Role of HDL_1 in cholesteryl ester uptake in rats

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Abstract It has been suggested that apoE may play a central role in reverse cholesterol transport in rats. By this hypothesis, cholesteryl esters (CE) accumulate in high density lipoprotein (HDL) particles, which acquire apoE at the expense of apoA-I, and the apoE targets them for rapid hepatic uptake. However, the pathway has not been directly assessed in vivo. We directly traced the metabolism of HDL_1 cholesteryl esters in rats. To do this, rat HDL_1 was labeled in its apoE and CE moieties, and HDL_2 free of apoE was labeled in its apoA-I and CE moieties; ^{14}C- or ^{3}H-labeled cholesteryl-oleyl ether traced the CE moieties and the ^{125}I- or ^{131}I-labeled N-methyltyramine cellobiose (NMTC) ligand traced the apolipoprotein moieties. The labeled HDLs were injected, plasma decays were followed, and tissues were examined after 24 h. ApoE tracer decayed from plasma 2.4-times faster than HDL_1 CE and 1.8-times faster than HDL_2 CE. HDL_1 CE decayed significantly more slowly than HDL_2 CE (0.75-times). As expected, hepatic uptake of HDL_2 CE was mostly by selective (indirect) uptake. However, hepatic uptake of HDL_1 CE was at a fractional rate significantly lower than that of HDL_2 CE (0.69-times), even though the uptake of apoE was much higher. The plasma decay of HDL_1 apoE evidently reflects in large part the uptake of apoE after transfer to other fractions, and it over-estimates the clearance of HDL_1 CE. Selective uptake plays the major role in hepatic HDL CE uptake in rats.—**Richard, B. M., and R. C. Pittman.** Role of HDL_1 in cholesteryl ester uptake in rats. *J. Lipid Res.* 1993. **34:** 571–579.

Supplementary key words apoE • apoA-I • N-methyltyramine cellobiose

HDL is believed to participate in the transport of cholesterol from extrahepatic tissues to the liver for excretion or reutilization. Much of this transport is as cholesteryl esters (CE) which are formed in plasma by the action of HDL-associated lecithin:cholesterol acyltransferase. Four pathways have been proposed to play a role in clearance of the resulting HDL CE from the circulation.

The pathway usually thought dominant in humans depends on plasma cholesteryl ester transfer activity. This activity is present in some species such as humans and rabbits, but not in others such as rats (1, 2). The activity mediates the net transfer of HDL CE to more buoyant fractions, apparently in exchange for triglycerides (3). The transferred CE are then cleared from the circulation in the more buoyant particles.

Another pathway thought to play a role in rats and perhaps other animals involves receptor recognition and cellular uptake of particles rich in apoE (4). As cholesterol taken from cells is esterified and CE accumulates in HDL, the particles acquire apoE at the expense of apoA-I. The apoE then targets the enlarged HDL particles (now termed "HDL_1") for uptake by the liver (5). Implicit in the hypothesis that this pathway plays a major or dominant role in hepatic uptake of HDL CE is the assumption that HDL_1-associated CE is cleared more rapidly from the circulation than is CE residing in other HDL pools, an assumption we have tested here. It is also possible that HDL_1 particles arise independently of the HDL_2 and HDL_3 pools, a possibility supported by the presence in peripheral lymph of particles rich in apoE (6).

Whatever the origin of the plasma apoE-rich HDL particles, it might be expected that those particles would play their greatest role in animals without plasma CE transfer activity but rich in apoE and HDL_1 particles. Rats, the subject of this study, are such a species. In rat plasma about 60% of total HDL cholesterol is carried in HDL_1 (7, 8), a class of particles differentiated from other HDL particles by their larger size, lower density, high apoE content, and low content of other apolipoproteins. There is evidence that the major source of HDL_1 in the rat is by conversion from the HDL_2 fraction (9).

A third pathway for uptake of HDL CE is by uptake of HDL particles without benefit of apoE. This uptake is generally thought not to play a quantitatively very important role, based mostly on the slow clearance of HDL apolipoproteins which reflects the slow clearance of HDL particles.

The final pathway for uptake of HDL CE is by direct, nonendocytotic uptake of the CE moiety without parallel uptake of HDL particles ("selective uptake"), a pathway studied by this laboratory (10). This pathway plays a major role in hepatic uptake of HDL CE in rats (11, 12). It even plays a quantitatively significant role in rabbits

Abbreviations: HDL, high density lipoprotein; NMTC, N-methyltyramine cellobiose; CE, cholesteryl ester; COEt, Cholesteryl oleyl ether; FCR, fractional catabolic rate.

which have very high levels of the competing CE "transfer pathway" (13).

All of the above pathways have been demonstrated in vitro, and all have been examined in some fashion in vivo. However, direct quantitative information on the rates of the various pathways in vivo is sparse. It is only with such measurements that the roles of the several pathways can be understood.

Rats offer a convenient model for in vivo tracer studies of lipoprotein metabolism. One reason for this is their lack of plasma cholesteryl ester transfer activity. Injected CE tracer remains with the originally labeled particles, simplifying experimental design and interpretation, allowing measurement of the rates of cholesteryl ester uptake by individual organs, and providing information on the mechanism of that uptake (10, 14, 15). Many workers have exploited the advantages of the rat model to explore HDL metabolism in a variety of ways (16). In the present study we have again taken advantage of this model system.

The present studies directly examined the rate and sites of uptake of CE and apoE tracers introduced in HDL_1 particles. The experimental design required the assumption that HDL_1 in rats is kinetically homogeneous, an assumption not challenged by evidence of metabolic heterogeneity that would change the conclusions of the study. The rates and sites of uptake of HDL_1 tracers were directly compared to those of CE and apoA-I tracers introduced in HDL depleted of particles with apoE. Because of the rapid transfer of apoE between lipoprotein fractions (6, 16), we have not assumed that HDL_1-associated apoE traces HDL_1 particles. To allow determination of rates of uptake by individual organs, intracellularly trapped tracers were used throughout.

METHODS

Apoprotein isolation and labeling

Rat apoE and apoA-I were isolated as previously described (17). To insure separation of the two apolipoproteins, heparin-Sepharose affinity chromatography was used as a final separation step (18). The purity of isolated apolipoproteins was checked by SDS-PAGE.

Purified apolipoproteins were labeled by covalent linkage of radioiodinated N-methyltyramine cellobiose (NMTC) (14, 17). Carrier-free $Na^{125}I$ and $Na^{131}I$ were from Amersham/Searle. More than 90% of the radioactivity migrated with the appropriate apoE or apoA-I band on SDS-PAGE. In some studies apoE was radioiodinated either by oxidative iodination using Iodogen (19) or by covalent attachment of the iodinated Bolton-Hunter reagent (N-succinimidyl-3-(4-hydroxyphenyl)propionate) (20).

Preparation of tracers of cholesteryl esters

[^{3}H]cholesteryl oleyl ether ([^{3}H]COEt) and [^{14}C]COEt were used as nonhydrolyzable markers of cholesteryl esters. These were prepared from [1,2-^{3}H]cholesterol (45.6 Ci/mmol) or [4-^{14}C]cholesterol (55 mCi/mmol) (Amersham/Searle) according to standard procedures as previously reported (21).

Isolation of HDL_1 and apoE-free HDL

Retired breeder Sprague-Dawley rats (275–300 g body weight) maintained on standard rat chow (Western Research Co, Orange, CA) were used for the isolation of HDL subfractions. Rats were fasted overnight and blood was collected from the abdominal aorta under ether anesthesia. Lipoproteins were isolated by standard ultracentrifugal methods (22) at 10°C using a Beckman 50.3 Ti rotor.

HDL_1 was prepared in the density range 1.06–1.09 g/ml. Serum was adjusted to 1.06 g/ml with solid KBr and centrifuged for 20 h at 42,000 rpm. The top fraction was washed at the same density, and then adjusted to d 1.09 g/ml and centrifuged again. The top fraction from this spin was harvested and washed at the same 1.09 g/ml density. The final HDL_1 preparation contained 91 ± 8% (n = 3) of total protein as apoE, with trace amounts of apoA-I and smaller apolipoproteins.

For the preparation of HDL depleted of particles with apoE, HDL were prepared in the density range 1.06–1.21 g/ml. The resulting preparation was treated by heparin affinity chromatography to remove particles containing apoE. In the final preparation, apoA-I accounted for almost 90% of total protein.

All HDL preparations were dialyzed against 0.15 M NaCl containing 0.05 mM EDTA and 0.02% NaN_3, pH 7.4, and stored in the cold. Preparations were dialyzed against phosphate-buffered normal saline just before use.

Preparation of doubly labeled HDLs

Both HDL_1 and apoE-free HDL particles were doubly labeled with intracellularly trapped tracers, as previously described (14). In brief, [^{3}H]COEt was incorporated into HDL_1 using the cholesteryl ester transfer protein procedure (23), and then ^{125}I-labeled NMTC–apoE (< 10% of the HDL_1 apoE mass) was exchanged into the particles; similarly, ^{131}I-labeled NMTC–apoA-I was exchanged into HDL particles that had been labeled with [^{14}C]COEt. The labeled lipoproteins were then reisolated by flotation at the high end of their original preparation density range. All procedures were carried out in the presence of 0.02% sodium azide and 1.5 mM DTNB to inhibit esterification of lipoprotein cholesterol.

In some studies, noted in the text, apoE was labeled by direct radioiodination or by attachment of the Bolton-Hunter ligand. These labeled apoE preparations were incorporated into HDL_1 as just described. In other cases, HDL_1 particles were labeled by direct reaction with the iodinated NMTC ligand, rather than by incorporation of

TABLE 1. Comparison of FCRs for apoE endogenous to HDL_1 and apoE exchanged into HDL_1

HDL_1 Tracer	Plasma FCR	
	ApoE	ApoA-I
	h^{-1}	
ApoE in *I-NMTC-HDL_1 (n = 11)	0.34 ± 0.08	0.19 ± 0.05
*I-NMTC-apoE exchanged into HDL_1 (n = 17)	0.29 ± 0.05	

labeled apoE; [^{3}H]COEt was then exchanged into the ^{125}I-labeled HDL_1. In these cases, 62 ± 13% of label was in apoE, and 9–30% was in apoA-I. Plasma decay of apoE specifically was determined after separation of apoE on PAGE-SDS gels.

Studies in rats

The in vivo tracer studies were conducted as previously described (10, 15). Female Sprague-Dawley rats, 225–250 g, fed standard chow, were fasted overnight prior to initiating the metabolic studies and during the studies. The labeled HDL preparation(s) (a total of 0.2–0.6 mg of protein in 0.2–0.6 ml of normal saline) was then injected into a tail vein. Blood samples were collected over the ensuing 24-h period and assayed for content of tracers. Plasma ^{125}I and ^{131}I were directly radioassayed, while ^{3}H and ^{14}C were assayed by scintillation spectroscopy after lipid extraction (24). The resulting data for each tracer were fitted to biexponential functions, and the plasma fractional catabolic rates (FCR) were calculated. The plasma FCR represents the fraction of the intravascular pool of tracer that irreversibly leaves the plasma compartment each hour.

After 24 h, the animals were anesthetized and then exsanguinated via the abdominal aorta. The vasculature was thoroughly perfused with normal saline containing EDTA to remove residual blood. Tissues were removed and either directly analyzed for ^{125}I and ^{131}I or analyzed for ^{3}H and ^{14}C after lipid extraction. The fraction of total uptake attributable to each organ was calculated as the amount of tracer recovered in that organ as a fraction of the total tracer recovered in all organs, urine, feces, and gut contents. Total uptake by liver was calculated including tracer that appearing in feces and gut contents (secondary to hepatic uptake), as previously described (14, 15). Inclusion of feces and gut contents resulted in a large correction for hepatic uptake of apolipoprotein tracers, but a minor correction for uptake of CE tracers.

In some cases, 2.5 ml of the terminal plasma was taken for sequential separation of lipoprotein fractions by ultracentrifugation (22). After dialysis of the fractions against normal saline containing 0.05 mM EDTA and 0.02% NaN_3, pH 7.4, the distribution of tracers among lipoprotein fractions was determined.

Statistical analysis

All data are expressed as mean ± standard deviation. The significance of differences was determined using a two-tailed, paired or unpaired Student's t test. A difference was considered significant at $P < 0.05$.

RESULTS

To trace the CE and apolipoprotein moieties of HDL_1 and apoE-free HDL, [^{3}H]- or [^{14}C]cholesteryl-oleyl ether (COEt) traced CE, and the covalently attached radioiodinated N-methyltyramine cellobiose ligand (^{125}I- or ^{131}I-labeled NMTC) traced the apolipoproteins (25). The validity of HDL-associated cholesteryl-oleyl ether as a tracer of HDL cholesteryl esters in rats has been examined (11), as has the use of reassociated *I-NMTC-apoA-I as tracer of HDL-associated apoA-I (11, 26). However, *I-NMTC-apoE reassociated with HDL_1 has not been validated as a tracer of endogenous HDL_1-associated apoE.

To examine this question, we first determined the effect of reassociation on the plasma decay kinetics of HDL_1-associated apoE. Two types of HDL_1 tracer particles were prepared: particles into which ^{125}I-labeled NMTC-apoE was exchanged, and particles in which the endogenous apoE was directly labeled with the ^{131}I-labeled NMTC ligand while resident on the particles. In the latter case, radioactivity in plasma apoE at each time point was determined after separation of apolipoproteins by SDS-PAGE. The results, shown in **Table 1,** indicate that the plasma fractional catabolic rates of endogenously and exogenously labeled apoE were not significantly different. All of the data subsequently shown are from experiments in which purified apoE was labeled and then transferred into HDL_1.

We also considered the possibility that the *I-NMTC ligand might alter the fate of the apoE to which it was attached. To do this, two types of doubly labeled HDL_1 particles were prepared: particles into which ^{125}-labeled NMTC-apoE and ^{131}I-labeled apoE (directly radioiodinated using the ICI method) were exchanged; particles into which ^{125}I-labeled NMTC-apoE and apoE labeled with the ^{131}I-Bolton-Hunter ligand (^{131}I-labeled hydroxyphenylpropionate) (20) were exchanged. These preparations were injected into rats and the plasma decays of the

TABLE 2. Plasma decay of HDL_1-associated apoE radiolabeled by various methods

Method of Labeling ApoE	Plasma FCR
	h^-_1
Experiment 1	
NMTC-apoE (n = 7)	0.30 ± 0.04
Directly iodinated (n = 7)	0.24 ± 0.03
Experiment 2	
NMTC-apoE (n = 6)	0.30 ± 0.03
Bolton Hunter apoE (n = 6)	0.28 ± 0.02

tracers were determined. The decays of apoE labeled with the NMTC ligand and the Bolton-Hunter ligand were not significantly different (**Table 2**), while the decay of directly radioiodinated apoE was somewhat slower ($P < 0.002$). These data do not disclose which tracer more precisely traced apoE, but the similarity in results for the two nonoxidative labeling methods implies that direct iodination may have reduced the rate of clearance of apoE, as suggested by Eisenberg (16). Whatever the case, the NMTC tracer did not underestimate the FCR for HDL_1-associated apoE. Furthermore, the presence of NMTC-labeled apoE did not affect the rate of clearance of HDL_1-associated CE tracer, which was the measurement central to the purpose of this study.

The plasma decay kinetics of the CE and apolipoprotein moieties of both HDL_1 and apoE-free HDL were determined over a 24-h period. **Table 3** shows two compilations of these data. The first represents an experiment in which HDL_1 and apoE-free HDL tracers were simultaneously injected into the same rats, and the second represents a wider group of animals including those in which tracers of the two HDL subfractions were not simultaneously injected. Both sets of data show that apoE injected in HDL_1 decayed from plasma much more quickly than did apoA-I injected in apoE-free HDL, as expected from previous studies (8) and as already shown in Table 1 above.

A more striking feature of the data is that the HDL_1-associated apoE tracer was removed from the circulation at a greater fractional rate than was HDL_1-associated CE tracer. As CE does not transfer between lipoprotein particles in the circulation of rats (27), the CE tracer marked the injected particles. (The HDL_1 CE tracer actually yielded a maximum rate for HDL_1 particle clearance because some fraction of the CE tracer was most likely taken up by the selective uptake pathway, as discussed below.) It is apparent that the rate of apoE tracer clearance was considerably greater than the rate of HDL_1-associated CE tracer clearance. This "excess" decay of apoE might be accounted for by clearance of free apoE unassociated with plasma lipoproteins (see below), or by transfer of apoE to other lipoproteins that are more rapidly cleared as previously suggested (16), or both. Whatever the mechanisms for clearance, the HDL_1-associated apoE tracer does not faithfully trace the decay of HDL_1 particles.

The data of Table 3 also show that the FCR for CE tracer introduced in apoE-free HDL was significantly greater than that for CE tracer introduced in HDL_1. Even acknowledging the interconversion of HDL subfractions, this indicates that clearance of CE from the HDL_1 pool was at a lower fractional rate than clearance from the pool of HDL without apoE. Apparently the rapid clearance of HDL_1 particles does not play a dominant role in uptake of HDL CE, as might be suggested by the reported rapid hepatic uptake of HDL_c tracers (28).

It should be noted that in the experiments of Table 3 the tracers were still predominantly associated with the HDL fraction at the end of the 24-h experiments. In all cases less than 10% of the remaining tracer was found at densities less than 1.05 g/ml.

To determine the roles of individual tissues in uptake of HDL_1, compared to apoE-free HDL, the tissue contents of the intracellularly trapped tracers were determined at termination of the 24-h plasma decay period. These results are reported for the same animals for which plasma decay data are shown in Table 3. The results of the tissue analyses are summarized in **Table 4** in terms of

TABLE 3. Plasma decay kinetics of tracers introduced in HDL_1 and in apoE-free HDL

HDL Subfraction	ApoE	ApoA-I	CE
		FCR (h^-_1)	
Animals simultaneously injected with doubly labeled HDL_1 and apoE-free HDL (n = 4)			
HDL_1	0.23 ± 0.01[a]		0.13 ± 0.01[c]
ApoE-free HDL		0.16 ± 0.01	0.16 ± 0.01
Data for all animals, including simultaneously injected			
HDL_1 (n = 17)	0.29 ± 0.05[b]		0.12 ± 0.02[d]
ApoE-free HDL (n = 9)		0.14 ± 0.02	0.16 ± 0.02

[a] Significantly greater than HDL apoA-I ($P < 0.001$ by two-tailed t-test for paired values).
[b] Significantly greater than HDL apoA-I ($P < 0.0001$ by two-tailed t-test for unpaired values).
[c] Significantly less than HDL CE ($P < 0.001$ by two-tailed t-test for paired values).
[d] Significantly less than HDL CE ($P < 0.0001$ by t-test for unpaired values).

TABLE 4. Specific activities of tissues in uptake of tracers introduced in HDL_1 and in apoE-free HDL (plasma FCR per gram)

Tissue	HDL_1 (n = 17)		ApoE-Free HDL (n = 9)	
	CE	ApoE	CE	ApoA-I
	fractional catabolic rate per gram ($10^3 \times h^{-1} \times g^{-1}$)			
Liver	15.1 ± 3.3	25.63 ± 4.54	22.04 ± 3.20	11.9 ± 2.77
Adrenal gland	27.3 ± 9.8	9.06 ± 3.58	30.65 ± 3.83	8.86 ± 2.65
Ovaries	22.9 ± 9.1	5.40 ± 1.86	15.37 ± 4.01	11.0 ± 4.4
Kidneys	0.23 ± 0.06	43.7 ± 14.2	0.48 ± 0.05	17.8 ± 3.44
Spleen	5.81 ± 2.14	10.2 ± 3.2	6.88 ± 1.97	17.2 ± 5.9
Lymphatic tissues	1.23 ± 0.75	1.50 ± 0.43	1.27 ± 0.44	1.96 ± 0.56
Small intestine	1.20 ± 0.33	1.02 ± 0.22	1.13 ± 0.25	1.89 ± 0.24
Lungs	1.20 ± 0.90	0.68 ± 0.52	1.17 ± 0.42	0.97 ± 0.44
Pancreas	0.29 ± 0.11	0.68 ± 0.22	0.35 ± 0.10	0.77 ± 0.26
Thymus	0.36 ± 0.18	0.38 ± 0.13	0.48 ± 0.24	0.70 ± 0.26
Large intestine	0.35 ± 0.10	1.13 ± 0.49	0.41 ± 0.11	1.84 ± 1.27
Heart	0.30 ± 0.09	0.26 ± 0.06	0.53 ± 0.14	0.42 ± 0.08
Skin	0.19 ± 0.08	0.23 ± 0.06	0.20 ± 0.08	0.01 ± 0.00
Stomach	0.29 ± 0.08	0.71 ± 0.28	0.30 ± 0.09	0.51 ± 0.24
Adipose	0.13 ± 0.06	0.13 ± 0.11	0.23 ± 0.08	0.03 ± 0.02
Muscle	0.05 ± 0.01	0.04 ± 0.02	0.04 ± 0.02	0.00 ± 0.00
Urine	0.00 ± 0.00	1.18 ± 0.29	0.00 ± 0.00	0.91 ± 0.20

specific activities for uptake of each tracer (the plasma FCR attributable to each gram of tissue), and in **Table 5** in terms of the fractional contribution of each tissue to whole-body uptake (percentage of total clearance).

Hepatic uptake of the CE tracer introduced in HDL_1 was at a lower fractional rate than was uptake of the apoE tracer introduced in the same particles (Table 4), indicating hepatic uptake of apoE independently of the uptake of the originally labeled HDL_1 particles. This result was not surprising in view of the plasma decay data (Table 3) and the expectation that the liver would account for most of the uptake of both the CE and apoE tracers (Table 5).

In contrast to the results for the CE and apolipoprotein tracers introduced in HDL_1, the hepatic uptake of CE introduced in apoE-free HDL was at a greater fractional rate than the uptake of the apoA-I tracer introduced in the same particles (0.022 $h^{-1} \cdot {}^{-1}$ vs. 0.012 $h^{-1} \cdot {}^{-1}$). Whereas we have previously shown that hepatic uptake of apoA-I reasonably traces the uptake of the originally labeled HDL particles in rats (11, 26), this indicates selective uptake of CE from HDL.

The liver took up CE tracer introduced in apoE-free HDL at a greater fractional rate than CE tracer introduced in HDL_1, a result in parallel with the plasma de-

TABLE 5. Contributions of individual tissues in uptake of tracers introduced in HDL_1 and in apoE-free HDL

Tissue	HDL_1 (n = 17)		ApoE-Free HDL (n = 9)	
	CE	ApoE	CE	ApoA-I
	contribution of tissue to total tracer uptake (%)			
Liver	74.77 ± 4.42	53.55 ± 7.43	80.72 ± 1.91	47.88 ± 6.28
Adrenal gland	1.45 ± 0.45	0.20 ± 0.06	1.34 ± 0.22	0.43 ± 0.09
Ovaries	2.37 ± 0.62	0.23 ± 0.05	1.19 ± 0.31	1.15 ± 0.09
Kidneys	0.36 ± 0.10	27.68 ± 7.10	0.53 ± 0.07	22.50 ± 4.44
Spleen	1.90 ± 0.44	1.38 ± 0.29	2.07 ± 0.25	5.95 ± 1.35
Lymphatic tissues	0.24 ± 0.12	0.12 ± 0.04	0.22 ± 0.05	0.40 ± 0.09
Small intestine	3.39 ± 1.25	1.18 ± 0.3	1.82 ± 0.37	3.52 ± 0.53
Lungs	0.96 ± 0.33	0.22 ± 0.05	0.93 ± 0.24	1.69 ± 0.53
Pancreas	0.21 ± 0.05	0.21 ± 0.04	0.22 ± 0.05	0.54 ± 0.09
Thymus	0.08 ± 0.05	0.04 ± 0.02	0.08 ± 0.04	0.14 ± 0.08
Large intestines	0.55 ± 0.24	0.73 ± 0.4	0.35 ± 0.09	1.78 ± 1.16
Heart	0.20 ± 0.04	0.07 ± 0.01	0.53 ± 0.07	0.24 ± 0.04
Skin	6.96 ± 2.7	3.39 ± 0.71	5.22 ± 1.29	0.38 ± 0.10
Stomach	0.30 ± 0.08	0.30 ± 0.11	0.24 ± 0.09	0.45 ± 0.15
Adipose	1.94 ± 0.89	0.79 ± 0.66	2.26 ± 0.81	0.27 ± 0.18
Muscle	4.27 ± 1.57	1.57 ± 0.54	2.50 ± 0.93	0.14 ± 0.17
Urine	0.03 ± 0.02	8.34 ± 2.34	0.04 ± 0.06	13.33 ± 4.45

cay data. Thus, the presence of apoE on HDL_1 particles did not dictate their clearance at a rate that was very fast compared to other processes for HDL CE uptake. As mentioned above, the rate of uptake of HDL_1 CE is a maximum estimate for uptake of HDL_1 particles as CE can be selectively taken up from particles with only apoE (17). However, we cannot allocate the HDL_1 CE uptake between selective uptake and particle uptake, as we have done for apoE-free HDL, because we have no measure of particle uptake in the case of HDL_1.

Kidney was second (behind the liver) in its contribution to uptake of both apoE and apoA-I, accounting for 28% of apoE and 23% of apoA-I clearance (Table 5). Both apolipoproteins were taken up with very little accompanying uptake of CE, a result like that previously reported for apoA-I (10). Presumably the high rate of uptake of the apoE tracer is due to glomerular filtration and tubular reabsorption of apoE unassociated with lipoprotein particles, as has been described for apoA-I (29).

While steroidogenic tissues made little contribution to whole body uptake of HDL_1 CE, the adrenal gland was the most active tissue per weight of tissue. As can be seen in Table 4, the fractional rate of uptake of CE was greater than that of apoE, in contrast to the results for liver where the fractional rate of uptake of apoE was greater. Adrenal was also the most active organ per weight in uptake of CE tracer introduced in apoE-free HDL, and this uptake was predominantly by selective uptake. It seems likely that most of the uptake of the CE from HDL_1 was also by selective uptake.

DISCUSSION

Previous studies in rats have examined the tissue uptake of CE tracer introduced into the pool of HDL depleted of particles containing apoE. Uptake of CE was mostly into liver, and most hepatic uptake was by the selective uptake pathway (10, 11, 26). Associated studies showed that the apoA-I uptake by liver closely measured uptake of the holo-HDL, excluding the possibility that hepatic selective uptake might actually represent the endocytotic uptake of HDL particles depleted of apoA-I tracer and enriched in unlabeled apoE (10, 17, 26). Direct interpretation of these earlier data would indicate that in rats only about one-third of hepatic HDL CE uptake is by uptake of HDL particles, including uptake both with or without the benefit of apoE.

However, these early experiments did not foreclose a dominant role for HDL_1 particles in transport of CE to the liver, if HDL_1 particles were to arise in part independently of the HDL_3-HDL_2 pools. That CE transport would be in addition to that traced using apoE-poor HDL CE if subfractions of HDL with apoE were metabolically dissimilar and did not mix. There is evidence that HDL_1 in rats does indeed arise predominantly from the HDL_3-HDL_2 pools (9), although other studies indicate the possibility that particles could enter into the HDL_1 pool without ever residing in the HDL_2-HDL_3 pool (30, 31). In dogs (32) and in humans (33) interstitial fluid contains large, apoE-rich particles that are not substantially present in plasma. The presence of such particles is consistent with a role for apoE in targeting CE in these particles for rapid hepatic uptake. If these particles were to be rapidly cleared without mixing with the HDL_1 pool, we would not see that here.

The major purpose of this study was to evaluate, as directly as possible, the role of HDL_1 in reverse cholesterol transport. HDL_1 was assumed to adequately measure the role of apoE in HDL CE uptake by the liver since the predominant portion of HDL CE associated with apoE resides in this fraction (7, 8). Thus, HDL_1 was assumed to be a metabolically uniform pool through which all CE destined to be cleared by an apoE-mediated mechanism passed. However, the standard caveat of such tracer studies applies: we would not have seen the consequences of a very small plasma pool of particles that mediated a high flux of CE but which did not communicate with the pool(s) that were traced.

Although we have assumed that the HDL_1 pool is metabolically homogeneous, the fraction is at least physically heterogenous and is comprised of a continuum of particle sizes. This does not necessarily signify pertinent metabolic heterogeneity. Although this question has not been directly addressed experimentally here, work to be reported elsewhere indicates that the plasma decay kinetics of CE and apoA-I tracers incorporated into five HDL subfractions are fairly similar for five HDL subfractions (including HDL_1) in spite of the fact that selective uptake and particle uptake vary with particle size in vitro (17, 34). Apparently the transfer of tracers between particles and the interconversion of particles between subfractions are sufficiently rapid as to make CE appear almost kinetically homogeneous throughout the HDL_1-HDL_2 system.

In tracing HDL_1 in rats, intracellularly trapped tracers of the CE and apoE moieties were used. These tracers were almost completely cleared irreversibly from plasma during the course of the experiments, allowing a theoretically correct determination of the contributions of various tissues to uptake of the tracers and their tracees (assuming that newly formed tracees entered first into the plasma compartment, which was the labeled compartment) (35). However, this did not disclose from which plasma pool(s) the tracers were actually cleared, or the mechanism(s) of that clearance.

One obstacle to determining the mechanisms of HDL_1 CE uptake in these studies was our inability to apportion that uptake between uptake of particles and selective uptake of CE, an apportionment we were able to accomplish

in the case of HDL without apoE. Because CE can be selectively taken up in vivo from particles with apoE as the only apolipoprotein (17), it seems probable that some fraction of HDL_1 CE uptake in vivo is by selective uptake.

A salient result of this study is that the fractional rates for plasma clearance and for hepatic uptake were lower in the case of CE tracer introduced in HDL_1 than in the case of CE tracer introduced in HDL without apoE. Apparently uptake of HDL_1 particles is not nearly as rapid as the clearance of HDL_1-associated apoE would seem to indicate. Furthermore, if it is accepted that some clearance of HDL_1 CE must be by selective uptake, then the role of apoE in HDL CE uptake diminishes further. All of this seems to indicate that apoE is not a dominant determinant of HDL CE clearance, even in rats where it seems most likely to play a prominent role.

However, some caution is appropriate in interpreting these results. HDL particles interconvert between subclasses, and the tracers were not necessarily cleared from the pools which they originally labeled, even when they remain with the neutral lipid core originally labeled. For example, HDL without apoE convert to HDL_1 in the circulation (9, 36). There is also conversion in the other direction, and particles residing in the HDL_1 pool of rats convert to HDL_2 particles (B. M. Richard and R. C. Pittman, unreported studies). This does not preclude our reaching the conclusion that CE are cleared from the pool of HDL without apoE at a more rapid rate than from the HDL_1 pool. In fact, interconversion of the HDL subclasses would tend to damp the real difference in turnovers, and tracer studies will underestimate the difference. Thus, the difference in clearance rates of CE tracer introduced in HDL_1 and in HDL_2 is a minimal difference, and CE removal from HDL_1 pool is at a lower rate than is apparent here, and CE removal from the pool of HDL without apoE is at a greater rate than is apparent here. Thus, although we cannot explicitly apportion the CE flux between the competing pathways, the data here do place a minimum value on the difference.

The rate of hepatic uptake of apoE tracer introduced in HDL_1 greatly exceeded that of CE tracer. As apoE can rapidly exchange between lipoprotein classes, part of the hepatic uptake of apoE tracer must have been from other lipoprotein pools (e.g., VLDL remnants) (9, 16). However, the large fraction of apoE tracer that was taken up by kidney may well represent another pathway. As with apoA-I tracer (29), it may reflect the glomerular filtration and tubular reabsorption of apoE unassociated with lipoproteins.

This study indicates that in rats apoE does not mediate the clearance of HDL_1 particles at such a rate as to dominate HDL CE flux to the liver, a conclusion also supported previously (36). This in no way precludes a major role for apoE in mediating uptake of HDL particles. It seems highly probable that HDL_1 particles are cleared more rapidly from plasma than are particles without apoE, as is the case in vitro (37, 38); in HepG2 human hepatoma cells, much of the uptake of HDL particles without apoE is in fact mediated by apoE secreted by the cultured cells (39, 40). Studies in whole animals support the conclusion from in vitro studies. For example, particles with many apoE moieties are rapidly cleared by liver in rats (28), and rats treated with ethinyl estradiol to increase apoB/E receptor activity exhibit an increased rate of hepatic uptake of HDL-associated apoA-I (39, 40).

If apoE plays such a prominent role in uptake of HDL particles, and HDL particles progressively enlarge by accumulation of CE in the circulation of rats, how can CE not be taken up at a more rapid rate from HDL_1 than from HDL without apoE? Two mechanisms offer at least a partial explanation. First, selective uptake from HDL_1 may decrease particle size, thereby decreasing apoE affinity and allowing return of the particles to the HDL_2 pool. Second, the fractional rate of selective uptake varies with HDL particle size, being greater for smaller particles regardless of the apoprotein composition (34). From this one would predict that selective uptake from the HDL_1 should be at a lower fractional rate than selective uptake from HDL particles without apoE. These factors in concert would make it possible for most particle uptake to be by receptor recognition of apoE, while most HDL CE uptake is by selective uptake from the HDL_2–HDL_3 pools.

The central conclusion of the present study is that HDL_1 does not play a dominant role in clearance of HDL CE into liver, even in rats. This conclusion is concordant with a kinetic modeling study in rabbits that also concluded that there was no more rapid uptake of CE from the HDL_1 pool than from the HDL_2–HDL_3 pool (13). These studies are complementary in that they invoke quite different assumptions in species at the two extremes of plasma cholesteryl ester transfer protein activity. Both studies, while not precluding a significant role for HDL_1 in reverse cholesterol transport, counter the hypotheses that HDL_1 particles are the major mediator of reverse cholesterol transport involving CE in the HDL fraction. We conclude that selective uptake must play the major role in reverse cholesterol transport in rats to the extent that HDL CE mediate that transport.

We thank Florencia Casanada and Simone Green for their valuable contributions to this study. This work was supported by Grant RT 399 from the Tobacco-Related Disease Research Program, University of California.

Manuscript received 3 June 1992 and in revised form 6 October 1992.

REFERENCES

1. Patnaik, N. M., A. Montes, L. B. Hughes, and D. B. Zilversmit. 1978. Cholesteryl ester exchange in human

plasma: isolation and characterization. *Biochim. Biophys. Acta.* **530:** 428–438.
2. Fielding, C. J., and P. E. Fielding. 1982. Cholesterol transport between cells and body fluids. *Med. Clin. North Am.* **66:** 363–373.
3. Morton, R. E., and D. B. Zilversmit. 1981. A plasma inhibitor of triglyceride and cholesteryl ester transfer activities. *J. Biol. Chem.* **256:** 11992–11995.
4. Mahley, R. W., T. L. Innerarity, S. C. Rall, Jr., and K. H. Weisgraber. 1984. Plasma lipoproteins: apolipoproteins structure and function. *J. Lipid Res.* **25:** 1277–1294.
5. Sherill, B. C., T. L. Innerarity, R. W. Mahley, and S. Eisenberg. 1980. Rapid clearance of the canine lipoproteins containing only the E apoprotein by a high affinity receptor. *J. Biol. Chem.* **255:** 1804–1807.
6. Sloop, C. H., L. Dory, and P. Roheim. 1987. Interstitial fluid lipoproteins. *J. Lipid Res.* **28:** 225–237.
7. Lusk, L. T., L. F. Walker, L. H. DuBien, and G. S. Getz. 1979. Isolation and partial characterization of high density lipoprotein HDL_1 from rat plasma by gradient centrifugation. *Biochem. J.* **183:** 83–90.
8. Weisgraber, K. H., R. W. Mahley, and G. Assmann. 1977. The rat arginine-rich apoprotein and its redistribution following injection of iodinated lipoproteins into normal and hypercholesterolemic rats. *Atherosclerosis.* **28:** 121–140.
9. Gavish, D., Y. Oshry, and S. Eisenberg. 1987. In vivo conversion of human HDL_3 to HDL_2 and apoE-rich HDL_1 in the rat: effects of lipid transfer protein. *J. Lipid Res.* **28:** 257–267.
10. Glass, C. G., R. C. Pittman, M. Civen, and D. Steinberg. 1985. Uptake of HDL-associated apoprotein A-I and cholesterol esters by 16 tissues of the rat in vivo and by adrenal cells and hepatocytes in vitro. *J. Biol. Chem.* **260:** 744–750.
11. Glass, C. G., R. C. Pittman, D. B. Weinstein, and D. Steinberg. 1983. Dissociation of tissue uptake of cholesterol from that of apoprotein A-I of rat high density lipoproteins: selective delivery of cholesterol to liver, adrenal and gonad. *Proc. Natl. Acad. Sci. USA.* **80:** 5435–5439.
12. Rininger, F., and R. C. Pittman. 1987. Regulation of the selective uptake of high density lipoprotein-associated cholesteryl esters. *J. Lipid Res.* **28:** 1313–1325.
13. Goldberg, D. I., W. B. Beltz, and R. C. Pittman. 1991. Evaluation of pathways for the cellular uptake of high density lipoprotein cholesterol esters in rabbits. *J. Clin. Invest.* **87:** 331–346.
14. Pittman, R. C., and C. A. Taylor, Jr. 1986. Methods of assessment of tissue sites of lipoprotein degradation. *Methods Enzymol.* **129:** 612–628.
15. Pittman, R. C., T. E. Carew, C. K. Glass, S. R. Green, A. C. Taylor, Jr., and A. D. Attie. 1983. A radioiodinated, intracellularly trapped ligand for determining the sites of plasma protein degradation in vivo. *Biochem. J.* **212:** 791–800.
16. Eisenberg, S. 1986. Plasma lipoprotein conversions. *Methods Enzymol.* **21:** 347–366.
17. Pittman, R. C., T. P. Knecht, M. S. Rosenbaum, and C. A. Taylor, Jr. 1987. A non-endocytotic mechanism for the selective uptake of high density lipoprotein-associated cholesterol esters. *J. Biol. Chem.* **262:** 2443–2450.
18. Weisgraber, K. H., and R. W. Mahley. 1980. Subfractionation of human high density lipoprotein by heparin-Sepharose affinity chromatography. *J. Lipid Res.* **21:** 316–325.
19. McFarlane, A. S. 1958. Efficient trace labeling of proteins with iodine. *Nature. (London)* **182:** 53–57.
20. Bolton, A. E., and W. M. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Application to radioimmunoassay. *Biochem. J.* **133:** 529–539.
21. Pool, G. L., M. E. French, R. A. Edwards, K. Huang, and R. H. Lumb. 1982. Use of radiolabeled hexadecyl cholesteryl ether as a liposome marker. *Lipids.* **17:** 448–452.
22. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
23. Hough, J. L., and D. B. Zilversmit. 1984. Comparison of various methods for in vitro cholesteryl ester labeling of lipoproteins from hypercholesterolemic rabbits. *Biochim. Biophys. Acta.* **792:** 338–347.
24. Dole, V. P. 1956. A relation between the non-esterified fatty acids in plasma and the metabolism of glucose. *J. Clin. Invest.* **18:** 150–154.
25. Stein, O., and Y. Halperin. 1980. Biological labeling of very low density lipoproteins with cholesterol linoleyl ether and its fate in the intact rat. *Biochim. Biophys. Acta.* **620:** 247–260.
26. Richard, B. M., and M. Pfeuffer. 1992. Transport of HDL cholesterol esters to the liver is not diminished by probucol treatment in rats. *Arterioscler. Thromb.* **12:** 862–869.
27. Barter, P. J., and J. I. Lally. 1978. The activity of an esterified cholesterol transferring factor in human and rat serum. *Biochim. Biophys. Acta.* **531:** 233–236.
28. Sherill, B. C., T. L. Innerarity, and R. W. Mahley. 1982. Rapid hepatic clearance of the canine lipoproteins containing only apoprotein E by a high affinity receptor: identity with chylomicron remnant transport process. *J. Biol. Chem.* **257:** 11442–11447.
29. Glass, C. G., R. C. Pittman, G. A. Keller, and D. Steinberg. 1983. Tissue sites of degradation of apoprotein A-I in the rat. *J. Biol. Chem.* **258:** 7161–7167.
30. Basu, S. K., M. S. Brown, Y. K. Ho, R. J. Havel, and J. L. Goldstein. 1981. Mouse macrophages synthesize and secrete a protein resembling apolipoprotein E. *Proc. Natl. Acad. Sci. USA.* **78:** 7545–7549.
31. Blue, M-L., D. L. Williams, S. Zucker, S. A. Khan, and C. B. Blum. 1983. Apolipoprotein E synthesis in human kidney, adrenal gland, and liver. *Proc. Natl. Acad. Sci. USA.* **80:** 283–287.
32. Dory, L., L. M. Boquet, R. L. Hamilton, C. H. Sloop, and P. S. Roheim. 1985. Heterogeneity of dog interstitial fluid (peripheral lymph) high density lipoproteins: implication for a role in reverse cholesterol transport. *J. Lipid Res.* **26:** 519–527.
33. Rudra, D. N., N. B. Myant, J. J. Pflug, and D. Reichl. 1984. The distribution of cholesterol and apoprotein A-I between the lipoproteins in plasma and peripheral lymph from normal human subjects. *Atherosclerosis.* **53:** 297–308.
34. Pittman, R. C., C. K. Glass, D. Atkinson, and D. M. Small. 1987. Synthetic high density lipoprotein particles: application to studies of the apoprotein specificity for selective uptake of cholesterol esters. *J. Biol. Chem.* **262:** 2435–2442.
35. Shipley, R. A., and R. E. Clark. 1972. Tracer Methods for In Vivo Kinetics, Academic Press, New York. 77–109.
36. Eisenberg, S., Y. Oshry, and J. Zimmerman. 1984. Intravascular metabolism of the cholesteryl ester moiety of rat plasma lipoproteins. *J. Lipid Res.* **25:** 121–128.
37. Pitas, R. E., T. L. Innerarity, and R. W. Mahley. 1980. Cell surface receptor binding of phospholipid-protein complexes containing different ratios of receptor-active and -

inactive E apoprotein. *J. Biol. Chem.* **255:** 5454–5460.

38. Innerarity, T. I., R. E. Pitas, and R. W. Mahley. 1980. Receptor binding of cholesterol-induced high density lipoproteins containing predominantly apoprotein E to cultured fibroblasts with mutations at the low-density lipoprotein receptor locus. *Biochemistry.* **19:** 4359–4365.
39. Windler, E., Y-S. Chao, and R. J. Havel. 1980. Regulation of the hepatic uptake of triglyceride-rich lipoproteins in the rats. *J. Biol. Chem.* **255:** 8303–8307.
40. Rininger, F., and R. C. Pittman. 1987. Mechanism of the cholesteryl ester transfer protein-stimulated uptake of high density lipoprotein-associated cholesteryl esters by HepG2 cells. *J. Biol. Chem.* **264:** 6111–6118.