Application of simultaneous spleen and liver perfusion to the study of reverse cholesterol transport

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1. A new method to isolate and perfuse the rat spleen and liver simultaneously with a common blood perfusate at high haematocrit was developed. The spleen was pre-labelled with [^3H]cholesterol, enabling reverse cholesterol transport from an extrahepatic tissue to the blood and thence to the liver and bile to be studied in a single preparation *in vitro*. 2. The presence of the liver significantly increased the release of [^3H]cholesterol from the spleen by 15%, compared with experiments where the spleen was perfused alone. 3. There was a substantial release of [^3H]cholesterol and cholesterol mass from the spleen to serum lipoproteins, the majority (80%) to high-density lipoprotein (HDL), in which cholesteryl ester accumulated. 4. The HDL

subfractions HDL₂ and HDL₃ (d 1.085–1.250) were most important for removal of cholesterol from the spleen, whereas HDL₁ and HDL₂ (d 1.050–1.125) were important for delivery of cholesterol to the liver, a net uptake of cholesteryl ester occurring only from these fractions. 5. Approximately half of the [^3H]cholesterol released by the spleen was recovered in erythrocytes. Also, in experiments utilizing a lipoprotein-free perfusate containing erythrocytes, a substantial quantity of [^3H]cholesterol was transported and/or exchanged into the liver and bile, indicating that erythrocytes play an important role in the equilibration of unesterified cholesterol between the tissues.

INTRODUCTION

In order for tissues to maintain cholesterol homeostasis, it is necessary for cholesterol to be transported from extrahepatic tissues to the liver for excretion, a process known as reverse cholesterol transport (Glomset, 1968). This process involves several stages, (i) the release of unesterified cholesterol from extrahepatic tissues to the components of the blood, (ii) a possible esterification or transfer of the cholesterol to other blood constituents, (iii) uptake by the liver, and (iv) a proportion of the uptake being excreted in bile as unesterified cholesterol or as bile acid. We have developed a combined spleen–liver preparation which enables investigations of the interaction of all these stages of reverse cholesterol transport, which has not been studied previously in a single system *in vitro*. Studies *in vivo* suggest that high-density lipoprotein (HDL) plays a major role in reverse cholesterol transport. Infusions of HDL can inhibit the development of atherosclerosis in rabbits (Badimon et al., 1989). Also, a cholesterol enrichment and conversion into less-dense HDL fractions is observed when human HDL₃ is injected into rats (Gavish et al., 1987), and into the circulation in rabbits after loading the tissues with cholesterol by intravenous infusion of native or acetylated human low-density lipoprotein (Miller et al., 1985). More recently, it has been shown *in vivo* that the uptake of HDL cholesteryl esters by rat parenchymal cells is more efficiently coupled to the formation of bile acids than is cholesteryl ester uptake from low-density lipoprotein (Pieters et al., 1991). Also, evidence from statistical studies in humans support a role for HDL in reverse cholesterol transport, since an inverse relationship between plasma HDL cholesterol concentration and the incidence of coronary heart disease has been demonstrated (Miller and Miller, 1975; Gordon and Rifkind, 1989).

A perfused rat spleen system has been used as a model for examination of the release of cholesterol to the blood from an extrahepatic tissue (Mindham and Mayes, 1989a; Mindham et

al., 1990). In these studies it was possible to label the spleen *in vivo* with [^3H]cholesterol to a high specific radioactivity, before isolation and perfusion. The spleen released substantial amounts of labelled (30%) cholesterol and cholesterol mass (up to 8%) during subsequent perfusion (Mindham et al., 1990). We have also reported (Mindham and Mayes, 1991a) the application of sequential perfusion of the liver with the perfusate from a labelled spleen perfusion. In the present study, we have extended this system by perfusing the liver simultaneously with a spleen, both preparations sharing a common perfusate. As well as studying reverse cholesterol transport by using defined HDL fractions, this system has allowed us to ascertain the effect of the presence of the liver on the rate of removal of cholesterol from the spleen. The introduction of the liver preparation allows collection of bile, and therefore the transport of cholesterol from the spleen to the blood, and thence to the liver and bile, can be assessed. By using a blood perfusate of high haematocrit, a physiological pO_2 and rate of flow can be used to maintain optimum oxygenation of the preparation, and therefore the results obtained under these conditions are likely to reflect the true rates of the physiological process of cholesterol transport between these organs *in vivo*.

EXPERIMENTAL

Animals and materials

Male Wistar rats were used as spleen and liver donors (350–370 g body wt.) and as blood donors (350–450 g body wt.). They were fed on a standard pelleted diet and kept under constant day length (12 h) and temperature (25 °C). [$1\alpha,2\alpha\text{-}^3\text{H}$]Cholesterol was obtained from Du Pont NEN, Stevenage, Herts., UK. BSA (fraction 5, cell-culture grade) and cholesterol oxidase were supplied by Sigma Chemical Co., Poole, Dorset, U.K., and 3α -hydroxysteroid dehydrogenase was from BCL, Lewes, E. Sussex,

Figure 1 Apparatus for simultaneous rat spleen and liver perfusion

A, syringe for introducing an air bubble; B, water-jacket bowl; BE, bile collection tube; BT, bubble trap; C, stopcock; CF, Clingfilm; E, oxygen electrode; F, poplin filter; FM, air-bubble flowmeter; H, humidifier; L, liver; N, gas exchanger; P, blood pumps; PT, pressure transducer; S, spleen; ST, stirrer; T, thermistor-thermometer.

U.K. Chemicals were obtained from Fisons, Loughborough, Leics., U.K., or Merck, Dagenham, Essex, U.K.

Perfusion apparatus

A diagram of the perfusion apparatus is shown in Figure 1. Tubing and connectors were of surgical-grade plastic and supplied by Portex, Hythe, Kent, U.K. Where tubing was in contact with pumps, silicone rubber tubing was used (Esco Rubber; Sterilin, Hounslow, Middx., U.K.). The spleen was placed in a purpose-made glass bowl incorporating a water jacket maintained at 37 °C, whereas the liver was perfused *in situ*, the carcass being supported on a board whose height was adjustable. The liver outflow could thereby be adjusted to a standard pressure of 2.5 cm below the liver. Blood from the reservoir was filtered through a cotton poplin filter before entering separate variable-flow-rate peristaltic pumps to spleen or liver. Narrow-bore standpipes or pressure transducers were employed to indicate the pressure required to maintain flow, and bubble-traps were present before the blood entered each organ. Additional pumps circulated blood from the reservoir to a gas-exchanger and oxygen electrode. Through the gas-exchanger was passed a mixture of humidified air/CO₂ (19:1) and O₂/CO₂ (19:1), the proportions of these gases being adjusted to maintain a perfusate pO₂ of 100 mmHg (13.3 kPa). The gas mixture was withdrawn under slight negative pressure. The whole apparatus was housed in a thermostatically controlled cabinet maintained at 37 °C. Thermistor probes (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.) were placed adjacent to the spleen and under the liver to monitor their

temperature. Further details in common with the systems for perfusing the spleen and liver alone are given in Mindham and Mayes (1989b, 1991a).

Labelling of donor spleens

[³H]Cholesterol (250 μCi) in ethanol (0.25 ml) was decreased in volume by a stream of nitrogen to approx. 30 μl. Then 1.5 ml of 0.9% NaCl and 1.5 ml of packed rat erythrocytes, ampicillin (0.1 mg/ml) and glucose (10 mg/ml) were added, and the erythrocyte mixture was incubated overnight for 15 h at 37 °C in a shaking water bath. After incubation, the mixture was centrifuged at 2000 g for 10 min, and the erythrocytes were washed with 0.9% NaCl. The erythrocytes were resuspended in 0.9% NaCl to give a haematocrit of 50%, and 1 ml was injected into the tail vein of a rat. The spleen was isolated approx. 4 h after injection, when it contained 4–10 μCi of [³H]cholesterol.

Isolation of spleen and liver

Details of the methods for the individual surgical isolation of the liver and spleen and the preparation of the whole-blood perfusate have been described previously (Mayes and Felts, 1966; Mindham and Mayes, 1989b). The liver was prepared initially. Briefly, a rat was anaesthetized with sodium pentobarbital (60 mg/kg body wt.) and the bile duct was cannulated with polyethylene tubing. The inflow cannula was tied into the portal vein, and the liver was flushed free of blood with modified Krebs–Henseleit (1932) bicarbonate buffer containing 3% (w/v) BSA, mixed plasma

amino acids (0.67 mg/ml) and glucose (2.5 mg/ml). The outflow cannula was tied into the inferior vena cava in the thorax and the liver transferred into the perfusion circuit containing 110 ml of perfusate. The spleen was prepared from another anaesthetized rat. After removing pancreatic and adipose tissue in the vicinity of the spleen, an inflow cannula was tied into the coeliac artery, whose branches were ligated apart from the splenic artery, and the spleen was flushed free of blood with the modified Krebs–Henseleit buffer. The outflow cannula was tied into the portal vein, whose branches were ligated apart from the splenic vein. The spleen was removed from the carcass, placed in the water-jacket bowl, and connected to the main perfusion system. It was perfused at 1 ml/min per g and the liver at 1.25 ml/min per g with the whole-blood perfusate. The perfusate was prepared from defibrinated rat blood dialysed against the modified Krebs–Henseleit buffer as described previously (Mindham and Mayes, 1989b) and diluted with serum to give a haematocrit of 30%.

Preparation of HDL fractions

HDL fractions were prepared from rat serum by ultracentrifugation in 6.5 ml polyallomer tubes (Kontron, Watford, Herts., U.K.) in a fixed-angle rotor (MFT-50.6). The serum was centrifuged sequentially after stepwise addition of solid KBr to obtain HDL fractions of density 1.050–1.085 (HDL₁), 1.085–1.125 (HDL₂) and 1.125–1.250 g/ml (HDL₃). Each centrifugation was for 24 h at 4 °C at 120 000 *g*. The top 1 ml fraction was harvested by using a tube slicer. The fractions were dialysed against Krebs–Henseleit bicarbonate buffer containing glucose (2.5 mg/ml) and mixed amino acids (0.67 mg/ml) before use in the perfusate.

Analytical methods

After perfusion, the spleen and liver were flushed with modified bicarbonate buffer and homogenized with 75 ml and 200 ml of chloroform/methanol (2:1, v/v) respectively. The perfusate was centrifuged to remove erythrocytes, and portions of the serum were ultracentrifuged at *d* = 1.006, 1.050, 1.085, 1.125, and 1.250 to enable the distribution according to density of cholesterol mass and radioactivity to be determined. Chloroform/methanol extracts obtained from the liver, spleen, portions of the perfusate and portions of the lipoproteins obtained by ultracentrifugation, were shaken with 0.4 vol. of 0.03 M HCl, and the chloroform layer was taken for analysis. The erythrocytes from 1 ml of perfusate were washed three times with 0.9% NaCl and extracted in 20 ml of chloroform/propan-2-ol (7:11, v/v) (Rose and Oklander, 1965). The total content of bile acids was determined enzymically by using 3 α -hydroxysteroid dehydrogenase (Turley and Dietschy, 1978) before the remainder of the bile was extracted in 20 ml of chloroform/methanol (2:1, v/v) and partitioned with 0.4 vol. of water. Radioactivity in top and bottom layers (representing the incorporation of ³H into bile acids and cholesterol respectively) was determined by liquid-scintillation counting. Cholesterol and cholesteryl ester in lipid extracts were separated by t.l.c. on silica gel G with hexane/diethyl ether/formic acid (40:10:1, by vol.). Unesterified cholesterol was determined enzymically (Trinder, 1969) in aqueous samples or in lipid extracts after they had been dried down and redissolved in propan-2-ol. Esterified cholesterol was measured by addition of cholesterol esterase reagent (System C; Boehringer, Lewes, E. Sussex, U.K.) to the same samples after completion of the initial estimation of unesterified cholesterol, followed by measurement

of the further increase in absorbance. Apolipoproteins were prepared for electrophoresis by a rapid and simple precipitation method that gave improved recovery (Mindham and Mayes, 1992) and analysed by SDS/PAGE by using methods described by Laemmli (1970) adapted for a slab-gel system (LKB 1001; Pharmacia, Milton Keynes, U.K.). The gels were stained with Coomassie Blue (R250; Sigma) and scanned at 550 nm with a Beckman model 25 spectrophotometer.

RESULTS

Effect of the presence of the liver on reverse cholesterol transport from the spleen

A comparison of the distribution of released [³H]cholesterol after perfusion with a whole-blood perfusate of the spleen alone or of the spleen perfused simultaneously with a liver is shown in Table 1. After 3 h of perfusion, the spleens perfused alone had released 28.3% of their [³H]cholesterol radioactivity, of which 15.0% was in erythrocytes and the remainder in serum lipoproteins. The presence of a liver not only significantly altered the total [³H]cholesterol released from the spleen, but also markedly affected its distribution. Thus the spleen–liver preparation showed a substantial release of [³H]cholesterol from the spleen which amounted to 32.6% of that initially present, which represented a 15% increase in the release observed (28.3%) when the spleen was perfused alone. There was a large uptake of [³H]cholesterol by the liver (10.7%) as well as a significant incorporation of ³H into bile (approx. 1% of the [³H]cholesterol in the liver), mainly as bile acids (87%) and the remainder as unesterified cholesterol. Of the [³H]cholesterol recovered in serum, significantly more was incorporated into esterified cholesterol in the spleen–liver preparation than with the spleen alone, and in addition [³H]cholesteryl ester was recovered in the very low-density lipoprotein (VLDL; *d* < 1.006) fraction (Figure 2). The uptake of ³H by the liver was accompanied by a corresponding decrease in radioactivity remaining in the perfusate, particularly in the serum lipoproteins. A comparison of the distribution of [³H]cholesterol in serum lipoproteins after perfusion (Figure 2) indicated that all the lipoproteins were labelled, particularly the HDL (*d* 1.050–1.250). However, the HDL₂ fraction in combined perfusions showed a marked disappearance of 59% of that remaining in the perfusate in experiments in which the spleen was perfused alone, indicating the role of this HDL fraction in the mediation of cholesterol uptake by the liver. In experiments in which the spleen was perfused alone (Table 1), a large quantity of radioactivity was recovered in erythrocytes, but in the spleen–liver preparations there was only a slight diminution of radioactivity recovered in erythrocytes, reflecting

Table 1 Comparison of the release of [³H]cholesterol from spleens perfused alone or with a liver

Results are expressed as percentage of initial radioactivity in spleen, and are means $\pm$ S.E.M. of five 3 h perfusions: * difference significant at *P* < 0.02; ** significant at *P* < 0.01.

Radioactivity incorporated into:	Spleen alone	Spleen + liver
Serum lipoproteins	13.3 $\pm$ 0.70	8.37 $\pm$ 0.57**
Erythrocytes	15.0 $\pm$ 0.50	13.5 $\pm$ 0.94
Cholesteryl ester	1.66 $\pm$ 0.13	2.12 $\pm$ 0.11**
Liver	—	10.7 $\pm$ 0.68
Bile	—	0.11 $\pm$ 0.01
Total	28.3 $\pm$ 1.2	32.6 $\pm$ 1.47*

Figure 2 Distribution according to density of serum lipoprotein [^3H]cholesterol after perfusion of the spleen alone or of the spleen and liver combined

Spleens were labelled initially with [^3H]cholesterol as described in the text. The spleen was perfused either alone or with a liver for 3 h, and the serum lipoproteins were separated by ultracentrifugation as described in the Experimental section. Left-hand columns, spleen alone; right-hand columns, spleen and liver combined. ■, Radioactivity incorporated into cholesteryl ester; the remainder of each column represents unesterified cholesterol. Values are means $\pm$ S.E.M. of five perfusions.

the ability of erythrocytes to take up or exchange unesterified cholesterol rapidly in the spleen, but a lessened ability, compared with lipoproteins, to pass this cholesterol on to the liver. When the spleen was perfused alone, there was an increase in both unesterified and esterified cholesterol mass in the perfusate of 0.997 ± 0.032 and 0.617 ± 0.025 mg (means $\pm$ S.E.M.) respectively. However, when the liver was present, the increase in esterified cholesterol was no longer observed, whereas the increase in unesterified cholesterol was more marked (3.09 ± 0.14 mg), indicating the ability of the liver to facilitate a net uptake of cholesteryl ester mass.

Reverse cholesterol transport in the combined perfusion system, using defined HDL fractions

When whole blood is used as perfusate, it is difficult to determine the relative contribution of serum HDL fractions to reverse cholesterol transport. This is due to several factors. Firstly, the liver is secreting cholesterol in HDL and VLDL, and also adding new apolipoproteins to the perfusate. At the same time it is removing unesterified and esterified cholesterol from HDL. Secondly, HDL fractions may be interconverted, e.g. HDL₃ to HDL₂, making observations difficult to interpret, and thirdly, erythrocytes, and therefore cholesterol, are continuously taken up by the spleen. Some of these problems can be overcome by perfusing the spleen–liver preparation with individual HDL subfractions added to a basal perfusate containing no lipoproteins (modified Krebs–Henseleit bicarbonate buffer containing 6% BSA, amino acids and glucose at the previously stated concentrations, made up to 30% haematocrit with washed rat erythrocytes). Therefore the spleen–liver preparation was perfused for 3 h with the basal perfusate alone or the basal perfusate to which was added HDL₁ (d 1.050–1.085), HDL₂ (d 1.085–1.125) or HDL₃ (d 1.125–1.250) fractions. The fractions of HDL were

Table 2 Cholesterol and cholesteryl ester contents of HDL fractions isolated from rat serum

Values for cholesteryl ester are expressed as μg of cholesterol/ml of serum. Results are means $\pm$ S.E.M. of five preparations.

HDL fraction (density, g/ml)	Unesterified cholesterol ($\mu\text{g}/\text{ml}$)	Cholesteryl ester ($\mu\text{g}/\text{ml}$)
HDL ₁ (1.050–1.085)	19.3 ± 0.9	90.4 ± 3.6
HDL ₂ (1.085–1.125)	40.2 ± 1.8	186.6 ± 5.5
HDL ₃ (1.125–1.250)	26.1 ± 2.2	50.5 ± 2.7

Table 3 Apolipoprotein (Apo) composition of HDL fractions

Lipoproteins were isolated from rat serum by ultracentrifugation, and apolipoproteins were prepared and separated by SDS/PAGE as described in the Experimental section. Gels were scanned at 550 nm after staining with Coomassie Blue. Values for total protein (means $\pm$ S.E.M. of four preparations) were determined by the method of Peterson (1977). No low-density lipoprotein was present in any of the fractions, as judged by the absence of apolipoprotein B-100.

HDL fraction (density, g/ml)	Percentage of total protein recovered as:				Total protein ($\mu\text{g}/\text{ml}$ of serum)
	Apo A-IV	Apo E	Apo A-I	Apo C	
HDL ₁ (1.050–1.085)	1.1	94.7	2.3	1.9	115 ± 3.1
HDL ₂ (1.085–1.125)	13.6	11.4	71.3	3.7	510 ± 9.8
HDL ₃ (1.125–1.250)	7.5	6.8	77.9	7.8	193 ± 8.7

Table 4 Distribution of [^3H]cholesterol released by the spleen after simultaneous spleen–liver perfusions with defined HDL fractions

The combined preparation was perfused for 3 h as described in the text. The HDL fractions were added to a basal perfusate of albumin–buffer plus erythrocytes at their normal serum concentrations. Results are means $\pm$ S.E.M. of four or five perfusions: ^csignificantly different from ^b and ^d ($P < 0.02$) and ^a ($P < 0.01$); ^dsignificantly different from ^a and ^b ($P < 0.01$).

Perfusate addition	Percentage of [^3H]cholesterol released by the spleen to:			
	Perfusate lipoproteins	Erythrocytes	Liver	Total
None	1.45 ± 0.26	8.88 ± 0.61	5.50 ± 0.55^a	15.8 ± 0.52
HDL ₁	1.69 ± 0.37	8.45 ± 0.72	6.31 ± 0.62^b	16.5 ± 0.76
HDL ₂	2.18 ± 0.41	10.14 ± 0.51	8.55 ± 0.84^c	20.9 ± 0.85
HDL ₃	1.88 ± 0.39	9.58 ± 0.73	11.84 ± 1.15^d	23.3 ± 1.25

prepared by ultracentrifugation of rat serum at the densities stated, and they were added to the basal perfusate at their normal serum concentrations. The cholesterol and apolipoprotein composition of the fractions added is shown in Tables 2 and 3. Most of the protein and cholesterol, particularly esterified cholesterol, was present in HDL₂. The apolipoprotein of the HDL of d 1.050–1.085 was virtually all apolipoprotein E (apo E) and had a composition similar to that of HDL₁ described in studies of zonal centrifugation of rat lipoproteins (Lusk et al., 1979). A lipoprotein of similar density (HDL_c) has been described in animals fed on a high-cholesterol diet (Mahley, 1978), which contains apo E as its only protein. Both HDL₂ and HDL₃ had similar apolipoprotein compositions to each other. Apolipoprotein A-I (apo A-I) was the most abundant protein, followed by apolipoprotein A-IV, apo E and apolipoprotein C.

Table 5 Bile production following simultaneous spleen–liver perfusion

Results are means $\pm$ S.E.M. of four perfusions: *significantly different from basal perfusate value ($P < 0.01$).

Perfusate	Total bile acid produced (μmol)	^3H Cholesterol (% of total) recovered in:		Percentage of liver uptake of ^3H recovered in bile acid plus cholesterol
		Bile acid	Bile cholesterol	
Whole blood	8.94 ± 0.91	$0.092 \pm 0.016^*$	$0.017 \pm 0.001^*$	1.02
Basal perfusate only	7.79 ± 1.10	0.052 ± 0.013	0.012 ± 0.0008	1.16
Basal perfusate + HDL ₁	8.52 ± 0.74	0.058 ± 0.014	0.012 ± 0.0006	1.11
Basal perfusate + HDL ₂	9.21 ± 1.26	$0.102 \pm 0.018^*$	$0.017 \pm 0.0010^*$	1.38
Basal perfusate + HDL ₃	7.96 ± 1.51	$0.110 \pm 0.022^*$	$0.018 \pm 0.0018^*$	1.08

Table 6 Changes in perfusate unesterified cholesterol and cholesteryl ester concentrations during simultaneous spleen–liver perfusions

The combined preparation was perfused for 3 h as described in the text with a basal perfusate containing no addition, or added HDL₁, HDL₂ or HDL₃. Values are means $\pm$ S.E.M. of 4 perfusions: *significantly different from zero time at $P < 0.01$ (paired Student *t* test).

Perfusate addition	Unesterified cholesterol ($\mu\text{g/ml}$)		Esterified cholesterol ($\mu\text{g/ml}$)	
	0 h	3 h	0 h	3 h
None	3.1 ± 0.3	$13.2 \pm 1.5^*$	0.0 ± 0.0	0.0 ± 0.0
HDL ₁	19.8 ± 1.15	$59.9 \pm 4.6^*$	88.1 ± 1.9	$63.6 \pm 2.4^*$
HDL ₂	45.6 ± 3.2	$98.4 \pm 5.1^*$	169 ± 2.4	$130 \pm 4.1^*$
HDL ₃	37.3 ± 2.3	$79.9 \pm 3.7^*$	76.3 ± 2.3	78.8 ± 3.6

After a 3 h perfusion, HDL₂ and HDL₃ were found to be the most potent removers of ^3H cholesterol from the spleen, some 20.9% and 23.3% respectively of that initially present in the spleen being removed during perfusion (Table 4). In addition, there was significantly more ^3H cholesterol recovered in the liver with these particular fractions, particularly with HDL₃. In contrast with HDL₁ only 16.4% of spleen ^3H cholesterol was removed, a value not significantly different from that of control perfusions using the basal perfusate of albumin/buffer with no added HDL. In all experiments, erythrocytes played a prominent part in transporting cholesterol from the spleen, since, after 3 h of perfusion, of the total ^3H cholesterol released by the spleen, some 8.5–10% was in erythrocytes. However, there was no significant difference between the final amount of radioactivity in either erythrocytes, or in the remainder of the perfusate, in any of the perfusates tested. There was significantly greater ^3H recovery in bile with HDL₂- and HDL₃-containing perfusates, in which there was a greater uptake of ^3H cholesterol by the liver (Table 5). Consequently, the amount of ^3H in bile was similar when expressed as a proportion of the ^3H cholesterol in the liver at the end of the experiment. It was noteworthy that HDL₁, which failed to increase liver uptake of ^3H cholesterol compared with the basal perfusate, also failed to increase incorporation of ^3H into bile acid.

There was a net increase in perfusate total cholesterol mass using all perfusates (Table 6). This was mostly as unesterified cholesterol, but a net removal of cholesteryl ester from the perfusate was observed in experiments utilizing HDL₁ and HDL₂. This removal would be by uptake into the liver, since, when the spleen is perfused alone, there is net accumulation of cholesteryl ester with these perfusates (Mindham et al., 1990). Figure 3

Figure 3 Distribution according to lipoprotein density of ^3H cholesterol released to perfusate in simultaneous spleen–liver preparations, perfused with defined HDL fractions

The spleen was pre-labelled *in vivo* with ^3H cholesterol and perfused along with a liver as described in the text. The defined HDL fractions were added, as indicated by the arrows, to a basal perfusate of modified Krebs–Henseleit buffer (see the text) at the concentrations present in serum. Added lipoprotein: (a) none; (b) HDL₂; (c) HDL₂; (d) HDL₃. The densities analysed were $d < 1.050$, $1.050\text{--}1.085$, $1.085\text{--}1.125$, $1.125\text{--}1.250$ and > 1.250 .

shows the distribution according to density of ^3H cholesterol remaining in the perfusate at 3 h. When no lipoprotein was present (Figure 3a), most of the radioactivity was recovered in the more dense $d = 1.125\text{--}1.250$ and $d > 1.250$ fractions. The appearance of cholesterol at these densities might be expected, since the liver is known to secrete HDL of low cholesteryl ester content in this density range (Hamilton et al., 1976). This secretion would also be expected to occur with the other perfusates where HDL was present and may contribute to the recovery of radioactivity at the more dense fractions than that of the added HDL (e.g. Figures 3b–3d). Additionally, in these experiments, when HDL₂ was the added subfraction (Figure 3c), the metabolism and uptake of cholesterol and other lipids from HDL₂ by the liver may result in a more dense particle being released back to the perfusate, i.e. HDL₃. In contrast, when the added HDL subfraction was HDL₃ (Figure 3d), there was a large recovery of cholesterol in HDL₂ density range, reflecting a

possible accumulation of cholesterol and conversion of HDL₃ into HDL₂ due to reverse cholesterol transport from the spleen.

DISCUSSION

The present investigations utilizing the combined spleen and liver perfusion system are the logical extension of our previous studies using spleen perfusion alone (Mindham et al., 1990), and the sequential perfusion of the spleen followed by the liver, using a common perfusate (Mindham and Mayes, 1991a). The combined system has enabled us to demonstrate that, when the liver is perfused simultaneously with the spleen, it causes a significant increase in the rate of removal of [³H]cholesterol from the labelled spleen. The reasons for this are not immediately apparent, but it is possible that, as the liver would be secreting nascent HDL (Hamilton et al., 1976), this would stimulate reverse cholesterol transport from the spleen. Also, the liver in clearing cholesterol and cholesteryl ester from HDL₂ appears to regenerate HDL₃, which would be available to take part in further reverse cholesterol transport from the spleen.

The experiments utilizing a blood perfusate show that the [³H]cholesterol in the HDL₂ fraction (*d* 1.085–1.125) was the only fraction significantly decreased as a result of simultaneous perfusion (Figure 2). Previous experiments utilizing individual HDL fractions with the perfused spleen alone (Mindham et al., 1990) indicated that both HDL₂ and HDL₃ are equally important for [³H]cholesterol removal from that organ. It is therefore probable that a large amount of [³H]cholesterol is released to HDL₃, which subsequently becomes less dense and upon analysis is recovered as HDL₂, accounting for the distribution when the spleen is perfused alone. However, when the spleen is perfused in the presence of the liver, the decrease in the HDL₂ fraction with insignificant changes in the other fractions must indicate that it is this fraction that is pre-eminent in donating [³H]cholesterol to the liver. During perfusion, there was an increase in perfusate unesterified cholesterol, which was more marked when the liver was present. The liver is known to secrete HDLs, which are rich in unesterified cholesterol (Hamilton et al., 1976), and this may account for the further increase in lipoprotein unesterified cholesterol. In addition, the appearance of [³H]cholesteryl ester in VLDL is likely to be the result of uptake of radioactive cholesterol by the liver, followed by esterification by acyl-CoA: cholesterol acyltransferase and its subsequent secretion in VLDL. This follows from the lack of ability to transfer cholesteryl ester directly from HDL to triacylglycerol-rich lipoproteins in the rat, due to the absence of cholesteryl ester transfer-protein activity (Barter and Lally, 1978).

Experiments utilizing defined fractions of HDL support the findings with whole-blood perfusates. Both HDL₂ and HDL₃ when added to a basal perfusate on their own removed similar amounts of [³H]cholesterol from the spleen. Also, there was a significantly greater uptake of [³H]cholesterol by the liver when the perfusate contained HDL₃ as its sole lipoprotein addition (*P* < 0.01). After perfusion, the density profile of radioactivity in perfusate lipoproteins indicated that a part of the HDL₃ had been converted into less-dense HDL₂ during perfusion, presumably as a result of uptake of cholesterol (Figure 3d). This agrees with the finding that cholesterol is taken up by the liver mainly from the HDL₂ fraction during whole-blood perfusions (Figure 2). In contrast, when HDL₂ was the added lipoprotein (Figure 3c), a large proportion of the labelled cholesterol was recovered in the HDL₃ density range, presumably as a result of uptake of cholesterol by the liver. There was a net increase in unesterified cholesterol mass in the perfusate when each fraction

of HDL was used on its own in the perfusate, and even when the basal perfusate was used alone. However, a net uptake of cholesteryl ester mass from the perfusate was observed in experiments utilizing HDL₁ and HDL₂, but not HDL₃, indicating that HDL₁ and HDL₂ facilitate the uptake of cholesteryl ester from the serum into the liver. The mechanisms may be different from each lipoprotein, HDL₁ with its high content of apo E reacting with the apo E receptor, and HDL₂ possibly using apo A-I as a ligand in hepatic recognition. The large net increase in unesterified cholesterol observed probably results from HDL and VLDL secretion by the liver and masks the uptake of unesterified cholesterol, as indicated by the data for radioactivity. It has to be considered that *in vivo* reverse cholesterol transport would occur from many other tissues besides the spleen, and the liver might therefore be expected to receive and take up more HDL cholesterol than the current investigations indicate.

In all experiments a large proportion of [³H]cholesterol was recovered in erythrocytes. When perfusions of the spleen alone are considered (Table 1), erythrocytes remove from the spleen a similar amount of ³H as the serum lipoproteins. However, in the presence of a liver the data show that the erythrocytes are less effective than lipoproteins at transporting or exchanging cholesterol with the liver. Experiments utilizing a basal perfusate with no added lipoproteins, or those with added HDL fractions, all show a similar amount of ³H in erythrocytes (Table 4), suggesting that the erythrocytes are capable of directly removing ³H from the spleen and are not dependent on the presence of lipoproteins to mediate uptake or exchange of cholesterol. Although in the present experiments some nascent lipoproteins may participate in this process, due to the presence of the liver, in other investigations (Mindham and Mayes, 1991b), we have shown that erythrocytes transport substantial quantities of [³H]cholesterol from the spleen in the absence of lipoproteins and the liver. Previous experiments in which livers have been perfused with [³H]cholesterol-labelled erythrocytes also support a role for erythrocytes in cholesterol transport to the liver and subsequent incorporation into bile constituents (Laker and Mayes, 1985; Mindham and Mayes, 1991a). The reason why erythrocytes are better at removing cholesterol from the spleen than in delivery of cholesterol to the liver may lie in the mechanisms involved in each tissue. The removal of unesterified cholesterol from extrahepatic tissues may be due to diffusion from the extrahepatic cell membrane to the erythrocyte membrane or lipoprotein particle, due to a concentration gradient (Rothblat et al., 1992), whereas uptake by the liver may be, in addition to diffusion, a receptor- or ligand-mediated process specific for HDL (Glass et al., 1985). Our results are compatible with this hypothesis.

Examination of the apolipoprotein composition of the three fractions of HDL studied (Table 3) indicate a similarity in distribution of the major apolipoproteins between HDL₂ and HDL₃, but a dissimilarity with HDL₁. This suggests a close metabolic relationship and a similar origin for HDL₂ and HDL₃, compatible with their interconversion in a cycle of lipidation and delipidation facilitating reverse cholesterol transport between extrahepatic tissues and the liver. Such a scheme, postulated by Nikkila et al. (1982), is supported by our recent investigations demonstrating that apo A-I is not taken up significantly by the liver during participation of HDL in reverse cholesterol transport (Mindham and Mayes, 1994). The origins and role of HDL₁ are more obscure. However, it appears to increase under conditions of cholesterol loading (Mahley, 1978) and, as demonstrated in the present work, it participates in the transfer of cholesteryl ester to the liver.

In summary, we have described a new combined perfusion system of the rat spleen and liver which enables the complete

process of reverse cholesterol transport to be studied. By pre-labelling the spleen with [^3H]cholesterol *in vivo*, it has been possible to demonstrate that the lipoproteins primarily responsible for reverse cholesterol transport from the spleen to the liver are HDL₂ and HDL₃, in which cholesteryl esters are formed. A direct role for the erythrocytes in the transport and equilibration of unesterified cholesterol between the tissues is also indicated by our results. There was a rapid exchange or transport of unesterified cholesterol between HDL and the liver particularly involving HDL₂ and a net uptake of cholesteryl ester from HDL₁ and HDL₂. There was also significant incorporation of radioactivity into bile, demonstrating the potential of this system for the study of the complete process of reverse cholesterol transport, i.e. transfer of cholesterol from an extrahepatic tissue to the blood, followed by uptake into the liver and excretion into bile.

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REFERENCES

- Badimon, J. J., Badimon, L., Galvez, A., Dische, R. and Fuster, V. (1989) *Lab. Invest.* **60**, 455–461
- Barter, P. J. and Lally, J. I. (1978) *Biochim. Biophys. Acta* **531**, 233–236
- Gavish, D., Oschry, Y. and Eisenberg, S. (1987) *J. Lipid Res.* **28**, 257–267
- Glass, C., Pittman, R. C., Civen, M. and Steinberg, D. (1985) *J. Biol. Chem.* **260**, 744–750
- Glomset, J. A. (1968) *J. Lipid Res.* **9**, 155–167
- Gordon, D. J. and Rifkind, B. M. (1989) *N. Engl. J. Med.* **321**, 1311–1316
- Hamilton, R. L., Williams, M. C., Fielding, C. J. and Havel, R. J. (1976) *J. Clin. Invest.* **58**, 667–680
- Krebs, H. A. and Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Laker, M. E. and Mayes, P. A. (1985) *Biochem. Soc. Trans.* **13**, 142–143
- Lusk, L. T., Walker, L. F., DuBien, L. H. and Getz, G. S. (1979) *Biochem. J.* **183**, 83–90
- Mahley, R. W. (1978) in *Disturbances in Lipid and Lipoprotein Metabolism* (Dietschy, J. M., Gotto, A. M. and Ontko, J. A., eds.), pp. 181–197, Williams and Wilkins, Baltimore
- Mayes, P. A. and Felts, J. M. (1966) *Proc. Eur. Soc. Study Drug Toxic.* **7**, 16–29
- Miller, G. J. and Miller, N. E. (1975) *Lancet* **i**, 16–19
- Miller, N. E., LaVigne, A. and Crook, D. (1985) *Nature (London)* **314**, 109–111
- Mindham, M. A. and Mayes, P. A. (1989a) *Protides Biol. Fluids* **36**, 401–407
- Mindham, M. A. and Mayes, P. A. (1989b) *Biochem. J.* **263**, 325–332
- Mindham, M. A. and Mayes, P. A. (1991a) *Biochem. J.* **279**, 503–508
- Mindham, M. A. and Mayes, P. A. (1991b) *Biochem. Soc. Trans.* **19**, 325S
- Mindham, M. A. and Mayes, P. A. (1992) *J. Lipid Res.* **33**, 1084–1088
- Mindham, M. A. and Mayes, P. A. (1994) *Biochem. Soc. Trans.* **22**, 107S
- Mindham, M. A., Mayes, P. A. and Miller, N. E. (1990) *Biochem. J.* **268**, 499–505
- Nikkila, E. A., Kuusi, T. and Taskinen, M. R. (1982) in *Metabolic Risk Factors in Ischemic Cardiovascular Disease* (Carlson, L. A. and Pernow, B., eds.), pp. 205–215, Raven Press, New York
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- Pieters, M. N., Schouten, D., Bakkeren, H. F., Esbach, B., Brouwer, A., Knook, D. L. and Van Berkel, T. J. C. (1991) *Biochem. J.* **280**, 359–365
- Rose, H. G. and Oklander, M. (1965) *J. Lipid Res.* **6**, 428–431
- Rothblat, G. H., Mahlberg, F. H., Johnson, W. J. and Phillips, M. C. (1992) *J. Lipid Res.* **33**, 1091–1097
- Trinder, P. (1969) *Ann. Clin. Chem.* **6**, 24–29
- Turley, S. D. and Dietschy, J. M. (1978) *J. Lipid Res.* **19**, 924–928