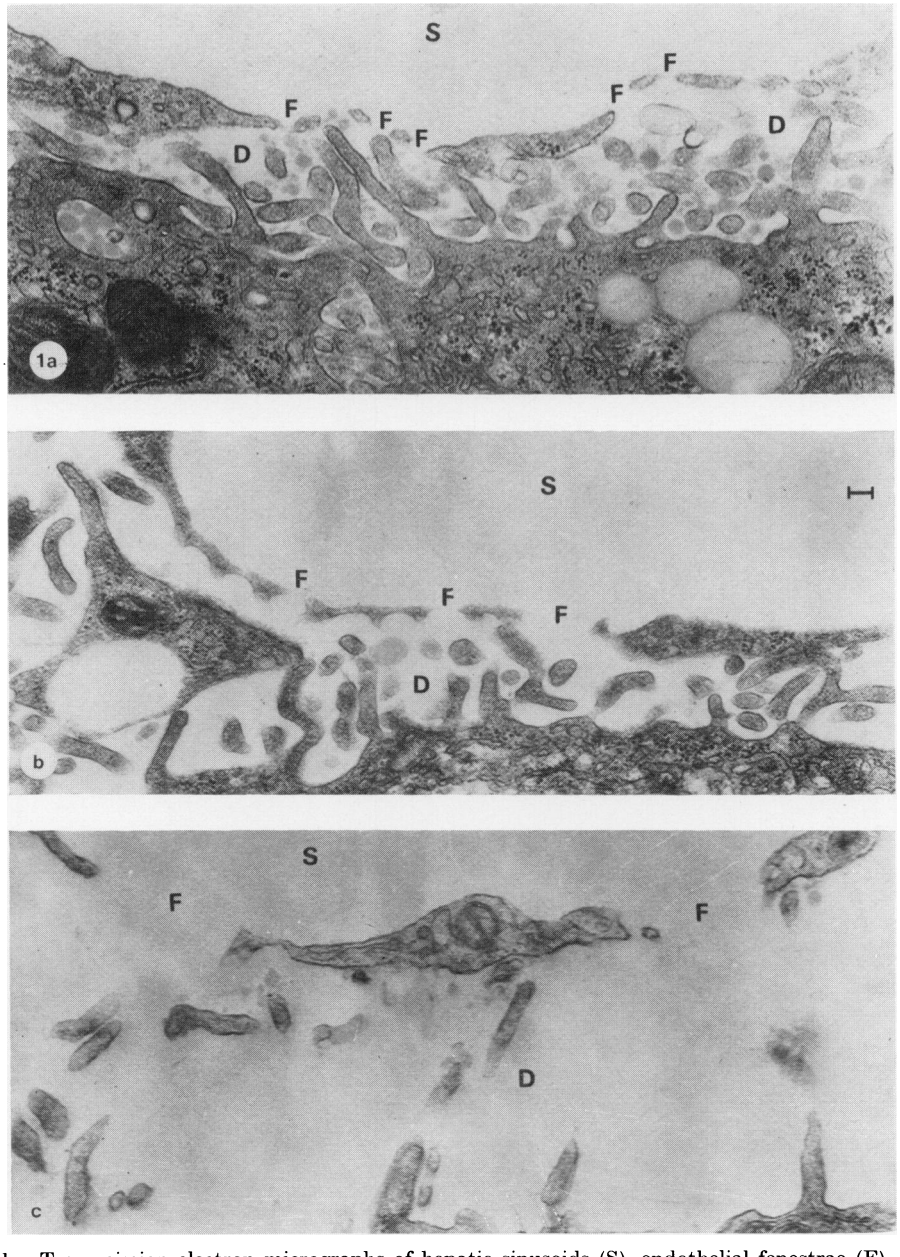

FIG. 1.—Transmission electron micrographs of hepatic sinusoids (S), endothelial fenestrae (F), and space of Disse (D) from rat livers perfused at 7 mmHg (a), 15 mmHg (b) and 34 mmHg (c) portal pressures, showing progressive increase in size of fenestrae. The bar represents 100 nm (mag × 27,000).

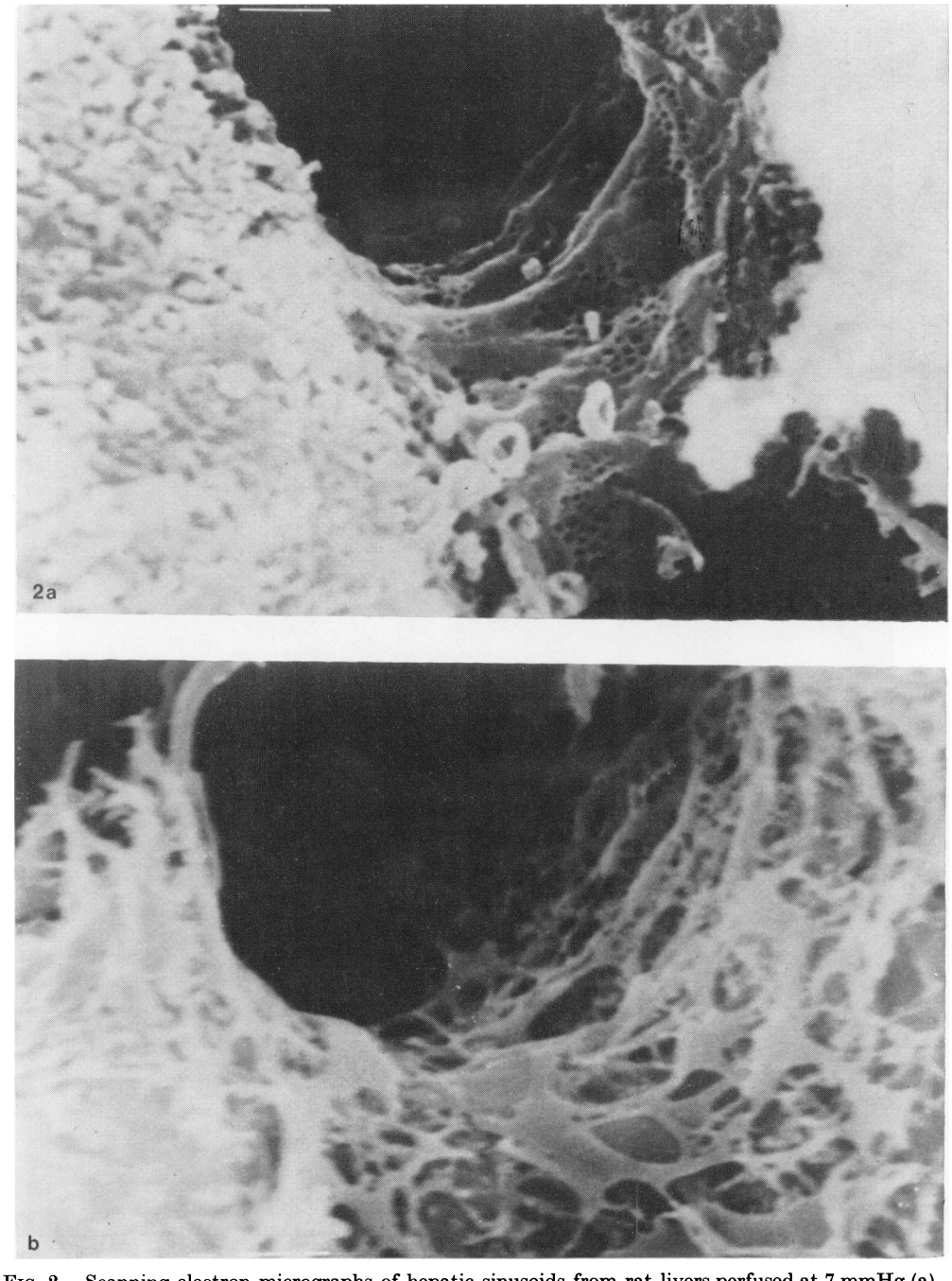

FIG. 2.—Scanning electron micrographs of hepatic sinusoids from rat livers perfused at 7 mmHg (a) and 34 mmHg (b) portal pressures. Small fenestrae are present in the endothelium of the top sinusoid, but gross dilatation or damage is present in the bottom endothelial fenestrae. The long bar represents 1000 nm (mag × 12,300).

TABLE II.—*The ratios of dpm in ^{3}H to ^{14}C trapped in livers following single-pass perfusion at different pressures through the portal vein of a mixture of lymph from donor rats containing mainly large chylomicrons labelled* in vivo *with ^{3}H-oleic acid and small chylomicrons labelled with ^{14}C-oleic acid*

	Perfused into livers	Trapped by livers at various pressures (mmHg)		
		7	20	34
Mean	25·6	8·2	14·4	22·8
Median	25·6	8·1	14·4	22·9
Range	—	7·2–9·4	12·5–16·2	22·2–23·2
n	1	8	8	4

DISCUSSION

The concurrent enlargement of endothelial pores and the increase in trapping of large chylomicrons when livers are perfused at high pressures adds support to the hypothesis that at physiological perfusion pressures the fenestrated sinusoidal endothelium acts as a mechanical sieve to lipoproteins of different sizes (Fraser *et al.*, 1978). If the reported preferential trapping of small compared to large chylomicrons perfused through livers at physiological pressures were a vital process, due to such factors as surface recognition, enzyme action or phagocytosis, it would seem unlikely to be almost abolished by increased pressures of perfusion (Table II).

The present paper has answered two questions posed by Nopanitaya *et al.* (1976), who have previously shown an increase in pore size of fenestrated sinusoidal endothelium following chronic hepatic venous obstruction. Our acute experiments have shown the increase in pore size to be related to increase in perfusion pressure rather than hypoxia. The dilated liver sieve has also been shown to have a decreased ability to seive chylomicrons of different sizes. Like these authors we believe from the evidence of our scanning electron micrographs (Fig. 2) that large fenestrations represent damaged endothelial extensions or sieve plates.

Since high pressures of perfusion (15, 20 and 34 mmHg) have been shown in this paper to dilate or damage the liver sieve and decrease its ability to sieve chylomicrons, it would seem prudent to confirm the integrity of endothelial sieve plates in future experiments designed to explore hepatic lipoprotein metabolism by perfusion techniques. Kunkel and Eisenmenger (1949) described portal pressures in rats of 7–12 mmHg as physiological, while Ryan *et al.* (1978) measured pressures of the order of 4 mmHg. In the present study pressures of 7–8 mmHg have been recorded both *in vivo* and during perfusion with the vena cava bisected. Pressures generally employed during liver perfusion have been higher; *e.g.* Miller *et al.* (1951) employed pressures of 15–18·5 mmHg, Hems *et al.* (1966) 15 mmHg and Sherrill and Dietchy (1978) 11 mmHg.

The perfusion of chylomicrons directly into portal vein is admittedly unphysiological, but has been used in this paper to demonstrate the sieving properties of sinusoidal endothelium. As stated previously, the use of naturally occurring large and small chylomicrons obtained from lymph or donor rats fed high- and low-fat diets labelled with different isotopes and injected concurrently into the liver overcomes some technical difficulties in the quantitation of trapping such as unevenness of perfusion, variations in liver weights and possible alterations of chylomicrons by ultracentrifugation (Fraser *et al.*, 1978).

The catabolism of circulating chylomicrons in normal rats is a two-stage process. Comparatively large chylomicrons transporting most dietary lipids after a fatty meal are first depleted of most of their triglycerides by lipoprotein lipase in tissues other than the liver. The resulting smaller triglyceride-depleted but cholesterol-rich chylomicron remnants are then rapidly removed from circulation by the liver (Redgrave, 1970). Anderson, Nervi and Dietschy (1977) have shown that cholesterol in large chylomicrons ciculates for a considerably longer period than that in small chylomicrons before its

uptake by the liver. It has been suggested that in normal rats the liver sieve shields hepatocytes from excess triglycerides in the form of large chylomicrons or large chylomicron remnants, but allows passage of smaller triglyceride-depleted remnants (Fraser *et al.*, 1978).

The findings in this paper have demonstrated that the ultrastructural liver sieve has been rendered less efficient at higher pressures of perfusion. It is tempting to speculate that when sinusoids are congested, as for example in chronic venous congestion, triglyceride-rich chylomicrons or large partially catabolized remnants may become trapped in the space of Disse, so contacting hepatocytes. The single-pass perfusion experiments used in this study only demonstrate the initial trapping of chylomicrons within the liver and do not examine their eventual catabolism. However the increased presentation of triglycerides to hepatocytes might be one factor in the pathogenesis of fatty change seen in the "nutmeg" liver of chronic venous congestion.

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