Reverse cholesterol transport in the rat

Studies using the isolated perfused spleen in conjunction with the perfused liver

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1. A new method combining the use of an isolated perfused extrahepatic tissue with a perfused liver was developed as a model system for the study of reverse cholesterol transport. Rat spleens, initially labelled *in vivo* with [³H]cholesterol, were perfused for 3 h with whole blood. The spleen was then replaced with an isolated rat liver, whose uptake of cholesterol from the spleen-derived blood and excretion of cholesterol into bile constituents were determined. 2. During spleen perfusion, a net release of cholesterol mass and radioactivity to lipoproteins was observed. 3. During liver perfusion, there was also a rapid exchange or transport of unesterified cholesterol between high-density lipoprotein (HDL) and the liver, in particular with HDL₂ ($d = 1.085\text{--}1.125$). 4. The liver showed an increased uptake of cholesteryl ester from serum that had previously been used in spleen perfusion. 5. Approximately half of the [³H]cholesterol released by the spleen was recovered in erythrocytes. During subsequent liver perfusion there was a substantial uptake of radioactivity from the erythrocytes, although less than that recorded from serum lipoproteins. 6. In all experiments there was significant excretion of [³H]cholesterol into bile; most (85 %) was in bile acids. Thus the complete process of reverse cholesterol transport is observed in this dual-perfusion system.

INTRODUCTION

The transfer of cholesterol from extrahepatic tissues to the liver, followed by its excretion in the bile as bile acid and cholesterol, has become known as reverse cholesterol transport. The proposal that high-density lipoprotein (HDL) participates in this process (Glomset, 1968) is supported by the inverse relationship observed between plasma HDL cholesterol concentration and the incidence of coronary heart disease (Rifkind, 1990). Also, there is evidence that a portion of the cholesterol of cultured cells may be removed by certain subclasses of HDL (Miller, 1989) and also by synthetic complexes of lipid and apolipoproteins resembling HDL (Stein *et al.*, 1986; DeLamatre *et al.*, 1986). Studies *in vivo* have demonstrated that infusions of HDL can inhibit the development of diet-induced 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 circulating HDL in rabbits after loading the tissues with cholesterol by intravenous infusion of native or acetylated human low-density lipoprotein (LDL) (Miller *et al.*, 1985). Additional evidence *in vivo* that HDL is the principal lipoprotein acceptor of cholesterol has been provided by studies of human peripheral lymph (Reichl *et al.*, 1982).

Recently, we have employed the rat spleen as a model for the study of reverse cholesterol transport from an intact extrahepatic tissue (Mindham & Mayes, 1989a; Mindham *et al.*, 1990). The spleen was labelled with radioactive cholesterol by making use of its ability to sequester labelled erythrocytes *in vivo*. In the present study, we have extended the system by the incorporation of an isolated liver which is perfused with the cholesterol-labelled blood derived from the spleen perfusion. Thus the additional liver preparation allows the collection of bile, enabling the assessment of the overall contribution of blood constituents to the complete process of reverse cholesterol transport, i.e. the

release of cholesterol by an extrahepatic tissue to the blood, followed by uptake into the liver and excretion into the bile as bile acid or unesterified cholesterol. The use of a whole-blood perfusate in these investigations ensures an adequate fuel supply and oxygenation of the organs while using a physiological pO_2 and rate of flow. Confirmation of this has been obtained from previous work where the metabolism of the organs was studied (Mindham & Mayes, 1989b; Laker & Mayes, 1979). Therefore results obtained under our conditions are likely to represent the physiological process of cholesterol transport between these organs *in vivo*.

EXPERIMENTAL

Animals and materials

Male Wistar rats (MRC strain) were kept under constant day length (12 h) and temperature (25 °C) and fed on a standard pelleted diet (MRC diet 86). Animals weighing 340–360 g were used as spleen and liver donors, and others weighing 350–450 g were used as blood donors. BSA (fraction 5) was supplied by ICN Biomedicals, High Wycombe, Bucks., U.K. [³H]Cholesterol was obtained from Amersham International, Amersham, Bucks., U.K. Cholesterol oxidase, silica gel G and other chemicals were obtained from BDH, Dagenham, Essex, U.K.

Labelling of spleens

[³H]Cholesterol (250 µCi) in acetone (0.1 ml) was added dropwise to 2.5 ml of stirred rat serum at 37 °C. A stream of N₂ was directed at the surface for 30 min to remove the acetone. Packed erythrocytes (2.5 ml), ampicillin (0.05 mg/ml) and glucose (10 mg/ml) were added, and the reconstituted blood was incubated for 15 h at 37 °C in a shaking water bath. After incubation, the blood was centrifuged at 2000 *g* for 10 min, the serum was removed, and the erythrocytes were washed twice with 0.9 % NaCl. A 1 ml portion of erythrocytes containing

Abbreviations used: VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; apo E, apolipoprotein E; apo A-I, apolipoprotein A-I.

Fig. 1. Distribution according to density of lipoprotein radioactivity before and after rat liver perfusion with [^3H]cholesterol-labelled blood derived from spleen perfusates

[^3H]Cholesterol-labelled spleens were perfused for 3 h with whole blood. The resulting blood was either incubated for 4 h at 37 °C (left-hand columns) or used as perfusate for an isolated liver for 4 h (right-hand columns); ■ represents radioactivity incorporated into cholesteryl ester. Incubation of the control blood for 4 h resulted in some equilibration of unesterified [^3H]cholesterol between the lipoprotein fractions. Values are means $\pm$ S.E.M. of 5 experiments.

approx. 35 μCi of [^3H]cholesterol at a haematocrit of 50 % in 0.9 % NaCl was injected into a tail vein of a rat. The spleen was isolated 3–4 h after injection, at which time the spleen had assimilated 15–20 % of the injected [^3H]cholesterol.

Method for perfusion of the spleen followed by perfusion of the liver

Details of the methods for the surgical isolation, apparatus for perfusion and preparation of the whole-blood perfusate have been described for the spleen (Mindham & Mayes, 1989b) and for the liver (Mayes & Felts, 1966). Briefly, the donor spleen was prepared from a rat anaesthetized with sodium pentobarbital (60 mg/kg body wt.), whose spleen had been labelled *in vivo* with [^3H]cholesterol. Pancreatic and adipose tissue in the vicinity of the spleen were removed and a cannula was tied into the coeliac artery. The spleen was flushed free of blood with modified Krebs & Henseleit (1932) bicarbonate buffer containing 3 % (w/v) BSA, mixed plasma amino acids and glucose, before an outflow cannula was tied into the portal vein. Once prepared, the spleen was connected into a recirculating perfusion circuit containing 110 ml of defibrinated rat blood at 30 % haematocrit and housed in a thermostatically controlled cabinet at 37 °C. The method of preparation of the blood perfusate avoided the use of heparin, which would otherwise cause the release and activation of lipoprotein lipase and consequent alteration of serum lipoproteins which might affect reverse cholesterol transport. The spleen was perfused at a flow rate of 1 ml/min per g, and the $p\text{O}_2$ was maintained at 100 mmHg (13.3 kPa). The donor liver was prepared from a rat anaesthetized with sodium pentobarbital. First the bile duct was cannulated, and then an inflow cannula was tied into the portal vein. The inferior vena cava was cut below the liver, and the liver was flushed free of blood with the modified Krebs & Henseleit (1932) buffer. An outflow cannula was tied into the inferior vena cava in the thorax and the abdominal vena cava was tied off. After removal of the spleen from the perfusion circuit, the liver preparation was transferred

to the thermostatically controlled cabinet and perfused *in situ* at approx. 1.5 ml/min per g with 90 ml of the [^3H]cholesterol-labelled blood resulting from spleen perfusion.

Analytical methods

After perfusion, spleen and livers were homogenized with 20 vol. of chloroform/methanol (2:1, v/v). Blood samples were centrifuged at 2000 g for 10 min, and portions of the serum ultracentrifuged at densities of 1.006, 1.050, 1.085, 1.125 and 1.25 g/ml to enable determination of mass and radioactivity of unesterified cholesterol and cholesteryl esters in lipoprotein fractions. Chloroform/methanol extracts prepared from the spleen, liver and portions of the perfusate and 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 were washed three times with 0.9 % NaCl, and extracted in 20 vol. of propan-2-ol/chloroform (11:7, v/v) (Rose & Oklander, 1965). Bile samples were extracted in 20 ml of chloroform/methanol (2:1, v/v), which was partitioned with 8 ml of distilled water. Cholesterol and cholesteryl esters in lipid extracts were separated by t.l.c. on silica gel G with hexane/diethyl ether/formic acid (40:10:1, by vol.) as the developing solvent. Radioactivity was assayed by liquid-scintillation counting. Dried lipid extracts and silica-gel bands from t.l.c. were counted for radioactivity with 18 ml of toluene-based scintillant containing 3 g of 2,5-diphenyloxazole/l and 0.25 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene/l. Incorporation of [^3H]cholesterol into bile acids was determined by counting radioactivity in 5 ml portions of the aqueous phase of the extracted bile with 12 ml of Instagel (Packard Instruments, Reading, Berks., U.K.). The chloroform extracts were evaporated under N_2 and redissolved in 0.2 ml of propan-2-ol for cholesterol estimation. Lipoprotein unesterified cholesterol in aqueous solutions and in propan-2-ol was determined enzymically by the method of Trinder (1969). Esterified cholesterol in the same samples was measured by addition of cholesterol esterase reagent

Table 1. Specific radioactivity and concentrations of cholesterol during sequential spleen and liver perfusion.

[^3H]Cholesterol-labelled spleens were perfused with whole blood as described in the text. After 3 h the spleen was removed and the perfusate used to perfuse an isolated liver for a further 4 h. The specific radioactivity of unesterified cholesterol is expressed relative to that in serum lipoproteins after spleen perfusion (22 d.p.m./nmol = 1.00). Values are means $\pm$ S.E.M. of 5 experiments. * Incubated for 4 h at 37 °C before analysis, i.e. for the same amount of time as the blood used for liver perfusion.

	Liver		Erythrocytes		Serum lipoproteins		
	Sp. radioactivity	Unesterified cholesterol: total (mg)	Sp. radioactivity	Unesterified cholesterol ($\mu\text{g/ml}$ of blood)	Sp. radioactivity	Unesterified cholesterol ($\mu\text{g/ml}$)	Cholesteryl ester ($\mu\text{g/ml}$)
Before spleen perfusion	—	—	0.0	383.2 $\pm$ 12.8	0.0	116.8 $\pm$ 4.3	284 $\pm$ 9.9
After 3 h spleen perfusion*	—	—	0.41	339.5 $\pm$ 9.0	1.00	127.4 $\pm$ 5.8	330 $\pm$ 10.4
After 3 h spleen perfusion plus 4 h liver perfusion	0.39	23.15 $\pm$ 1.1	0.25	367.4 $\pm$ 6.8	0.26	167.7 $\pm$ 4.7	291 $\pm$ 8.7

(System C; Boehringer, Lewes, E. Sussex, U.K.) after completion of the initial estimation of unesterified cholesterol, followed by measurement of the further increase in absorbance. Total protein was measured by a modification of the Lowry method (Peterson, 1977). Apolipoproteins were analysed by SDS/PAGE as described by Laemmli (1970). The gels were stained with Coomassie Blue (R250; Sigma Chemical Co., Poole, Dorset, U.K.) and scanned at 550 nm by a Beckman (model 25) spectrophotometer.

RESULTS

Reverse cholesterol transport from the spleen

In the first stage of all experiments, labelled spleens were perfused for 3 h. Each spleen initially contained a similar quantity of radioactivity (mean content, 4.4×10^6 d.p.m. of [^3H]cholesterol). There was a substantial release of [^3H]cholesterol to both erythrocytes and serum lipoproteins, which amounted to 28.3 % of that originally present in the spleen. This comprised a release of 13.3 ± 0.7 (S.E.M.) to serum lipoproteins and 15.0 ± 0.5 % to erythrocytes. Most (80 %) of the [^3H]cholesterol recovered in serum lipoproteins was in HDL ($d = 1.050\text{--}1.25$), of which 12.5 % was cholesteryl ester (Fig. 1). Incorporation of

Fig. 3. Disappearance of radioactivity from [^3H]cholesterol-labelled serum or erythrocytes derived from spleen perfusates upon uptake by perfused rat liver

Livers were perfused for 1 h with [^3H]cholesterol-labelled serum obtained after blood perfusion of [^3H]cholesterol-labelled spleens. The serum was reconstituted as blood with fresh unlabelled erythrocytes (Expt. B). Livers were also perfused with [^3H]cholesterol-labelled erythrocytes obtained after blood perfusion of the labelled spleens. The erythrocytes were reconstituted as blood with fresh unlabelled serum (Expt. C). ■, Serum of Expt. B; □, erythrocytes of Expt. B; ○, erythrocytes of Expt. C; ●, serum of Expt. C. Samples were kept at 37 °C until the 60 min time interval, when all were analysed simultaneously. Results are means $\pm$ S.E.M. of 4 experiments. The right-hand bars indicate the radioactivity recovered in the liver at 60 min in Expts. B and C.

Fig. 2. Disappearance of radioactivity from [^3H]cholesterol-labelled blood derived from spleen perfusates upon uptake by perfused rat liver

Livers were perfused for 4 h with [^3H]cholesterol-labelled blood obtained by blood perfusion of a [^3H]cholesterol-labelled spleen. ■, [^3H]Cholesterol in erythrocytes; ●, [^3H]cholesterol in serum lipoproteins. Values are means $\pm$ S.E.M. of 4–6 experiments, except where stated in parentheses. The right-hand bars indicate the radioactivity recovered in liver (L) and bile (B) at 4 h.

radioactivity into cholesteryl ester was limited almost entirely to the HDL fractions, and in addition the specific radioactivity of cholesterol in HDL, particularly in the $d = 1.125\text{--}1.25$ fraction, was greater than in other lipoprotein fractions. During perfusion with whole blood (Table 1), both unesterified and esterified cholesterol concentrations in serum increased significantly ($P < 0.01$). The apolipoprotein composition of lipoproteins before and after spleen perfusion did not change, and indicated a lack of lipoprotein synthesis or degradation by the spleen.

Uptake of [^3H]cholesterol by the liver

The cholesterol-labelled blood (90 ml), resulting from spleen perfusion (having a mean [^3H]cholesterol content of 7100 d.p.m./ml in serum lipoproteins and 5800 d.p.m./ml of blood in erythrocytes), was used in studies with the isolated liver in which

Table 2. Composition of bile following liver perfusion with [³H]cholesterol-labelled blood

Livers were perfused as described in the text with labelled whole blood for 3 h* or for 1 h (A). Livers were also perfused for 1 h with perfusates (B and C) which were derived from labelled whole blood. Values are means $\pm$ S.E.M. of 5 experiments* or 4 experiments (A, B and C).

Perfusate	Total bile acid produced (μ mol)	Percentage of perfusate [³ H]cholesterol in:		Percentage of liver uptake of [³ H]cholesterol in:		Ratio [³ H]-bile acid:bile cholesterol
		Bile acid	Bile cholesterol	Bile acid	Bile cholesterol	
Labelled whole blood*	9.82 $\pm$ 1.5	0.93 $\pm$ 0.07	0.16 $\pm$ 0.03	2.19 $\pm$ 0.17	0.32 $\pm$ 0.03	6.5:1
(A) Labelled whole blood	2.75 $\pm$ 0.18	0.33 $\pm$ 0.01	0.04 $\pm$ 0.00	0.46 $\pm$ 0.02	0.06 $\pm$ 0.01	7.9:1
(B) Labelled serum + unlabelled erythrocytes	2.6 $\pm$ 0.10	0.24 $\pm$ 0.01	0.04 $\pm$ 0.00	0.50 $\pm$ 0.03	0.07 $\pm$ 0.01	6.9:1
(C) Labelled erythrocytes + unlabelled serum	2.48 $\pm$ 0.41	0.09 $\pm$ 0.01	0.02 $\pm$ 0.00	0.50 $\pm$ 0.07	0.13 $\pm$ 0.02	4.0:1

Fig. 4. Changes in serum cholesterol and cholesteryl ester concentrations upon liver perfusion with blood derived from perfused spleens

Livers were perfused, as described in the text, for 1 h with: A, whole blood from spleen perfusion; B, serum from spleen perfusion reconstituted with fresh erythrocytes; C, normal serum reconstituted with spleen-perfused erythrocytes. Values for *P* represent significance of change in cholesterol or cholesteryl ester concentrations within groups. Values are means $\pm$ S.E.M. of 4 experiments. Zero-time samples were kept at 37 °C until the 60 min time interval, when all samples were analysed simultaneously.

uptake of [³H]cholesterol by the liver and excretion in bile was determined. Livers were perfused for either 4 h or 1 h.

When the liver was perfused for 4 h with [³H]cholesterol-labelled blood there was initially a rapid net uptake of radioactivity from both serum lipoproteins and erythrocytes, amounting to a total of $42.6 \pm 2.4\%$ (S.E.M.) of the radioactivity in the blood, comprising 26% from serum lipoproteins and 17% from erythrocytes (Fig. 2). After 1 h of perfusion there was no further net change in perfusate [³H]cholesterol content. Analysis of the distribution of radioactivity according to density revealed that most (73.4%) of the [³H]cholesterol taken up by the liver from lipoproteins was derived from the HDL₂ fraction (*d* 1.085–1.125) (Fig. 1). Determination of radioactivity in bile indicated the liver had excreted approx. 1% of the initial perfusate radioactivity and approx. 2% of the [³H]cholesterol taken up by the liver, mainly as [³H]bile acids (85%) and the remainder as unesterified [³H]cholesterol.

Throughout the perfusion, the liver secreted lipoproteins to the perfusate, e.g. very-low-density lipoprotein (VLDL) and HDL, in addition to taking up [³H]cholesterol derived from the labelled lipoproteins produced by the spleen. Therefore measurements of cholesterol mass and apolipoprotein composition of the perfusate are a resultant of both these processes. After 4 h of liver perfusion (Table 1) there was a significant increase in serum unesterified cholesterol content, from 127 μ g/ml to 168 μ g/ml ($P < 0.01$), and a decrease in cholesteryl ester, from a mean of 330 μ g/ml to 291 μ g/ml ($P < 0.02$). During liver perfusion there was no net change in apolipoprotein A-I (apo A-I) or apolipoprotein E (apo E) contents of HDL, which had mean values of 940 and 79 μ g/ml respectively. In addition, at termination of liver perfusion, the specific radioactivity of unesterified cholesterol in the liver was greater than that of the serum lipoproteins or erythrocytes, indicating a net uptake of labelled cholesterol or cholesteryl ester by the liver (Table 1).

Comparative roles of erythrocytes and lipoproteins in reverse cholesterol transport

In order to determine the relative importance of erythrocytes and lipoproteins to the transport or exchange of cholesterol with the liver and its subsequent excretion in bile as bile acids or cholesterol, the following procedure was adopted. [^3H]Cholesterol-labelled blood, formed during spleen perfusion, was separated by centrifugation into radioactive erythrocytes and radioactive serum, each of which were reconstituted with unlabelled fresh serum or erythrocytes respectively. Both perfusates which resulted contained similar quantities of labelled cholesterol. Livers were perfused with each of these perfusates or with the original unseparated [^3H]cholesterol-labelled blood. The experimental time for liver perfusions was decreased to 1 h, since our previous experiments indicated no further net change in perfusate radioactivity after 1 h. Also, a shorter time would diminish exchange of unesterified cholesterol between serum lipoproteins and erythrocytes.

The uptake of [^3H]cholesterol by the liver was greater from serum lipoproteins (49 %) than from erythrocytes (28 %) (Fig. 3). Although the total radioactivity detected in bile was greater from the labelled lipoproteins, the radioactivity in bile acids was similar in both series of experiments when expressed as a proportion of [^3H]cholesterol taken up by the liver (Table 2). However, the proportion secreted as unesterified cholesterol was not the same. Changes in serum cholesterol concentrations during liver perfusion are detailed in Fig. 4. In all cases there was a statistically significant increase in unesterified cholesterol. However, there was a significant difference in the treatment by the liver of serum cholesteryl ester between experiments in which the serum had been used previously in spleen perfusions and those which used fresh serum. Thus in experiments utilizing spleen-labelled serum (Expts. A and B) there was a small decrease in cholesteryl ester concentration, whereas in experiments with fresh serum (Expt. C) there was a significant increase in cholesteryl ester.

DISCUSSION

Spleens loaded with [^3H]cholesterol *in vivo* upon perfusion with blood *in vitro* release substantial quantities of radioactivity to both erythrocytes and serum lipoproteins. The release to erythrocytes could not have been due to the release of intact erythrocytes used to label the spleen *in vivo*, since we have shown in previous experiments, utilizing spleens loaded with ^{51}Cr -labelled erythrocytes, that the erythrocytes sequestered are rapidly metabolized and not released back intact to the perfusate (Mindham *et al.*, 1990). Therefore the substantial quantity of [^3H]cholesterol found in erythrocytes demonstrates that erythrocytes provide a route for the rapid exchange or transport of cholesterol between tissues. Unesterified cholesterol would therefore be expected to be transported from the spleen via the erythrocytes when the chemical potential gradient is favourable.

Analysis of [^3H]cholesterol in serum lipoproteins indicated that most of the radioactivity removed from the spleen was recovered in the HDL density range. The specific radioactivity of cholesterol was also much greater in HDL, and in the $d = 1.125$ – 1.21 fraction in particular. This fraction may therefore contain the primary lipoprotein acceptor of cholesterol from the spleen, or may be capable of a more rapid unesterified cholesterol exchange than that of other lipoproteins present in the perfusate. A net increase in the unesterified cholesterol and cholesteryl ester content of serum was observed after spleen perfusion, demonstrating that a transfer in cholesterol mass as well as in

radioactivity was taking place (Table 1). Part of this increase results probably from the continual sequestration of erythrocytes by the spleen throughout perfusion (Mindham *et al.*, 1990), followed by transport of cholesterol back out of the spleen to the lipoproteins and erythrocytes. In addition, a net transfer of unesterified cholesterol from erythrocytes to lipoproteins can occur, especially if the unesterified cholesterol concentration in lipoproteins is decreased by cholesteryl ester formation (Murphy, 1962; Glomset, 1972).

When livers were perfused with [^3H]cholesterol-labelled blood derived from spleen perfusions, there was a rapid uptake of radioactivity from both serum lipoproteins and erythrocytes. The distribution according to density of lipoprotein radioactivity before and after rat liver perfusion indicated that most of the uptake by the liver was derived from HDL, and in particular from HDL in the range $d = 1.085$ – 1.125 , corresponding to HDL₂. Determination of the specific radioactivity of cholesterol in lipoproteins and the liver indicated that there was an accumulation of the labelled cholesterol by the liver (Table 1). Also, there was a net uptake of cholesteryl ester mass, amounting to a 12 % decrease in the perfusate of livers perfused for 4 h. In contrast, an increase in serum cholesteryl ester was observed when, after spleen perfusion, labelled erythrocytes were reconstituted with fresh unlabelled serum and used as perfusate for the liver (Fig. 4). If it is assumed that cholesteryl ester production was the same in all three groups of experiments (Fig. 4), it shows that the liver has a preference for the removal of cholesteryl ester from serum which has been subjected to prior passage through the spleen. A particular species of lipoprotein may be elaborated during passage through the spleen, which is being targeted specifically for cholesteryl ester removal by the liver. During liver perfusion there was a net increase in unesterified cholesterol in lipoproteins in all experiments. This is due most likely to the liver secreting significant amounts of lipoproteins to the perfusate (Mayes & Felts, 1966; Laker & Mayes, 1979). The appearance of [^3H]cholesteryl ester in lipoproteins other than HDL (e.g. in VLDL) indicates that some of the radioactivity removed by the liver was esterified by acyl-CoA:cholesterol acyltransferase and then secreted in VLDL. It cannot have occurred as a result of a combination of lecithin:cholesterol acyltransferase and cholesteryl ester transfer-protein activities on unesterified cholesterol, since the latter activity is absent from rat blood (Barter & Lally, 1978; Mindham *et al.* 1990).

In all experiments with perfused livers, there was a significant incorporation of [^3H]cholesterol into the bile as bile acid and unesterified cholesterol. When [^3H]cholesterol-labelled erythrocytes derived from spleen perfusions were separated and reconstituted with unlabelled serum and then used as perfusate in isolated livers, labelled bile acids and unesterified cholesterol were secreted into the bile. This result confirmed the finding by Laker & Mayes (1985), that erythrocyte unesterified cholesterol is a significant source of these major bile constituents. Although erythrocytes transport approximately the same amount of [^3H]cholesterol from the spleen as the serum lipoproteins, they transport or exchange only half as much [^3H]cholesterol to the liver (Fig. 3). Nevertheless, the results indicated that [^3H]cholesterol taken up by the liver from erythrocytes is equally capable of conversion into bile acid and excretion in bile as that derived from serum lipoproteins, and implies that cholesterol is entering the same bile acid precursor pool.

No significant change in the concentrations of apo A-I or apo E were observed after spleen or liver perfusion. This result is compatible with the finding that the perfused spleen does not synthesize or secrete new apolipoproteins during reverse cholesterol transport (Mindham & Mayes, 1991). However, as the liver is one of the major organs for formation and secretion of

apolipoproteins, it is not possible to comment on the extent of the apolipoprotein uptake which may have accompanied cholesteryl ester removal by the liver. Evidence for the disassociation of the metabolism of apo A-I from cholesteryl ester in HDL has been provided by studies *in vivo* in the rat utilizing HDL doubly labelled with non-degradable tracers (Glass *et al.*, 1985) and in the removal of apo E-deficient HDL by the perfused rat liver (Arbeeny *et al.*, 1987).

In summary, our data demonstrate that in the rat the primary lipoprotein acceptor of unesterified cholesterol from the spleen resides in HDL (d 1.085–1.25), in which cholesteryl esters accumulate. During liver perfusion there is a rapid exchange or transport of unesterified cholesterol between the HDL and the liver, in particular with HDL₂ (d = 1.085–1.125). In addition, there is a net uptake of cholesteryl ester by the liver, which shows an increased propensity to remove cholesteryl ester from serum that has been used previously in spleen perfusion. There is a significant incorporation of radioactivity in bile, demonstrating the potential of this dual perfusion system for the study of the complete process of reverse cholesterol transport, i.e. transport of cholesterol from an extrahepatic tissue to the blood, followed by uptake into the liver and excretion into bile.

The expert technical assistance of Robert Collins and Michael Avella and the financial support of the British Heart Foundation are appreciated.

REFERENCES

- Arbeeny, C. M., Rifichi, V. A. & Eder, H. A. (1987) *Biochim. Biophys. Acta* **917**, 9–17
- Badimon, J. J., Badimon, L., Galvez, A., Dische, R. & Fuster, V. (1989) *Lab. Invest.* **60**, 455–461
- Barter, P. J. & Lally, J. I. (1978) *Biochim. Biophys. Acta* **531**, 233–236
- DeLamatre, J., Wolfbauer, G., Phillips, M. C. & Rothblat, G. H. (1986) *Biochim. Biophys. Acta* **875**, 419–428
- Gavish, D., Oschry, Y. & Eisenberg, S. (1987) *J. Lipid Res.* **28**, 257–267
- Glass, C., Pittman, R. C., Civen, M. & Steinberg, D. (1985) *J. Biol. Chem.* **260**, 744–750
- Glomset, J. A. (1968) *J. Lipid Res.* **9**, 155–167
- Glomset, J. A. (1972) *Am. J. Clin. Nutr.* **23**, 1129–1136
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Physiol. Chem.* **210**, 33–66
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Laker, M. E. & Mayes, P. A. (1979) *Biochem. Pharmacol.* **28**, 2813–2827
- Laker, M. E. & Mayes, P. A. (1985) *Biochem. Soc. Trans.* **13**, 142–143
- Mayes, P. A. & Felts, J. M. (1966) *Proc. Eur. Soc. Study Drug Toxic.* **7**, 16–29
- Miller, N. E. (1989) (ed.) *High Density Lipoproteins and Atherosclerosis II*, Elsevier Science Publishers, Amsterdam
- Miller, N. E., LaVille, A. & Crooke, D. (1985) *Nature (London)* **314**, 109–111
- Mindham, M. A. & Mayes, P. A. (1989a) *Protides Biol. Fluids* **36**, 401–407
- Mindham, M. A. & Mayes, P. A. (1989b) *Biochem. J.* **263**, 325–332
- Mindham, M. A. & Mayes, P. A. (1991) *Biochem. Soc. Trans.* **19**, 325S
- Mindham, M. A., Mayes, P. A. & Miller, N. E. (1990) *Biochem. J.* **268**, 499–505
- Murphy, J. (1962) *J. Lab. Clin. Med.* **60**, 86–109
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–350
- Reichl, D., Rudra, D. N., Myant, N. B. & Pflug, J. J. (1982) *Atherosclerosis* **44**, 73–84
- Rifkind, B. M. (1990) *Am. J. Cardiol.* **66**, 3A–6A
- Rose, H. G. & Oklander, M. (1965) *J. Lipid Res.* **6**, 428–431
- Stein, O., Stein, Y., LeFevre, M. & Roheim, P. S. (1986) *Biochim. Biophys. Acta* **878**, 7–13
- Trinder, P. (1969) *Ann. Clin. Chem.* **6**, 24–29

Received 7 May 1991/10 June 1991; accepted 17 June 1991