Reverse cholesterol transport in the isolated perfused rat spleen

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1. A method has been developed which enables the rat spleen to be loaded *in vivo* with [³H]cholesterol to a high specific radioactivity using cholesterol-labelled erythrocytes. The erythrocytes were shown to be rapidly degraded by the spleen and not released intact during subsequent perfusion. 2. When labelled spleens were perfused with whole blood or serum, lipoproteins in the high-density lipoprotein (HDL) range were shown to be the principal lipoprotein vehicles for the removal of cholesterol, the specific radioactivity of cholesterol being much greater in the HDL fractions than in other lipoproteins, particularly in the *d* 1.175–1.210 fraction. 3. The formation of [³H]cholesteryl ester was restricted to the major HDL fractions. 4. Experiments utilizing individual HDL fractions added to a basal perfusate indicated that HDL₁ (*d* 1.050–1.085) was of less importance in the removal of cholesterol from the spleen than HDL subfractions of higher density. Also, a decrease in density of the lipoproteins was observed during perfusion, concurrent with uptake of cholesterol, especially in the *d* 1.085–1.125 subfraction. 5. When [³H]cholesterol-labelled spleens were perfused with whole blood, about half of the radioactivity released was detected in erythrocytes, indicating a rapid exchange or transport of cholesterol. Thus erythrocytes could play an important role in the transfer of unesterified cholesterol when the chemical potential gradient is favourable.

INTRODUCTION

The relationship between atherosclerosis, coronary heart disease and plasma low-density lipoprotein (LDL) cholesterol has been firmly established in metabolic, genetic and epidemiological studies (Stokes & Mancini, 1988). Also, an independent inverse relationship between plasma high-density lipoprotein (HDL) cholesterol concentration and incidence of coronary heart disease has been well documented (Miller, 1989). It is generally accepted that LDL acts as a carrier of cholesterol to tissues, and that HDL has a role in the transfer of cholesterol from extrahepatic cells to the liver, prior to its conversion to bile acids and excretion in the bile, a process usually referred to as reverse cholesterol transport (Reichl & Miller, 1986). There is abundant evidence that certain subclasses of HDL have the ability to remove cholesterol from cells in culture (Miller, 1989). Studies *in vivo* have been much less numerous. In intact rabbits, cholesterol enrichment and a decrease in the density of circulating HDL were observed after tissues had been acutely loaded with cholesterol by intravenous infusion of native or acetylated human LDL (Miller *et al.*, 1985). In the same species, infusions of HDL were recently shown to inhibit the development of diet-induced atherosclerosis (Badimon *et al.*, 1989). In humans, the cholesterol that is mobilized from adipocytes during restriction of energy intake has been shown to enter plasma HDL in preference to other lipoproteins (Nestel & Miller, 1978). Other evidence *in vivo* that HDL is the principal acceptor of cell cholesterol has been provided by studies of lipoproteins in human peripheral lymph (Reichl *et al.*, 1982).

Both approaches to the study of reverse cholesterol transport that have been used to date have limitations. Studies in intact animals are difficult to interpret because of the complexity of the system. Thus the sources of cholesterol in plasma lipoproteins are difficult to identify due to continuous uptake and secretion of lipoprotein components by the various tissues. Studies in tissue culture have provided valuable data at the cellular level, but may not accurately reflect events in the extracellular matrix of tissues,

where lipoproteins are subjected to a sieving effect and probably also undergo considerable remodelling due to lipid transfer, particle fusion and the action of lipases (Reichl & Miller, 1986).

Some of these problems may be overcome by the use of organ perfusion. Therefore we have developed an isolated intact organ system using the rat spleen as a model for the study of cholesterol transport from an intact extrahepatic tissue (Mindham & Mayes, 1989). The spleen was chosen as it is the main site of destruction of erythrocytes and thrombocytes in man and in the rat (Clark, 1988) and consequently obtains large quantities of cholesterol from cell membranes, which must be re-transported from the organ in order to prevent cholesterol accumulation. The ability of the spleen to sequester erythrocytes was exploited to enable its labelling with radioactive cholesterol *in vivo*. The cholesteryl ester content of control non-perfused rat spleens was only 0.022 ± 0.002 mg (S.E.M. of six results), representing no more than 1% of total cholesterol. As this value did not change during perfusion and esterification of [³H]cholesterol was negligible, the data obtained represent the transport of cholesterol from a total cholesterol pool that is not significantly influenced by the cycle of esterification and hydrolysis that is prominent in some other tissues (Brown & Goldstein, 1983).

The isolated rat spleen preparation which we have employed has been used previously under similar conditions to study the metabolism of the spleen (Mindham *et al.*, 1987; Mindham & Mayes, 1989). The results from these studies provided the information necessary to ensure an adequate fuel supply and oxygenation of the preparation. Thus results obtained under these conditions are likely to represent accurately the process of cholesterol transport from this organ *in vivo*.

EXPERIMENTAL

Animals and materials

Male Wistar rats were kept under constant day length (12 h) and temperature (25 °C) and fed a standard pelleted diet. Animals

Abbreviations used: LDL, low-density lipoprotein; HDL, high-density lipoprotein; apo, apolipoprotein.

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weighing 340–360 g and 350–450 g were used as spleen donors and blood donors respectively. BSA (fraction 5) was supplied by ICN Biomedicals, High Wycombe, Bucks., U.K. [$1\alpha,2\alpha$ - ^3H]Cholesterol and sodium [^{51}Cr]chromate were obtained from Amersham International, Amersham, Bucks., U.K. Cholesterol oxidase and other chemicals were obtained from BDH, Dagenham, Essex, U.K.

Methods of perfusion

Details of the methods for surgical isolation of the spleen, apparatus for perfusion and preparation of the whole blood perfusate have been described previously (Mindham & Mayes, 1989). Briefly, the donor spleen was prepared from a rat anaesthetized with sodium pentobarbital (60 mg/kg body wt.). After removing pancreatic and adipose tissue in the vicinity of the spleen, an inflow cannula was tied into the coeliac artery, and the spleen was flushed free of blood with Krebs & Henseleit (1932) bicarbonate buffer containing 3% (w/v) BSA. The outflow cannula was tied into the portal vein. Once prepared, the spleen was transferred to a thermostatically controlled cabinet at 37 °C and connected into a recirculating perfusion circuit containing approx. 50 ml of perfusate. The method of preparation of the blood perfusate avoided the use of heparin, which would cause the release and activation of lipoprotein lipase and a consequent alteration of serum lipoproteins which might affect reverse cholesterol transport. When whole defibrinated rat blood was used as the perfusate, the flow rate was 1 ml/min per g, and the $p\text{O}_2$ was maintained at 100 mmHg (13.3 kPa). In experiments that did not have erythrocytes in the perfusate, the flow rate was increased to 4 ml/min per g, and the $p\text{O}_2$ was raised to 500 mmHg (66.5 kPa).

Preparation of HDL fractions

HDL fractions were prepared from rat serum by ultracentrifugation in 6.5 ml cellulose tubes (Kontron, Watford, Herts., U.K.) in a fixed-angle rotor (MFT-50.6) of an MSE Europa 55 ultracentrifuge. The serum was centrifuged sequentially after stepwise addition of solid KBr to raise d progressively to 1.050, 1.085, 1.125 and 1.210. Each centrifugation was at 4 °C for 24 h at 120 000 g . The top 1 ml fraction was harvested by cutting the tubes with a tube slicer. Each fraction was 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.

Labelling of donor spleens

[^3H]Cholesterol (100 μCi) in acetone (0.1 ml) was added dropwise to 2.5 ml of stirred rat serum at 37 °C. A stream of N_2 was directed at the surface to remove the acetone. After 30 min, 2.5 ml of packed erythrocytes, ampicillin (0.05 mg/ml) and glucose (10 mg/ml) were added, and the reconstituted blood was incubated overnight for 15 h at 37 °C in a shaking water bath. In some experiments the erythrocytes were labelled with ^{51}Cr , in addition to [^3H]cholesterol, by adding 100 μCi of sodium [^{51}Cr]chromate in 50 μl of 0.9% NaCl (Van Kampen *et al.*, 1966). After incubation, the blood was centrifuged at 2000 g for 10 min, and then the erythrocytes were washed twice with 0.9% NaCl. A 1 ml portion of erythrocytes, at a haematocrit of 50% in 0.9% NaCl, was injected into the tail vein of a rat. The spleen was isolated 4–5 h after injection.

Analytical methods

After perfusion, the spleen was homogenized with 75 ml of chloroform/methanol (2:1, v/v). The perfusate was centrifuged to remove erythrocytes, and portions of the serum were centrifuged at the densities specified to enable determination of

mass and radioactivity in unesterified cholesterol and cholesteryl esters. Chloroform/methanol extracts prepared from the spleen, portions of the perfusate and the lipoproteins obtained by ultracentrifugation were shaken with two-fifths of their volume of 0.03 M-HCl, and the chloroform layer taken for analysis. The erythrocytes, when present, 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). Cholesterol and cholesteryl ester in lipid extracts were separated by t.l.c. on silica gel G (Merck Chemicals; supplied by BDH) using hexane/diethyl ether/formic acid (40:10:1, by vol.) as the developing solvent. Lipid extracts were blown down and redissolved in 0.2 ml of propan-2-ol for cholesterol estimation. Unesterified cholesterol in aqueous solutions and in propan-2-ol was determined enzymically by the method of Trinder (1969). In aqueous solutions, 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. In solutions of propan-2-ol, cholesteryl ester was determined by the method of Rudel & Morris (1973).

Radioactivity was assayed by liquid scintillation counting. Dried lipid extracts and silica gel bands from t.l.c. were counted with 18 ml of toluene-based scintillant, containing 3 g of 2,5-diphenyloxazole/l and 0.25 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene/l.

Total protein was measured by a modification of the Lowry method (Peterson, 1977). Apolipoproteins were analysed by PAGE in the presence of SDS as described by Laemmli (1970). Lipoproteins were first delipidated by addition of 0.1 ml of 3.6 M-sodium deoxycholate to 1 ml of the lipoproteins prepared by ultracentrifugation. After standing for 15 min at room temperature, 0.1 ml of 4.4 M-trichloroacetic acid was added. The precipitated apolipoproteins were pelleted by centrifugation at 3000 g for 20 min. The apolipoproteins were then redissolved in 0.25 ml of sample buffer, neutralized by dropwise addition of 0.5 M-NaOH until the indicator Bromophenol Blue turned to blue, incubated for 5 min at 80 °C, and finally made up to 0.5 ml with distilled water. The sample buffer consisted of 0.125 M-Tris buffer (pH 6.8) containing 41 mg of SDS/ml, 0.02 mg of Bromophenol Blue/ml, 20% (v/v) glycerol and 10% (v/v) 2-mercaptoethanol. The samples, containing up to 2 mg of protein/ml, were layered in wells in the stacking gel and run at a constant current of 40 mA at 10 °C. The gels were stained with Coomassie Blue (R250; Sigma Chemical Co., Poole, Dorset, U.K.).

RESULTS

Labelling of the spleen with [^3H]cholesterol

The tissue distribution of [^3H]cholesterol, following injection of labelled erythrocytes into the tail vein of rats, was determined at intervals by measuring the radioactive content of erythrocytes, serum, spleen and liver in rats anaesthetized with sodium pentobarbital (60 mg/kg body wt.). The spleen and liver were flushed free of blood with Krebs & Henseleit bicarbonate buffer containing 3% (w/v) BSA, introduced by means of a cannula inserted in the coeliac artery. The results (Fig. 1) showed that there was a rapid accumulation of [^3H]cholesterol in both spleen and liver, with a concurrent decrease of radioactivity in blood erythrocytes. The radioactive content of the spleen was at its highest level at 5 h after injection, when it represented 21% of the injected dose, after which it decreased. The radioactive content of the liver and plasma were at their maxima several hours after this.

In order to ascertain whether the uptake of [^3H]cholesterol by

Fig. 1. Distribution of radioactivity in tissues after intravenous injection of [^3H]cholesterol-labelled erythrocytes in rats

A 1 ml portion of labelled erythrocytes was injected into the tail vein of rats. The radioactive content of erythrocytes (Δ), spleen ($\bullet$) and liver ($\blacksquare$) were determined at intervals as described in the text.

the spleen represented sequestration of erythrocytes, and whether these were released intact into the perfusate during subsequent perfusion, the erythrocytes were labelled with ^{51}Cr in addition to [^3H]cholesterol. In contrast with [^3H]cholesterol, ^{51}Cr is firmly bound to protein in the erythrocytes. Therefore ^{51}Cr radioactivity detected in erythrocytes during subsequent perfusion of the spleen would be due to the release of intact erythrocytes used to label the organ. Spleens isolated at 4 h after injection contained a similar proportion of ^{51}Cr (15.5%) to the uptake recorded for [^3H]cholesterol. During subsequent perfusion for 3 h, only 1.5% of the ^{51}Cr sequestered by the spleen *in vivo* was released in erythrocytes. In contrast, the perfusate serum was found to contain a substantial amount of radioactivity (44.8%), indicating that a rapid degradation of the injected erythrocytes had taken place in the spleen.

Reverse cholesterol transport to whole blood and serum perfusates

In two series of experiments, [^3H]cholesterol-labelled spleens were perfused with whole blood at 30% haematocrit or with serum for 3 h. A substantial amount of [^3H]cholesterol was released from the spleen at a constant rate into either perfusate (Fig. 2). When the perfusate was serum, 97.5% of the [^3H]cholesterol released was recovered in serum lipoproteins ($d < 1.210$). When the perfusate was whole blood, only 48.7% was recovered in serum lipoproteins, and the remainder was in erythrocytes. However, the specific radioactivity of cholesterol in the erythrocytes was only one-quarter of that in lipoproteins. Thus when whole blood was replaced by serum as perfusate, the total radioactivity recovered from lipoproteins was increased by 2-fold. However, the proportional distribution of [^3H]cholesterol among the lipoprotein fractions, separated according to density, was similar in both series of experiments (Fig. 3). HDL fractions (d 1.063–1.210) were found to contain 78% of the radioactivity released by the spleen. Furthermore, the specific radioactivity of cholesterol in the HDL, particularly in the d 1.175–1.210 fraction, was much greater than in other lipoprotein fractions. A similar proportion of [^3H]cholesteryl ester was detected in the d 1.075–1.175 and d 1.175–1.210 fractions of serum, perfused either with or without erythrocytes, which in total represented 12.7% of the [^3H]cholesterol released into the serum lipoproteins. No [^3H]cholesteryl ester was present in lipoproteins of $d < 1.075$.

Fig. 2. Release of [^3H]cholesterol from labelled perfused rat spleens

Spleens were perfused for 3 h with whole blood or serum. $\bullet$, Release of radioactivity to whole blood; $\circ$, release of radioactivity to serum; $\square$, release of radioactivity to erythrocytes in whole blood; Δ , release of radioactivity to serum lipoproteins in whole blood. Values are means $\pm$ S.E.M. for four perfusions.

Fig. 3. Distribution of radioactivity released to serum lipoproteins from [^3H]cholesterol-labelled spleens

Spleens were perfused for 3 h with either serum ($\square$) or whole blood ($\blacksquare$) after labelling with [^3H]cholesterol, and the lipoproteins were separated according to density by ultracentrifugation. Radioactivity incorporated into cholesteryl ester is indicated (hatched). Values are means $\pm$ S.E.M. of four perfusions.

During perfusion with whole blood, total erythrocyte cholesterol significantly ($P < 0.05$) decreased by a mean of 3.5% ($540 \pm 33 \mu\text{g}$, mean $\pm$ S.E.M.) and the cholesterol content of the spleen (when compared with non-perfused controls) increased significantly from 2.45 ± 0.08 to $2.86 \pm 0.11 \text{ mg}$ ($P < 0.01$). These changes presumably reflected a continuous sequestration of erythrocytes throughout the time of perfusion.

Reverse cholesterol transport to defined HDL fractions

HDL fractions of d 1.050–1.085, 1.085–1.125 and 1.125–1.210 were prepared by ultracentrifugation (see the Experimental section). The compositions of these three fractions differed considerably (Table 1). The majority of cholesteryl ester was in the d 1.085–1.125 fraction, which also contained the greatest quantity of total apolipoprotein. The fractions of d 1.050–1.085

Table 1. Composition of HDL fractions isolated from rat serum

The HDL fractions were prepared and analysed as described in the Experimental section. Values for cholesteryl ester are expressed as μg of cholesterol.

<i>d</i>	Unesterified cholesterol ($\mu\text{g}/\text{ml}$ of serum)	Cholesteryl ester ($\mu\text{g}/\text{ml}$ of serum)	Total apolipoprotein ($\mu\text{g}/\text{ml}$ of serum)
1.050–1.085	25.4 ± 1.0	83.7 ± 4.2	122.0 ± 4.9
1.085–1.125	36.1 ± 1.9	175.3 ± 6.7	417.6 ± 16.6
1.125–1.210	24.5 ± 1.8	70.8 ± 3.1	274.7 ± 13.2

Fig. 4. Apolipoprotein composition of HDL fractions isolated from rat serum and used in perfusions of rat spleen

Lipoprotein fractions were obtained by ultracentrifugation and delipidated as described in the Experimental section. Apolipoproteins were separated by discontinuous PAGE in the presence of SDS using a 10% resolving gel and a 3% stacking gel. Proteins were stained with Coomassie Blue. Apolipoproteins were derived from the following lipoprotein fractions: lane A, *d* 1.050–1.085; lane B, *d* 1.085–1.125; lane C, *d* 1.125–1.210; lane D, standard proteins.

and 1.125–1.210 contained lesser amounts of both unesterified and esterified cholesterol, although proportionally more protein was associated with the more dense fraction. The HDL of *d* 1.050–1.085 contained only apolipoprotein E (apo E) (Fig. 4), and had a composition similar to that of HDL₁ described in studies of zonal centrifugation of rat lipoproteins (Lusk *et al.*, 1979). In addition, HDL_c, a lipoprotein of similar density containing a high proportion of cholesterol, which also contains apo E as its only apolipoprotein, is found in large quantities in animals fed on a high cholesterol diet (Mahley, 1978). The relative proportions of apolipoproteins in the *d* 1.085–1.125 and 1.125–1.210 fractions were similar, and both contained apo A-I as the most abundant protein constituent. In addition, these fractions contained substantial quantities of apo A-IV, and smaller quantities of apo E, apo A-II and C apolipoproteins.

The lipoprotein fractions prepared by ultracentrifugation were added to a basal perfusate at their normal serum concentrations (see Table 1). The basal perfusate consisted of Krebs & Henseleit bicarbonate buffer containing BSA (60 mg/ml), glucose (2.51 mg/ml), mixed amino acids (0.67 mg/ml) and oleic acid (0.5 $\mu\text{mol}/\text{ml}$). Previous investigations (Mindham & Mayes, 1989) had indicated that this perfusate provided sufficient substrates for optimal spleen metabolism.

In a series of experiments [^3H]cholesterol-labelled spleens were perfused for 3 h with the basal perfusate alone, or with the basal perfusate to which was added one of the HDL fractions prepared by ultracentrifugation. Experiments utilizing the basal perfusate alone indicated that 8.8% of the [^3H]cholesterol in the spleen was released during 3 h perfusions (Fig. 5). A substantial quantity of [^3H]cholesterol from the spleen, representing approx. 17% of the initial radioactivity present, was recovered from both the *d* 1.085–1.125 and the *d* 1.125–1.210 fractions. The HDL fraction of *d* 1.050–1.085 removed only 12.4% of radioactivity. In addition, there was a net release of cholesterol mass into all of the perfusates (Table 2). Again this was most marked with the *d* 1.085–1.125 and *d* 1.125–1.210 fractions, where the unesterified cholesterol content of the perfusate increased by approx. 10% during the perfusion. [^3H]Cholesteryl ester was observed to accumulate only in the *d* 1.085–1.125 fraction, and represented only 2.7% of the total radioactivity released by the spleen.

Fig. 5. Release of radioactivity from labelled perfused rat spleen to defined HDL fractions

The following lipoprotein fractions were added to a basal perfusate: $\square$, *d* 1.085–1.125; $\blacksquare$, *d* 1.125–1.210; $\blacklozenge$, *d* 1.050–1.085; $\diamond$, basal perfusate alone. Results are means $\pm$ S.E.M. from four perfusions.

Table 2. Release of cholesterol mass in perfusions of rat spleen

Spleens were perfused for 3 h with perfusates of different compositions. The basal perfusate consisted of modified Krebs & Henseleit buffer, plus additions as described in the text, to which defined fractions of lipoproteins were added at concentrations present in serum. Values are means $\pm$ S.E.M. from four perfusions. Values for cholesteryl ester are expressed as μg of cholesterol.

Perfusate	Net increase in perfusate:		
	Unesterified cholesterol (μg)	Cholesteryl ester (μg)	Total cholesterol (μg)
Whole serum	105.8 ± 6.2	68.4 ± 2.9	174.2
Basal perfusate alone	91.1 ± 5.5	1.4 ± 0.2	92.5
Basal perfusate + <i>d</i> 1.050–1.085 lipoproteins	80.6 ± 3.5	-9.1 ± 0.1	71.5
Basal perfusate + <i>d</i> 1.085–1.125 lipoproteins	184.5 ± 11.2	22.1 ± 0.1	206.6
Basal perfusate + <i>d</i> 1.125–1.210 lipoproteins	144.0 ± 7.4	-8.1 ± 0.1	135.9

Fig. 6. Distribution according to lipoprotein density of radioactivity released from $[^3\text{H}]$ cholesterol-labelled spleens perfused with defined HDL fractions

The defined HDL fractions were added to a basal perfusate of modified Krebs & Henseleit buffer at the concentrations present in serum. (a) No added lipoprotein; (b), (e) d 1.050–1.085 added; (c), (f) d 1.085–1.125 added; (d), (g) d 1.125–1.210 added. After 3 h perfusion, the densities analysed were A, $d < 1.050$; B, d 1.050–1.085; C, d 1.085–1.125; D, d 1.125–1.210; E, $d > 1.21$. The release to the basal perfusate (a) has been subtracted in parts (e), (f) and (g). No apolipoproteins could be detected, with the methods described, in the density fractions analysed in Fig. 6(a).

An analysis according to lipoprotein density of $[^3\text{H}]$ cholesterol released by the spleen when perfused with defined lipoprotein fractions is shown in Fig. 6. The majority of the $[^3\text{H}]$ cholesterol released to the basal perfusate was observed in the d 1.125–1.210 and the $d > 1.21$ fractions (Fig. 6a). The release recorded to the HDL fractions contains a component of the release to the basal perfusate (Figs. 6b–6d). This component may be subtracted to give an apparent net release to each of the added lipoprotein fractions (Figs. 6e–6g). The distribution of radioactivity after this subtraction indicates that the majority of HDL that did not remain in the same density range as that added was found in less dense fractions following perfusion. This was most marked on addition of the d 1.085–1.125 fraction, for which 49% of $[^3\text{H}]$ cholesterol remained at d 1.085–1.125, 40% moved into lighter fractions and 10% to more dense fractions. The corresponding data for cholesterol mass were 61%, 31% and 8%

Table 3. Specific radioactivity of unesterified cholesterol in different perfusate components at the termination of perfusion of $[^3\text{H}]$ cholesterol-labelled rat spleens

Spleens were perfused for 3 h with each of the perfusates indicated. The final mean specific radioactivity of spleen cholesterol was 920 d.p.m./ μg . Values are means $\pm$ S.E.M. from four experiments. * Added to modified Krebs & Henseleit buffer.

	Total unesterified cholesterol (μg)	Specific radioactivity of unesterified cholesterol (% of final specific radioactivity of spleen)
Spleen	2610	100
Erythrocytes (component of whole blood)	15750	3.2 ± 0.2
Serum (component of whole blood)	3780	13.0 ± 0.7
Serum (without erythrocytes)	5400	16.9 ± 0.6
d 1.050–1.085 serum fraction*	1221	19.6 ± 1.2
d 1.085–1.125 serum fraction*	1808	30.4 ± 2.6
d 1.125–1.210 serum fraction*	1247	46.4 ± 3.7
Modified Krebs & Henseleit buffer alone	278	68.7 ± 4.9

respectively. The extent to which the results can be corrected by removing the component of the release to the basal perfusate is problematic, since the added HDL and the basal perfusate may compete for the available cholesterol. Thus it is likely that the subtraction in the higher density fractions (columns D and E, Fig. 6) will be of greater magnitude than that actually occurring, and this is indicated by the negative data in columns D and E in Figs. 6(e) and 6(f).

DISCUSSION

The injection of $[^3\text{H}]$ cholesterol-labelled erythrocytes is shown to be an effective method for loading the spleen with cholesterol *in vivo* to a high specific radioactivity (Table 3). The present studies *in vivo*, and perfusion experiments utilizing spleens loaded with ^{51}Cr -labelled erythrocytes, indicated that a substantial proportion of the erythrocytes is taken up into the spleen and metabolized rapidly, since a substantial amount of ^{51}Cr was released back to the perfusate serum proteins. The uptake of ^{51}Cr -labelled erythrocytes paralleled the uptake of cholesterol-labelled erythrocytes.

The subsequent reverse transport of cholesterol which we have observed from the spleen into whole blood or serum provides direct confirmation in an intact physiological system that the HDL density range contains the principal lipoprotein vehicle for this process. We found that the specific radioactivity of cholesterol was much greater in the HDL fractions than in other lipoproteins, and in particular in the d 1.175–1.210 fraction. This result demonstrates either that these particles include the primary acceptors of cell cholesterol in the spleen or that their unesterified cholesterol exchanges with membrane cholesterol more rapidly than does that of the other lipoproteins that were present in the perfusate. It is also of interest that the specific radioactivity of unesterified cholesterol in the apo-E-rich d 1.063–1.075 fraction, corresponding to HDLc, was not elevated, unlike that in the remainder of the HDL. This fraction has been implicated in reverse cholesterol transport from cultured macrophages (Brown & Goldstein, 1983), but there was no evidence of this in the present work, in spite of the fact that the spleen is known to contain a high concentration of macrophages (Bohnsack &

Brown, 1986). The formation of [^3H]cholesteryl ester was restricted to the major HDL fractions, confirming that esterified cholesterol derived from the lecithin:cholesterol acyltransferase reaction is incorporated into HDL (Barter *et al.*, 1981), and that cholesteryl ester exchange protein activity is absent from rat blood (Barter & Lally, 1978).

A substantial quantity of [^3H]cholesterol was found in erythrocytes. This indicates that erythrocytes provide a route for a rapid exchange or transport of cholesterol and suggest that when the chemical potential gradient is favourable, unesterified cholesterol would be expected to be transported from the spleen via the erythrocytes.

Studies utilizing the full complement of lipoproteins and erythrocytes do not provide unequivocal information about the primary acceptors of cholesterol among the blood constituents, because of the exchange of unesterified cholesterol that occurs between them. Therefore we undertook further studies with defined HDL fractions added to a basal perfusate. The composition of the HDL fractions used was similar to that employed in other studies (Lasser *et al.*, 1973; Lusk *et al.*, 1979; Van't Hooft *et al.*, 1981). During 3 h perfusions there was a significant release of radioactivity and cholesterol mass to all of the HDL fractions tested. However, in contrast with experiments utilizing whole blood or serum, the rate of release of [^3H]cholesterol to the basal perfusate and HDL fractions declined during the period of perfusion. This was most marked with the basal perfusate, where there was no added lipoprotein present, and with the d 1.050–1.085 fraction, which removed less radioactivity and cholesterol mass than the denser fractions, suggesting that this fraction has a lower capacity for the removal of cholesterol. Two reasons for the relatively high proportion of [^3H]cholesterol released into the basal perfusate, which did not contain any detectable apolipoproteins either before or after perfusion, are suggested by the work of Mendel & Kunitake (1988). They showed that cholesterol-containing membrane fragments were released into the medium of cultured fibroblasts. It is possible that, in our work, membrane fragments of high specific radioactivity were released by the spleen during phagocytosis of the labelled erythrocytes. In addition, the above investigators showed that efflux of cholesterol was increased by the presence of albumin, a finding which we can confirm. New formation of [^3H]cholesteryl ester was detected only in the added d 1.085–1.125 fraction, where the amount formed was only one-fifth of that in whole blood or serum. This may be due to a partial loss of lecithin:cholesterol acyltransferase activity during preparation of the HDL fraction, or to the fact that some activity resides in the d > 1.210 fraction which was not present during these perfusions.

Analysis according to lipoprotein density following perfusions with defined HDL fractions indicated a decrease in density of a proportion of the HDL added. This is compatible with an increase in the cholesterol content of these fractions arising from reverse cholesterol transport (see Table 2).

Measurements of cholesterol mass, in parallel with measurements of radioactivity, showed a net movement into the perfusate in all experiments. Erythrocytes showed a decrease in cholesterol mass, probably due in part to sequestration by the spleen throughout the perfusion, and in part to transfer of unesterified cholesterol to HDL and esterification therein by lecithin:cholesterol acyltransferase (Murphy, 1962; Glomset, 1972). A comparison of the specific radioactivities of cholesterol in the components of the perfusate and spleen following perfusion is shown in Table 3. In general there was a decrease in specific radioactivity as the initial total perfusate cholesterol content increased. Thus, as would be expected on the basis of isotope dilution, experiments utilizing whole blood or serum containing

a substantial mass of cholesterol exhibited a lower specific radioactivity in perfusate cholesterol than perfusates containing less endogenous cholesterol. The linear increase in the rate of [^3H]cholesterol released by the spleen in the former experiments is an indication that equilibration of ^3H was still proceeding rapidly at termination of perfusion. In contrast, there was a marked decrease in the rate of release of [^3H]cholesterol to the basal perfusate, particularly in the final 1 h of perfusion (Fig. 5), in which the cholesterol had a specific radioactivity of nearly 70% of that in spleen. It is of particular significance that the spleen released greater quantities of radioactivity and cholesterol mass to the HDL fraction of d 1.125–1.210, and at a higher specific radioactivity, than to the d 1.050–1.085 fraction, despite the fact that these two fractions contained a similar total amount of cholesterol. This result indicates that the d 1.125–1.210 fraction, and to a lesser extent the d 1.085–1.125 fraction, of HDL have an increased propensity for reverse transport of unesterified cholesterol in addition to the general ability around this density range to synthesize cholesteryl ester. Whether these two properties are related is not apparent from the present work.

If the radioactivity derived from labelled erythrocytes was uniformly distributed throughout spleen cholesterol, the net gain in perfusate cholesterol would be expected to be of the order of 230 μg for the basal perfusate and 760 μg for the experiments with whole serum, which removed the most radioactivity. In all cases the net gain in perfusate cholesterol was less than predicted (Table 2), indicating that the cholesterol pool in the sequestered erythrocytes had not fully equilibrated with the remainder of the spleen cholesterol.

The formation of cholesteryl ester in HDL removes unesterified cholesterol from the exchangeable pool, and may have been important in the removal of cholesterol from the spleen, by maintaining a potential gradient of cholesterol between it and the perfusate. In the rat, cholesteryl esters produced by the lecithin:cholesterol acyltransferase reaction *in vivo* are quantitatively retained in HDL, but in species possessing cholesteryl ester transfer protein activity, transfer of cholesteryl ester from HDL to apo-B-containing lipoproteins, followed by uptake of the latter into tissues possessing LDL receptors (apo B,E receptors), promotes further recycling of cholesterol.

The present results have demonstrated a rapid exchange of cholesterol between spleen, serum lipoproteins and erythrocytes. Studies *in vivo* have indicated that the same occurs when blood flows through other organs, although the rate of exchange shows important regional differences (Chobanian & Hollander, 1962; Wilson, 1970). The unesterified cholesterol of lipoproteins, erythrocytes and organs whose cholesterol is rapidly exchangeable behaves as a single pool (Wilson, 1970). Net reverse transport of cholesterol from tissues through plasma to the liver can only be assured by introducing non-equilibrium steps, such as cholesteryl ester formation by lecithin:cholesterol acyltransferase, formation of bile acids in the liver and secretion of cholesterol into the bile.

In summary, our data demonstrate that in the rat the primary acceptor of unesterified cholesterol from the spleen resides in the d 1.175–1.210 fraction of HDL, that cholesteryl esters derived from this accumulate principally in the d 1.075–1.175 fraction, and that these processes are associated with a decrease in the density of HDL particles. Although we have no evidence that the results are not of general applicability, they cannot be assumed to reflect processes in organs whose ultrastructure and cellular composition differ from those of the spleen. Also, they provide no information on the later stages of reverse cholesterol transport, whereby cholesterol is delivered to hepatocytes. Current evidence indicates that this occurs by multiple routes involving several

classes of lipoproteins (Reichl & Miller, 1986), and possibly also erythrocytes (Laker & Mayes, 1985).

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REFERENCES

- 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
- Barter, P. J., Ha, Y. C. & Calvert, G. D. (1981) *Atherosclerosis* **38**, 165–175
- Bohnsack, J. F. & Brown, E. J. (1986) *Annu. Rev. Med.* **37**, 49–59
- Brown, M. S. & Goldstein, J. L. (1983) *Annu. Rev. Biochem.* **52**, 223–261
- Chobanian, A. V. & Hollander, W. (1962) *J. Clin. Invest.* **41**, 1732–1737
- Clark, M. R. (1988) *Physiol. Rev.* **68**, 503–554
- Glomset, J. (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. (1985) *Biochem. Soc. Trans.* **13**, 142–143
- Lasser, N. L., Roheim, P. S., Edelstein, D. & Eder, H. A. (1973) *J. Lipid Res.* **14**, 1–8
- Lusk, L. T., Walker, L. F., DuBien, L. H. & 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. & Ontko, J. A., eds.), pp. 181–197, American Physiological Society, Bethesda, MD
- Mendel, C. H. & Kunitake, S. T. (1988) *J. Lipid Res.* **29**, 1171–1178
- Miller, N. E. (ed.) (1989) *High Density Lipoproteins and Atherosclerosis*, vol. 2, Elsevier Science Publishers, Amsterdam
- Miller, N. E., La Ville, A. & Crook, D. (1985) *Nature (London)* **314**, 109–111
- Mindham, M. A. & Mayes, P. A. (1989) *Biochem. J.* **263**, 325–332
- Mindham, M. A., Mayes, P. A. & Miller, N. E. (1987) *Biochem. Soc. Trans.* **15**, 1142–1143
- Murphy, J. (1962) *J. Lab. Clin. Med.* **60**, 86–109
- Nestel, P. J. & Miller, N. E. (1978) in *High Density Lipoproteins and Atherosclerosis* (Gotto, A. M., Miller, N. E. & Oliver, M. F., eds.), pp. 51–54, Elsevier Science Publishers, Amsterdam
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–350
- Reichl, D. & Miller, N. E. (1986) *Clin. Sci.* **70**, 221–231
- Reichl, D., Rudra, D. N., Myant, N. B. & Pflug, J. J. (1982) *Atherosclerosis* **44**, 73–84
- Rose, H. G. & Oklander, M. (1965) *J. Lipid Res.* **6**, 428–431
- Rudel, L. L. & Morris, M. D. (1973) *J. Lipid Res.* **14**, 364–366
- Stokes, J. & Mancini, M. (eds.) (1988) *Hypercholesterolemia: Clinical & Therapeutic Implications*, *Atherosclerosis Reviews*, vol. 18, Raven Press, New York
- Trinder, P. (1969) *Ann. Clin. Chem.* **6**, 24–29
- Van Kampen, E. J., Reinkingh, W. A. & Heerspink, W. (1966) *Clin. Chim. Acta* **13**, 52–60
- Van't Hooft, F. M., Van Gent, T. & Van Tol, A. (1981) *Biochem. J.* **196**, 877–885
- Wilson, J. D. (1970) *J. Clin. Invest.* **49**, 655–665

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