Regulation of the selective uptake of high density lipoprotein-associated cholesteryl esters

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Abstract We have previously shown in rats that the cholesteryl ester component of high density lipoproteins (HDL) is taken up at a greater fractional rate than is the apolipoprotein A-I component (selective uptake) by liver and steroidogenic tissues. Selective uptake was also exhibited by cultured cells from these organs as well as by a wider range of cells in vitro (e.g., rat and human fibroblasts). We report here regulation of this pathway according to the cholesterol status of cells. Uptake of HDL cholesteryl esters by rat fibroblasts was decreased by prior loading of the cells with cholesterol, even while uptake of HDL-associated apoA-I actually increased. At high levels of cholesterol, the two were taken up about in parallel, i.e., selective uptake was suppressed. A similar regulation of selective uptake in primary rat hepatocytes in culture was not observed. To examine regulation of selective uptake in vivo, hypocholesterolemia was induced in rats using either 4-aminopyrazolo[3,4-d]pyrimidine or 17α-ethinyl estradiol. Rat HDL, doubly labeled in both the apoprotein A-I and cholesteryl ester moieties with intracellularly trapped tracers, were injected into untreated and treated rats. The plasma decay kinetics and the tissue sites of uptake were then determined. Hypocholesterolemia increased the plasma fractional catabolic rates of both tracers. Selective uptake was observed in tissues of treated rats that did not exhibit selective uptake in untreated rats (muscle, adipose tissue, and skin). Similarly, hypocholesterolemia increased the contribution of selective uptake to total HDL cholesteryl ester uptake by adrenal and ovary. In contrast, regulation of selective uptake by liver could not be demonstrated under these conditions. Thus, selective uptake of HDL cholesteryl esters can be regulated in extrahepatic tissues of rats in vivo and in vitro, suggesting a role for selective uptake in the maintenance of cholesterol homeostasis in these tissues.—**Rinninger, F., and R. C. Pittman.** Regulation of the selective uptake of high density lipoprotein-associated cholesteryl esters. *J. Lipid Res.* 1987. **28:** 1313–1325.

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It has been proposed that high density lipoproteins (HDL) play an important role in the transport of excess cholesterol from extrahepatic tissues to liver for reutilization or excretion into bile (1, 2). At least in the rat, HDL also delivers cholesterol to the adrenal gland, the gonads, and perhaps to other tissues (3). Thus, HDL may play a dual role in these animals.

The in vivo sites of irreversible uptake of individual HDL components in rats have been studied in this laboratory (4–6). These studies have shown that the liver and steroidogenic tissues take up the cholesteryl ester moiety of HDL at a greater fractional rate than the apoprotein A-I (apoA-I) moiety, i.e., cholesteryl esters are taken up without parallel uptake of HDL-associated apoA-I (selective uptake). Liver accounted for most of the cholesteryl ester uptake in excess of apoA-I uptake, and steroidogenic tissues accounted for much of the rest (6). These results are consistent with a role for the selective uptake of HDL cholesteryl esters in the return of excess cholesterol from extrahepatic tissues to the liver, as well as in the delivery of cholesterol to steroidogenic tissues. These in vivo results have been confirmed in vitro using primary cultures of rat hepatocytes and adrenal cells (6). In addition, selective uptake of cholesteryl esters has been observed in a wider range of cell types, including rat and human fibroblasts (7). Because selective uptake was exhibited only by liver and steroidogenic tissues in vivo, this suggested that the pathway might be down-regulated in some tissues of normal rats.

To test this hypothesis we carried out experiments both in vitro and in vivo, which are reported here. HDL-associated cholesteryl ester were traced with a non-hydrolyzable, intracellularly trapped [^{3}H]cholesteryl-oleyl ether (5, 8); the major apolipoprotein component of HDL, apoA-I, was traced with an intracellularly trapped label (9, 10), ^{125}I-labeled N-methyl-tyramine-cellobiose-apoA-I (^{125}I-NMTC-apoA-I). We show that selective uptake by cultured rat fibroblasts can be down-regulated by prior incubation with free cholesterol in the medium. In

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; hLDL, human low density lipoprotein; Lac-LDL, lactosylated human LDL; apoA-I, apolipoprotein A-I; 4-APP, 4-aminopyrazolo[3,4,-d]pyrimidine; ethinyl estradiol, 17α-ethinyl estradiol; NMTC, N-methyl-tyramine-cellobiose; ^{125}I-NMTC-apoA-I, apoA-I radiolabeled by covalent attachment of the NMTC ligand; FCR, fractional catabolic rate; PBS, phosphate-buffered saline.

contrast, no such regulation was seen in primary rat hepatocytes. Regulation of the selective uptake pathway in vivo was studied in rats made hypocholesterolemic by treatment with either 4-aminopyrazolo[3,4-d]pyrimidine (4-APP) (11, 12) or 17α-ethinyl estradiol (ethinyl estradiol) (13–15). Selective uptake in steroidogenic tissues was enhanced by drug treatment, and some tissues that did not exhibit selective uptake in normal rats did so in hypocholesterolemic rats.

METHODS

Lipoprotein preparations

Rat HDL was isolated in the density range 1.09–1.21 g/ml from the plasma of overnight-fasted, retired breeder female Sprague-Dawley rats using sequential preparative ultracentrifugation according to standard techniques (16). HDL particles containing apoprotein E were depleted by heparin-agarose affinity chromatography (17, 18).

Apoprotein A-I was isolated and labeled as previously described (6, 19). Briefly, HDL was delipidated using ethanol–diethyl ether and chromatographed on a Sephacryl S-200 column (Pharmacia, Piscataway, NJ). Purity of the apoA-I preparations was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (20). ApoA-I was labeled by covalent attachment of the intracellularly trapped ^{125}I-labeled N-methyl-tyramine-cellobiose (^{125}I-NMTC) ligand (9, 10).

An ether analogue of cholesteryl esters, [^{3}H]cholesteryl-oleyl ether, was prepared (21) and incorporated into HDL from a donor liposomal preparation using partially purified human plasma cholesteryl ester transfer protein (22) as previously described (7, 23). The donor liposomes were separated from HDL by ultracentrifugation at a salt density of d 1.063 g/ml. ^{125}I-NMTC-apoA-I was then associated with the ^{3}H-labeled HDL by exchange (37°C, 24 hr). Unbound apoprotein was removed by centrifugation at a salt density of 1.21 g/ml at 100,000 *g* for 48 hr. The supernatant fraction containing the doubly labeled HDL was dialyzed against phosphate-buffered saline at pH 7.4 (PBS) containing EDTA (0.3 mM), and sterile-filtered (0.45 μm) before use.

Human low density lipoproteins (hLDL, d 1.019–1.063 g/ml) and human high density lipoproteins (HDL, d 1.063–1.21 g/ml) were prepared from pooled plasma of healthy human donors by sequential preparative ultracentrifugation according to standard methods (16). All lipoproteins were dialyzed against PBS containing EDTA (0.3 mM) before use.

To prepare lactosylated LDL (Lac-LDL), lactose was covalently conjugated with human LDL by reductive amination using sodium cyanoborohydride (Sigma, St. Louis, MO) as previously described (24). Lac-LDL was then exhaustively dialyzed against PBS containing EDTA (0.3 mM) before use. Lac-LDL was radioiodinated according to the method described by Bilheimer, Eisenberg, and Levy (25). Degradation of Lac-LDL was determined according to the total radioiodine appearing in the medium as products soluble in trichloroacetic acid (final concentration 10% w/v).

Synthetic HDL

Synthetic HDL was prepared as described in detail elsewhere (26). Briefly, cholesteryl oleate (Sigma), egg yolk phosphatidylcholine (Sigma), trace amounts of [^{3}H]cholesteryl-oleyl ether and [^{14}C]sucrose octaoleate were mixed and the solvents were evaporated under a stream of nitrogen. The [^{14}C]sucrose octaoleate, used as a tracer of the particle per se, was synthesized as reported (7). Buffer (0.1 M KCl, 10 mM Tris, 1 mM EDTA, 0.025% NaN_3) was added to the dried lipids. The mixture was then sonicated for 30 min at 49–52°C, using a sonifier (Heat Systems, Plainview, NY, model W 185) equipped with a macro tip at a power setting of 100 watts. The temperature was lowered to 39–42°C, and ^{125}I-NMTC-apoA-I in 2.5 M urea was added gradually over 5–7 min with continued sonication. The resulting preparation was fractionated by gel filtration chromatography on 6% agarose and then applied to density gradient ultracentrifugation. Particles in the density range from 1.06–1.21 g/ml were selected for the in vivo studies with rats. It has been shown that these particles resemble rat HDL closely in terms of their physical and metabolic characteristics in vivo and in vitro (26).

Experiments in vitro

Primary rat hepatocytes were prepared by recirculating liver perfusion with collagenase (Sigma, 150 units/mg, 0.7 mg/ml) as previously described (27). After isolation, hepatocytes were plated (3×10^{6}/plate) in fibronectin-coated, 60-mm plastic culture dishes (Lux, Naperville, IL.) in arginine-free Dulbecco's modified Eagle's medium (DME, Gibco, Grand Island, NY), containing 5% (v/v) heat-inactivated neonatal calf serum (Biocell, Carson, CA) and gentamycin (50 mg/l, Sigma). Viability of the cells was >97% as judged by trypan blue exclusion under the phase contrast microscope. Four hours after plating, the monolayers were washed with PBS and then incubated overnight in serum-free DME containing bovine serum albumin (5 mg/ml, Sigma), gentamycin (50 mg/l), and the additions indicated in the figures and tables.

Adult rat skin fibroblasts were grown in DME containing fetal calf serum (10% v/v, Gibco) and gentamycin (50 mg/l). Cells were plated (10^{5}/plate) in 60-mm plastic culture dishes (Lux). Near confluency (4–5 days), the cells were washed with PBS and then incubated in serum-free DME containing albumin (5 mg/ml, Sigma), gentamycin

(50 mg/l), and other additions as indicated in the figure legends.

In some experiments, free cholesterol (tissue culture grade, Sigma), 25-hydroxycholesterol (cholesten-3β-25-diol, Steraloids Inc. Wilton, NH) or mevalonolactone (DL-mevalonic acid lactone, Sigma) were added to the serum-free culture medium in ethanol for overnight incubation before subsequent assay of HDL uptake. In all plates, including controls, the ethanol concentration was 1% (v/v). The effect of 1% ethanol on HDL uptake was examined in each experiment and found to be either minimal or not detectable.

After overnight incubation in the presence or absence of additions (cholesterol, 25-hydroxycholesterol, hLDL, Lac-LDL, etc.) the hepatocyte or fibroblast monolayers were washed four times with PBS. Assay for uptake of double-labeled HDL was then carried out as previously described (7). Briefly, DME containing doubly labeled rat HDL (20 μg protein/ml) and bovine serum albumin (5 mg/ml) was added (1.5 ml/dish). The cells were incubated for 4 hr at 37°C, the medium was removed, and the cells were washed four times with PBS. Medium containing unlabeled human HDL (100 μg of HDL protein/ml) was then added for a 2-hr "chase" period at 37°C. After the chase period, the medium was removed, and the cells were washed once with PBS. The cells were then released from the plates with trypsin/EDTA solution (trypsin 0.5 g/l, EDTA 0.2 g/l, Irvine Scientific, Irvine, CA). Trypsin activity was quenched by the addition of PBS containing an excess of albumin (50 mg/ml). The cell suspensions were transferred to tubes and the dishes were rinsed with PBS. The cells were pelleted at 2000 *g* for 10 min, the supernates were discarded, and the cell pellets were washed with PBS. After a second centrifugation the pellets were dissolved in NaOH (0.1 N) with sonication. Aliquots were taken for protein determination, for direct ^{125}I radioassay, and for assay of ^{3}H after lipid extraction (6, 28).

The cell content of each intracellularly trapped tracer is reported as the apparent uptake of HDL particles (expressed in terms of HDL protein) that would account for the observed tracer uptake.

For the determination of cell cholesterol mass, parallel sets of dishes were incubated overnight in the presence of the indicated additions to the medium. After this incubation the cells were washed six times with PBS, and then harvested as described for the HDL uptake assay except that the chase period was deleted. The cell pellets were sonicated in water, and aliquots were taken for protein determination and for lipid extraction according to the method of Bligh and Dyer (29).

Experiments in vivo

Experiments to determine the plasma fractional catabolic rates and the rates of uptake of HDL tracers by various tissues of rats were carried out as previously described (6). Female Sprague-Dawley rats with body weights between 180 and 250 g on the day of the experiment were used for all experiments. All animals were fasted for 16 hr (overnight) before the experiments.

On the morning of the experiment, each rat was fitted with a catheter in an external jugular vein. Animals were fasted throughout the study period but had free access to drinking water. Doubly labeled HDL or synthetic HDL preparations were injected through the catheter. Periodic blood samples of 0.1 ml were withdrawn over a 24-hr period. In no case was more than 5% of the animals blood volume removed during the study interval. Serum samples were directly radioassayed for ^{125}I and radioassayed for ^{3}H (^{3}H and ^{14}C in some cases) after lipid extraction (28). Computer analysis using an interactive curve peeling program was used to fit a least-squares multiexponential curve to each set of plasma decay data and to calculate fractional catabolic rates (FCR) according to the model of Matthews (30).

The animals were anaesthetized 24 hr after tracer injection and exsanguinated, and the vasculature was thoroughly perfused with PBS (approximately 200 ml). Tissues, gut contents, feces, and urine were collected, weighed, homogenized, and radioassayed. In the case of adipose tissue, muscle, and skin, tissue samples were taken and literature values were used to estimate total organ weight. Radioiodine was assayed directly in a gamma counter. ^{3}H and ^{14}C were assayed by liquid scintillation spectroscopy of lipid extracts (6, 28).

Parallel to the turnover and uptake studies, other rats treated in an identical way were used as blood donors for the analysis of the plasma lipoproteins. At a time parallel to the time of tracer injection into the other animals, blood was drawn from the abdominal vena cava of these animals under light ether anaesthesia. HDL was isolated in the density range 1.07–1.21 g/ml. The HDL was then further fractionated by equilibrium density gradient ultracentrifugation into seven density ranges using a mixed sucrose/salt gradient (31). Density, protein, and total cholesterol concentrations were determined for each subfraction.

To lower plasma lipids, rats were subcutaneously injected in the morning of 4 consecutive days with 17α-ethinyl estradiol (Sigma), 5 mg/kg body weight (14, 15) dissolved in propylene glycol (Sigma). Control animals were treated with the solvent alone. No injections were given on the day of the experiment. Injections were done under light ether anaesthesia.

Other animals were injected intraperitoneally with 4-aminopyrazolo[3,4-d]pyridimine (4-APP, Sigma), 10 mg/kg body weight (11, 12), on the morning of the day before the experiment and on the day of the experiment itself. Control animals were injected with the carrier solution alone (PBS, pH 7.4). Injections were done under light ether anaesthesia.

Chemical assays

Cholesterol was assayed according to the method by Gamble et al. (32) as modified by Daniels et al. (33). Assay of 25-hydroxycholesterol by this method was about as efficient as assay of cholesterol (>95%). Protein was assayed according to the method described by Lowry (34).

Statistical analysis

All data are expressed as mean ± SD. Statistical significance was determined with the Student's *t*-test for paired data.

RESULTS

Regulation of selective uptake of HDL cholesteryl esters in cultured cells

To study regulation of selective uptake in vitro, the cell cholesterol of cultured rat fibroblasts was increased by incubation (16 hr) with varying amounts of free cholesterol added to the culture medium. Uptake of HDL by the cells was then determined in a subsequent assay using rat HDL that had been labeled in the cholesteryl ester moiety with [^{3}H]cholesteryl-oleyl ether and in the apoprotein moiety with ^{125}I-NMTC-apoA-I. Results of a representative experiment are shown in **Fig. 1A**. Uptake of ^{125}I-NMTC-apoA-I from doubly labeled HDL increased progressively as the cholesterol content of the cells increased. In contrast, uptake of HDL associated [^{3}H]cholesteryl ether decreased with increasing cellular cholesterol levels.

It should be noted that in Fig. 1 and elsewhere the uptake of each of the HDL tracers is shown in terms of apparent particle uptake, which is calculated in terms of apparent HDL protein uptake. In other words, uptake is shown as the amount of HDL particle uptake that would be necessary to account for the tracer observed in the cells. Expressed in these terms, holoparticle uptake is represented by equal uptake of the two tracers. In the experiment shown in Fig. 1A, uptake of the two tracers did approach the same value at high levels of cellular cholesterol, indicating that holoparticle uptake could account for most of the cholesteryl ether tracer uptake observed under these conditions.

The absolute rate of uptake of the cholesteryl ether tracer by rat fibroblasts did not always decrease with increasing cellular cholesterol, although it did most commonly as shown in Fig. 1A. In some experiments it changed little or actually increased. In six experiments in which cells were exposed to 100 μg of cholesterol/ml for 16 hr before uptake was measured, selective uptake (apparent HDL particle uptake indicated by [^{3}H]cholesteryl ether uptake, minus that indicated by ^{125}I-NMTC-apoA-I uptake) decreased from 0.27 ± 0.13 to 0.10 ± 0.07 μg of HDL protein/mg cell protein/4 hr ($P < 0.05$); particle uptake (indicated by ^{125}I-NMTC-apoA-I uptake) increased with

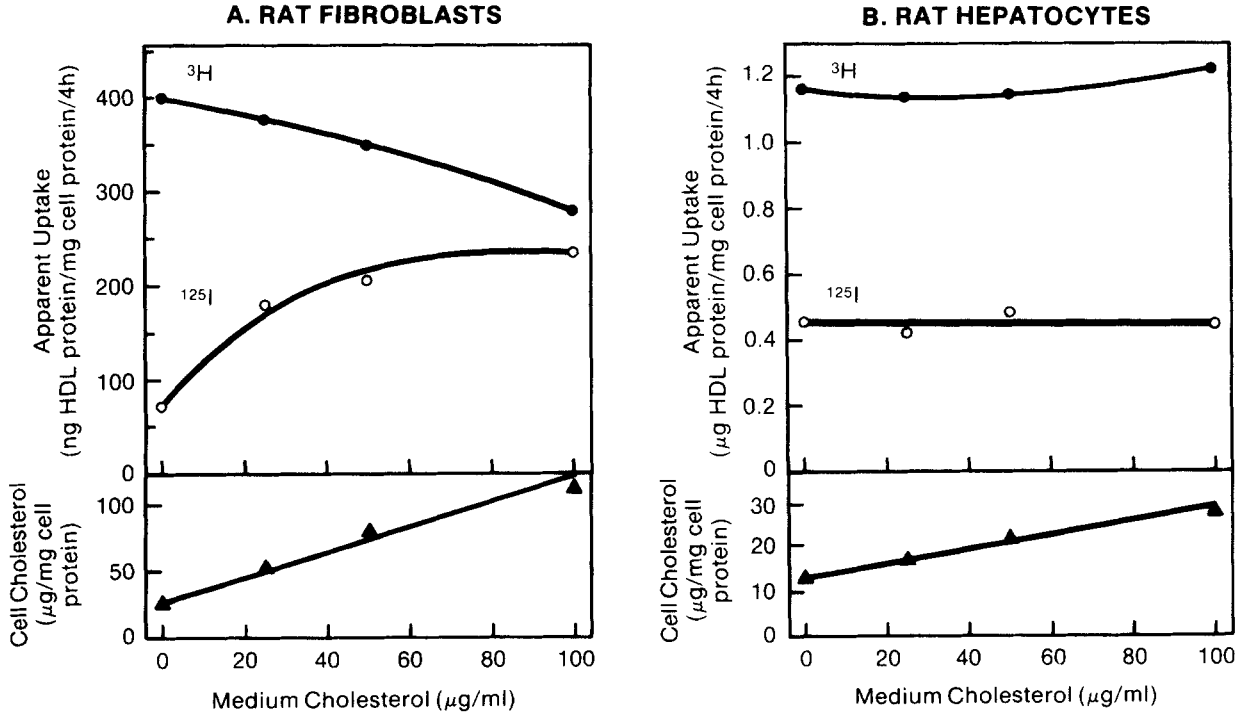

Fig. 1. Effect of increasing cell cholesterol content on HDL uptake by rat fibroblasts and hepatocytes. Cells were incubated for 16 hr with the indicated concentrations of cholesterol in the medium, and then fresh medium containing doubly labeled HDL (20 μg of protein/ml) was introduced for a 4-hr uptake period as described in Methods. Each point is the average of triplicate plates.

much greater variability from 0.058 ± 0.022 to 0.98 ± 1.48 μg of HDL protein/mg cell protein/4 hr. In all cases the *ratio* of the uptake of [^{3}H]cholesteryl ether to the uptake of ^{125}I-NMTC-apoA-I decreased and approached unity (6.1 ± 2.6 before and 1.7 ± 1.1, $P < 0.01$, after exposure to cholesterol in the medium). Thus, selective uptake was in every case down-regulated by increasing cellular cholesterol, even when total uptake of the ether tracer of cholesteryl esters actually increased. This suggests that the uptake of HDL cholesteryl esters represents the sum of two processes: holoparticle uptake, which is reflected by apoA-I uptake (7) and which increases with increasing cell cholesterol; and selective uptake, which decreases with increasing cellular cholesterol. Thus, on increasing cholesterol levels, the cholesteryl ester uptake that is mediated by particle uptake may sometimes increase more than cholesteryl ester uptake that is mediated by selective uptake decreases.

In contrast to the results obtained in fibroblasts, we were not able to demonstrate regulation of selective uptake in primary cultures of rat hepatocytes, as shown in Fig. 1B. As in the fibroblast experiments, attempts were made to increase the cell cholesterol during a 16-hr loading incubation, and uptake of the doubly labeled rat HDL was then measured in a subsequent assay under standard conditions (7). As shown in Fig. 1B, a relatively modest increase in the cholesterol content of hepatocytes was achieved under conditions of exposure to free cholesterol that resulted in a more dramatic increase in the cholesterol content of fibroblasts. No regulation of the uptake of either cholesteryl esters or apoA-I by hepatocytes was observed under these conditions.

Other approaches were also used in an attempt to elicit the regulation of selective uptake in hepatocytes. One such approach was to expose hepatocytes to 25-hydroxycholesterol, a cholesterol analogue that effectively regulates LDL metabolism in some systems (35, 36), and which also has been shown to lead to increased HDL binding by bovine vascular endothelial cells (37). Total cell sterol (including 25-hydroxycholesterol, which reacts as well as cholesterol in the assay system) increased modestly on addition of 25-hydroxycholesterol (from 12.7 to 19.8 μg of cholesterol/mg of protein at 50 μg/ml of sterol in the medium). However, as in the case of loading with cholesterol, no regulation of either cholesteryl ester or apoA-I uptake was observed. Another approach was to expose the cells to mevalonolactone, a cholesterol precursor that can increase cell cholesterol levels (36). Mevalonolactone (10 mM) either alone or in combination with 25-hydroxycholesterol (50 μg/ml), in which case cell cholesterol levels were increased from 12.7 to 24.5 μg of cholesterol/mg protein, also failed to down-regulate selective uptake. Other studies involved the uptake of lipoprotein cholesterol. Human LDL was used, as well as lactosylated human LDL (Lac-LDL) which is degraded by a receptor-mediated, lysosomal mechanism (24, 38). Lac-LDL was rapidly taken up by the hepatocytes; in a 2-hr assay at 50 μg of Lac-LDL protein/ml, uptake was 1.9 μg of protein/hr per mg of cell protein. This represents a delivery to the cells of more than 4 μg of cholesterol/hr per mg of cell protein. In spite of this high uptake rate, cell cholesterol levels were not increased after incubation with 100 μg Lac-LDL protein per ml for 16 hr, and selective uptake was not down-regulated. Human LDL (200 μg/ml), alone or in combination with Lac-LDL (100 or 200 μg/ml), increased cell cholesterol levels from 12.5 to as high as 18.5 μg/mg of cell protein, but did not reduce selective uptake.

Regulation of the selective uptake of HDL cholesterol esters in vivo

Previous in vivo studies have shown that only liver and the steroidogenic tissues of normal rats take up HDL-associated [^{3}H]cholesteryl ether at a greater fractional rate than the HDL-associated ^{125}I-NMTC-apoA-I (5, 6). However, studies in vitro have shown that fibroblasts (and thus perhaps other extrahepatic, nonsteroidogenic cells) also have the capacity for selective uptake of cholesteryl esters (7). This suggested that selective uptake by some cells might be down-regulated in vivo in normal rats.

To test this hypothesis, we examined the possibility that selective uptake would be up-regulated in hypocholesterolemic rats. To lower plasma cholesterol levels, rats were treated with 4-APP, a drug that reduces hepatic lipoprotein secretion (11, 12). This treatment reduced plasma cholesterol levels by about 90% **(Table 1)**.

The plasma decay kinetics and the rates of uptake of HDL by individual tissues were determined in both treated and untreated rats. HDL labeled with ^{125}I-NMTC-apoA-I and [^{3}H]cholesteryl-oleyl ether was intravenously injected, periodic blood samples were withdrawn, and tissues were taken for radioassay 24 hr after tracer injection when more than 90% of the tracer had been irreversibly cleared from the circulation.

The plasma fractional catabolic rates of both tracers were increased by 4-APP treatment (Table 1). The activities of selected tissues in uptake of the HDL tracer are shown in **Table 2**. These tissues account for about 90% of total HDL cholesteryl ester uptake in both treated and untreated animals, but for only about 60% of apoA-I uptake. This difference is largely due to exclusion of data for kidney which accounts for about 25% of apoA-I uptake but virtually no cholesteryl ester uptake, a result previously reported (4). The activities of the various tissues are expressed as organ fractional catabolic rates per gram of tissue. This represents the fraction of the plasma pool of the traced HDL component cleared per hour per gram of tissue (39).

All tissues of rats treated with 4-APP took up both HDL tracers at greater fractional rates than did the tissues of control animals (Table 2). However, the fractional rates of uptake of the two tracers were not increased

TABLE 1. Effect of 4-APP and ethinyl estradiol treatment on the plasma fractional catabolic rates of doubly labeled HDL in rats

Treatment	Plasma Cholesterol	Plasma Fractional Catabolic Rate		n
		^{125}I	^{3}H	
	mg/dl	*hr*$^{-1}$		
Control	70.6 ± 16.3	0.158 ± 0.051	0.162 ± 0.017	9
4-APP	6.3 ± 1.1	0.459 ± 0.077	0.436 ± 0.103	4
Ethinyl estradiol	11.0 ± 0.0	0.443 ± 0.120	0.710 ± 0.100	3

in parallel in all tissues. This can be seen in Table 2 in terms of the differences in the ratio of the fractional rates of uptake of the two tracers in control and treated animals. In the adrenal gland the ratio of fractional rates increased from 5.3 to 11.3, and in ovary from 3.0 to 5.9, indicating that the contribution of selective uptake to total uptake of HDL cholesteryl esters in these steroidogenic tissues was greater in 4-APP-treated rats than in control animals. More to the point of the hypothesis to be tested, tissues that did not display selective uptake of cholesteryl esters in untreated rats (uptake ratio near 1) did so in treated rats (uptake ratio >1). Skin, skeletal muscle, and adipose tissue of rats treated with 4-APP exhibited a clearly higher rate of cholesterol ester uptake from HDL than of apoA-I, while these tissues of untreated rats did not. Thus, drug treatment elicited selective uptake in some tissues. There was no significant change in the fractional contribution of selective uptake to total uptake by the liver, a result consonant with the apparent absence of regulation in hepatocytes in vitro.

To examine the possibility that the results observed on treatment of rats with 4-APP were the consequence of a drug side effect independent of altered plasma lipoprotein levels, we studied rats treated with another drug, ethinyl estradiol, which also lowers plasma cholesterol levels but by a different mechanism (14, 15). In pharmacologic doses, this drug induces hepatic lipoprotein receptors and so increases hepatic lipoprotein uptake and catabolism (15).

Ethinyl estradiol also effectively lowered plasma cholesterol levels, as shown in Table 1. As in the case of 4-APP, the plasma fractional catabolic rates for both HDL tracers were increased in the treated rats. In further parallel to the 4-APP studies, the organ fractional catabolic rates of both tracers increased in all tissues (**Table 3**). Also in this case, the contribution of selective uptake to total uptake of cholesteryl esters was increased in the adrenal gland and ovary, as indicated by increased ratios of the organ fractional catabolic rates for the two HDL tracers. In skin, muscle, and adipose tissue, selective uptake of cholesteryl esters was elicited, as was the case with 4-APP.

It can be seen in Table 3 that the ratio of cholesteryl ether to apoA-I uptake was less than 1 for the adipose

TABLE 2. Effect of 4-APP treatment on the uptake of HDL-associated ^{125}I-NMTC-apoA-I and [^{3}H]cholesteryl-oleyl ether by rat tissues

Tissue	Fractional Catabolic Rate per g of Tissue		Uptake Ratio $^{3}H/^{125}I$
	^{125}I	^{3}H	
	hr^{-1} × *g*$^{-1}$ × *10*^{3}		
Control (n = 5)			
Liver	4.09 ± 1.14	9.48 ± 0.74	2.3 ± 0.5
Adrenals	7.80 ± 2.73	41.7 ± 11.0	5.3 ± 1.4
Ovaries	8.16 ± 3.24	24.5 ± 9.9	3.0 ± 0.9
Adipose	0.120 ± 0.04	0.152 ± 0.05	1.3 ± 0.5
Skin	0.172 ± 0.05	0.176 ± 0.05	1.0 ± 0.2
Muscle	0.036 ± 0.01	0.033 ± 0.005	0.92 ± 0.5
4-APP (n = 4)			
Liver	7.83 ± 3.86	17.4 ± 8.5	2.2 ± 0.5
Adrenals	47.9 ± 17.2	540 ± 44	11.3 ± 3.6
Ovaries	36.6 ± 6.3	217 ± 74	5.9 ± 2.2
Adipose	0.381 ± 0.08	1.68 ± 0.98	4.4 ± 2.1
Skin	0.376 ± 0.06	1.50 ± 0.01	4.0 ± 1.3
Muscle	0.093 ± 0.05	0.207 ± 0.06	2.2 ± 1.0

TABLE 3. Effect of ethinyl estradiol treatment on the uptake of HDL-associated ^{125}I-NMTC-apoA-I and [^{3}H]cholesteryl-oleyl ether by rat tissues

Tissue	Fractional Catabolic Rate per g of Tissue		Uptake Ratio $^{3}H/^{125}I$
	^{125}I	^{3}H	
	$hr^{-1} \times g^{-1} \times 10^{3}$		
Control (n = 3)			
Liver	5.58 ± 0.64	16.8 ± 2.5	3.0 ± 0.2
Adrenals	9.73 ± 3.23	45.6 ± 8.6	4.7 ± 0.8
Ovaries	7.00 ± 1.55	27.0 ± 8.6	3.9 ± 0.6
Adipose	0.450 ± 0.07	0.225 ± 0.03	0.50 ± 0.07
Skin	0.240 ± 0.07	0.264 ± 0.14	1.1 ± 0.3
Muscle	0.080 ± 0.03	0.128 ± 0.06	1.6 ± 0.2
Ethinyl estradiol (n = 3)			
Liver	28.8 ± 12.8	53.7 ± 9.6	1.9 ± 0.5
Adrenals	56.2 ± 31.4	540 ± 236	9.6 ± 4.0
Ovaries	29.8 ± 8.8	229 ± 80	7.7 ± 0.9
Adipose	1.73 ± 0.90	1.90 ± 0.94	1.1 ± 0.1
Skin	0.530 ± 0.18	1.42 ± 0.42	2.7 ± 0.2
Muscle	0.210 ± 0.05	0.479 ± 0.27	2.3 ± 0.9

tissue of control, mock-injected animals. Such a low ratio was not observed in any uninjected animals, nor in mock-injected rats in the 4-APP experiments. The different result in this case may have been a consequence of the stress of four daily injections of the ethinyl estradiol carrier. Indeed, these animals had very little adipose tissue. In spite of this low ratio of fractional uptake rates in the control rats, the ratio did increase with drug treatment, consistent with an increased dependency of the adipose tissue on selective uptake.

In the case of the liver, the organ FCR for the apoA-I tracer was increased by ethinyl estradiol treatment to 5.15 times control animals, while the organ FCR for the cholesteryl ester tracer was increased to only 3.2 times control (Table 3). This indicates that selective uptake made a *smaller* fractional contribution to total HDL cholesteryl ester uptake by liver in these drug-treated rats than in control rats. However, this does not necessarily denote a decreased capacity for selective uptake. Such a result could be explained by an increased hepatic holoparticle uptake via apoB/E receptors (14, 15, 40). In fact, the increase in the fractional rate of particle uptake, as indicated by apoA-I uptake, was far greater in the case of ethinyl estradiol treatment than in the case of 4-APP treatment (an increase of 23×10^{-3} plasma pools/hr per g, as compared to 3.7×10^{-3} for 4-APP at about the same plasma HDL cholesterol levels).

We have previously shown that denser HDL particles donate cholesteryl esters to the selective uptake pathway of mouse adrenal cells at a greater fractional rate than do more buoyant HDL particles (26). Therefore, HDL heterogeneity could influence the rate of selective uptake in vivo. In view of this possibility, we asked whether treatment of rats with 4-APP or ethinyl estradiol modified the density distribution of the HDL particles as well as lowering HDL cholesterol levels. To do this, HDL from control and treated rats was separated by equilibrium density gradient ultracentrifugation in a mixed sucrose salt gradient (31), as shown in **Fig. 2**. Both drug treatments resulted in a shift in the peak density for HDL cholesterol to a slightly higher density. More material appeared in

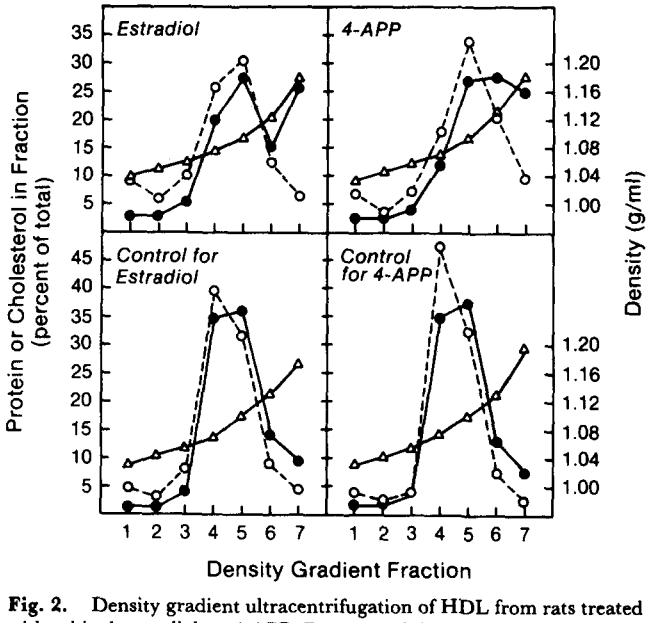

Fig. 2. Density gradient ultracentrifugation of HDL from rats treated with ethinyl estradiol or 4-APP. Rats were injected with the indicated drug or the corresponding carrier solution as described in Methods. Plasma HDL was then isolated by ultracentrifugation in the density range 1.07-1.21 g/ml. The HDL was further fractionated into seven density ranges by density gradient ultracentrifugation as described in Methods. Symbols used: (●), percent of total protein recovered in the fraction; (○), percent of total cholesterol recovered in the fraction; (△), density of the fraction.

fractions of higher density than the normal predominant HDL_2-like pattern of rats. While the tracer HDL injected into both control and drug treated rats was prepared from normal rats, it could not be excluded that the change in density profile due to drug treatment played a role in the apparent regulation of selective uptake. This is particularly true for the apoA-I tracer, since this apoprotein readily exchanges between particles and a large fraction of labeled apoA-I uptake by tissues undoubtedly occurred from particles of endogenous HDL.

We circumvented this problem by measuring selective uptake independently of the mobile coat apoproteins using synthetic HDL particles (24, 41). In this case selective uptake was measured in terms of the differential rates of uptake of two core lipids that remain with the injected tracer particles (7, 26). The synthetic particles, described in detail elsewhere (26), were made with egg lecithin, cholesteryl oleate, and apolipoprotein A-I. Cholesteryl oleate was, as usual, traced by the nonhydrolyzable, intracellularly [^{3}H]cholesteryl-oleyl ether. The other lipid core marker, which traced the particle core per se, was [^{14}C]sucrose octaoleate, a nonexchangeable, nonhydrolyzable (42) marker that is taken up only by particle uptake (26). Our usual ^{125}I-NMTC-apoA-I tracer was also incorporated for purposes of comparison. The synthetic particles, with these tracers incorporated, closely resemble rat HDL in terms of equilibrium density profile, particle size, and metabolic behavior, both in vivo and in vitro (26).

We injected these synthetic HDL particles into control and ethinyl estradiol-treated rats. The plasma FCRs of all three tracers were significantly increased in hypocholesterolemic rats **(Table 4)**; the FCRs for the ^{125}I-NMTC-apoA-I and that for the [^{3}H]cholesteryl-oleyl ether were not significantly different from those observed with authentic HDL. The uptake rates of these two tracers by individual tissues were also very similar to those obtained with authentic HDL. All tissues of the rats on drug treatment took up the three tracers at greater fractional rates than did the tissues of untreated animals, as shown in **Table 5**. In adrenal gland, the contribution of selective uptake to total uptake of cholesteryl esters again increased on drug treatment. This was true whether the fractional rate of cholesteryl ether uptake was expressed relative to uptake of apoA-I or relative to uptake of the nonexchangeable synthetic HDL core marker. Also in agreement with the results with authentic HDL, ethinyl estradiol treatment induced the selective uptake of cholesteryl esters by adipose tissue, skin, and muscle. Selective uptake by adipose tissue and skin was similar, whether determined relative to the uptake of apoA-I or relative to the uptake of [^{14}C]sucrose octaoleate. The increased organ FCR for uptake of [^{14}C]sucrose octaoleate by liver confirmed the increased holoparticle uptake induced in this organ by ethinyl estradiol treatment that was suggested by the increased apoA-I uptake from authentic HDL.

DISCUSSION

We have shown that selective uptake of cholesteryl esters by cultured rat fibroblasts can be down-regulated by uptake of free cholesterol from the medium. This occurs even in the face of an increased uptake of HDL particles, as indicated by the increased uptake of HDL-associated apoA-I (Fig. 1). In these experiments, conditions were similar to those under which Oram, Brinton, and Bierman (43) and Brinton et al. (44) showed increased saturable HDL binding by human fibroblasts and smooth muscle cells in response to increased cell cholesterol levels; this binding was associated with apoprotein uptake (44). We also observed an increase in cell-associated tracer that could be released from the cells by a 2-hr "chase" period and subsequent trypsin treatment (data not shown), which may be taken as a measure of HDL binding. Clearly, the reduced selective uptake of cholesteryl esters observed in cholesterol-loaded cells, which occurs even in the face of increased HDL binding and increased particle uptake, indicates that selective uptake is not a necessary consequence of total reversible cell association. It is possible that selective uptake is a necessary consequence of HDL binding to a special subset of sites that are down-regulated on increasing cellular cholesterol levels but which are masked by increased binding to other sites. Alternatively, selective uptake may be regulated independently of binding, whether or not uptake occurs from a special subset of binding sites.

TABLE 4. Effect of ethinyl estradiol treatment of rats on the plasma FCR of synthetic HDL labeled with ^{125}I-NMTC-apoA-I, [^{3}H]cholesteryl-oleyl ether, and [^{14}C]sucrose octaoleate

Treatment	Plasma Cholesterol	Plasma Fractional Catabolic Rate			n
		^{125}I	^{3}H	^{14}C	
	mg/dl		*hr^{-1}*		
Control	85.7 ± 10.1	0.154 ± 0.02	0.176 ± 0.02	0.0896 ± 0.01	3
Ethinyl estradiol	9.5 (7.0, 12.0)	0.538 (0.621, 0.455)	0.762 (0.841, 0.682)	0.413 (0.518, 0.307)	2

TABLE 5. Effect of ethinyl estradiol treatment on the uptake of synthetic HDL labeled with ^{125}I-NMTC-apoA-I, [^{3}H]cholesteryl-oleyl ether, and [^{14}C]sucrose octaoleate by rat tissues

Tissue	Fractional Catabolic Rate per g of Tissue			Uptake Ratio	
	^{125}I	^{3}H	^{14}C	^{3}H/^{125}I	^{3}H/^{14}C
	$hr^{-1} \times g^{-1} \times 10^{3}$				
Control (n = 3)					
Liver	9.5 ± 1.0	18.4 ± 2.6	6.7 ± 0.9	1.9 ± 0.1	2.7 ± 0.3
Adrenals	11.2 ± 0.89	91.3 ± 19.1	11.2 ± 1.4	8.2 ± 2.2	8.2 ± 0.8
Adipose	1.00 ± 0.54	0.700 ± 0.26	0.517 ± 0.13	0.70 ± 0.11	1.4 ± 0.3
Skin	0.225 ± 0.05	0.291 ± 0.04	0.222 ± 0.06	1.3 ± 0.21	1.3 ± 0.2
Muscle	0.079 ± 0.03	0.066 ± 0.01	[a]	0.84 ± 0.67	[a]
Ethinyl estradiol (n = 2)					
Liver	35.3 (37.5, 33.1)	46.3 (49.5, 43.0)	32.3 (42.2, 22.4)	1.3	1.4
Adrenals	58.7 (57.8, 59.5)	1492.0 (1533, 1450)	115.0 (125, 104)	25.4	13.0
Adipose	1.82 (2.37, 1.27)	3.85 (4.88, 2.82)	1.72 (2.16, 1.27)	2.1	2.2
Skin	0.595 (0.62, 0.57)	1.92 (2.19, 1.64)	0.688 (0.785, 0.591)	3.2	2.8
Muscle	0.163 (0.211, 0.115)	0.399 (0.368, 0.430)	[a]	2.4	[a]

[a] Insufficient data.

In contrast to the readily demonstrated regulation of selective uptake in rat fibroblasts, such an effect could not be shown in primary rat hepatocytes. Increases in cell cholesterol levels due to exposure to free cholesterol in the medium, as well as receptor-dependent loading of the hepatocytes with lipoprotein cholesterol, failed to regulate selective uptake in these cells. However, it was more difficult to increase the cell cholesterol of hepatocytes than of fibroblasts. Liver cells can process cholesterol in ways that fibroblasts cannot, for instance by conversion to bile acids or secretion of lipoproteins. In fact, we were not able to increase cholesterol levels in hepatocytes to the same level as achieved in fibroblasts. Because of this limited cholesterol loading, it could not be concluded that the capacity to regulate selective uptake is absent from cultured rat hepatocytes. It is clear, however, that these cells are resistant to regulation even in the face of high cholesterol uptake and significant increases in cell cholesterol level.

Studies of HDL metabolism in vivo supported the results of the in vitro studies. Most significantly, the drug treatments induced selective uptake in skin, muscle, and adipose tissue, which did not exhibit selective uptake in control rats. These tissues provide the most unqualified evidence that selective uptake in vivo is regulated by lipoprotein levels. However, tissue cholesterol levels change little, if at all, in most tissues (except steroidogenic tissues and liver) of rats after 4-APP treatment (11), and it might thus be argued that drug effects other than lipoprotein lowering might explain increased selective uptake in these tissues. However, the similarity of results obtained by the use of two drugs that act by different mechanisms and which apparently have in common only their effects on plasma lipoprotein levels, coupled with the observation that selective uptake can be regulated in vitro according to cell cholesterol status, suggest that cholesterol availability is the factor modulating selective uptake in skin, muscle, and adipose tissue of the drug-treated rats.

Hypocholesterolemia induced in rats by treatment with 4-APP or ethinyl estradiol also resulted in an increased contribution of selective uptake to total uptake of HDL cholesteryl esters by steroidogenic tissues. Selective uptake by mouse adrenal tumor cells is increased both by ACTH treatment and decreased by cholesterol loading using hLDL (T. P. Knecht and R. C. Pittman, unpublished results); it is not clear whether the ACTH effect is mediated through or is independent of cell cholesterol status. Thus the increased selective uptake in rats treated with 4-APP or ethinyl estradiol could reflect the sum of an increased trophic hormone effect and a direct effect of lowered plasma lipoprotein levels, and we cannot separate the two. The same considerations apply to the ovary. Whatever the mechanism(s) of regulation, the maintenance of about normal levels of HDL cholesteryl ester uptake by steroidogenic tissues occurred predominantly by increased selective uptake. However, this does not appear to account for most adrenal cholesterol flux in rats treated with 4-APP. Dietschy, Spady, and Stange (45) have reported a high rate of endogenous cholesterol synthesis in rat adrenal glands which increases dramatically on treatment of the animals with 4-APP (46). This endogenous synthetic rate is very high compared to the uptake of HDL cholesteryl esters determined here. Thus endogenous synthesis seems to be a more important source of adrenal cholesterol than HDL cholesteryl esters in rats treated with 4-APP.

Just as a high rate of cholesterol uptake in vitro failed to elicit down-regulation of selective uptake by hepatocytes, so lowering plasma cholesterol levels in vivo failed to provide evidence for up-regulation of selective uptake by liver. However, interpretation of these in vivo results is not straightforward. First, hepatic uptake in hypocholesterolemic rats was measured at about 10% of the normal plasma HDL level. Although the increased fractional rate of selective uptake in hypocholesterolemic rats appears explicable solely in terms of this changed substrate concentration, as judged from the kinetics of uptake by cultured rat hepatocytes (7), and although in the case of 4-APP treatment the fractional rate of hepatic selective uptake increased only in proportion to uptake by other pathways, nonetheless there is still some uncertainty as to whether there may have been some change in selective uptake capacity on drug treatment. Second, both 4-APP and ethinyl estradiol undoubtedly have effects other than simply lowering plasma lipoprotein levels (12, 13, 47); we used drugs that act by different mechanisms (11, 12, 15) in order to mitigate the possibility that effects on HDL metabolism were secondary to other drug effects. Nonetheless, both drugs do increase hepatic lipid levels (12, 13, 47). Treatment with 4-APP suppresses hepatic lipoprotein secretion, leading to accumulation of triglycerides and, to a lesser extent, cholesterol in the liver (12); ethinyl estradiol increases receptor-mediated uptake of lipoproteins, leading to accumulation predominantly of cholesterol in the liver (13–15). Thus it might be argued that up-regulation of hepatic selective uptake should not be expected under either drug treatment because liver cholesterol levels actually increased. On the other hand, there was no evidence that selective uptake *decreased* due to this increase in cholesterol content. In rats treated with 4-APP the ratio of HDL cholesteryl ester uptake to apoA-I uptake was not changed, while the decreased ratio in rats treated with ethinyl estradiol was explicable in terms of increased particle uptake. Thus there was no evidence for regulation of hepatic selective uptake in vivo, just as there was none for regulation of selective uptake by cultured hepatocytes. But, as in the studies in vitro, it is still possible that regulation can be elicited under some conditions.

Another difficulty in interpreting the effects of drug treatment on HDL metabolism in vivo was that both drugs altered the HDL density gradient profiles, whereas the injected tracers were carried in HDL taken from normal rats. We have previously shown that selective uptake by mouse adrenocortical tumor cells is a function of particle density (26), and such effects of HDL heterogeneity could affect our in vivo results. While the cholesteryl ether tracer surely remained with the particle in which it was injected because of the lack of cholesteryl ester transfer activity in rat plasma (48), the apoA-I tracer just as surely exchanged, at least to some extent, with endogenous HDL particles. Therefore, to an unknown degree we were measuring selective uptake in terms of cholesteryl ester

TABLE 6. Effect of 4-APP and ethinyl estradiol treatment on the rate of delivery of HDL cholesterol esters to tissues of rats

Tissue	HDL Cholesteryl Ester Uptake[a]		Fraction Taken up by Selective Pathway[b]	
	Untreated	Treated	Untreated	Treated
	μg of cholesterol × hr^{-1} × g^{-1}		%	
4-APP				
Liver	40	7.2	55	58
Adrenals	163	205	82	92
Ovaries	95	76	67	83
Adipose	0.53	0.64	13	76
Skin	0.68	0.57	6	73
Muscle	0.12	0.074	12	62
Ethinyl estradiol				
Liver	67	28	66	46
Adrenals	184	287	78	90
Ovaries	110	123	74	87
Adipose	0.91	1.00	[c]	[c]
Skin	1.05	0.77	9	63
Muscle	0.50	0.26	37	56

The values shown were calculated from the data of Tables 3 and 4.

[a] Calculated as: total ester cholesterol per ml plasma × rat weight × 0.0405 (plasma volume per g) × 0.8 (assumed fraction of cholesteryl esters in the HDL pool) × plasma FCR attributable to organ (Table 3 for 4-APP or Table 4 for ethinyl estradiol)/organ weight.

[b] Derived from Tables 3 and 4 as: (^{3}H uptake − ^{125}I uptake)/^{3}H uptake.

[c] Not calculated.

uptake from one class of particles to apoA-I uptake from another. To get around this problem, we evaluated the effect of drug treatment on selective uptake measured in terms of two tracers that remain with the particles in which they are injected. To do this we used synthetic particles to incorporate the [^{14}C]sucrose octaoleate marker of the core per se (26). The agreement of the [^{3}H]cholesteryl-oleyl ether and ^{125}I-NMTC-apoA-I tracers in these particles to the same tracers carried in authentic HDL supported the validity of the synthetic particle model. The general agreement of selective uptake measured in terms of the two lipid tracers with selective uptake measured in terms of the cholesteryl ether and apoA-I tracers confirmed the up-regulation of selective uptake in various tissues on drug treatment.

It can be calculated from the data presented here, assuming that our tracers adequately traced their endogenous tracees, that in hypocholesterolemic animals actual mass delivery of HDL cholesteryl esters to the extrahepatic tissues was not much changed from that in untreated animals, despite the drastic reduction in plasma cholesterol levels **(Table 6)**. This maintenance of approximately normal cholesteryl ester uptake was in large part at the expense of uptake by the liver. In those tissues able to compensate for the decreased plasma cholesterol levels, the compensation was due to increased selective uptake (indicated by a greater increase in the fractional rate of cholesteryl ester uptake than of apoA-I uptake), and not to particle uptake which actually decreased in each case. Thus, as shown in Table 6, selective uptake accounted for only a minor fraction of total HDL cholesteryl ester uptake by muscle, adipose tissue, and skin in control rats, but for the preponderance of uptake in those tissues of treated animals. Of course we do not know the contribution of selective uptake to the total provision of cholesterol to extrahepatic tissues. For example, Dietschy et al. (45) and Andersen and Dietschy (46) have shown increased de novo cholesterol synthesis in some tissues of rats treated with 4-APP which appears to be much greater than the cholesterol provided by the selective uptake pathway. Induction of apoB/E receptors may also play a role.

It can be determined from the data described here, or from data previously published (6, 26), that liver accounts for about 65% of whole-body HDL cholesteryl ester uptake of normal rats and that more than half of that occurs by the selective uptake pathway; about 90% of cholesteryl esters taken up in excess of apoA-I is taken up by the liver. Thus, in the normal rat, the pathway functions predominantly to direct HDL cholesteryl esters to the liver. By definition, this selective uptake occurs without parallel uptake of apoA-I (4) and in fact without uptake of the HDL particle per se (7, 26). To the extent that these HDL cholesteryl esters arise from free cholesterol which leaves extrahepatic cells and is then esterified by plasma lecithin:cholesterol acyltransferase (1), the pathway must contribute to the return of "excess" cholesterol from extrahepatic tissues to the liver, i.e., it must contribute to "reverse cholesterol transport" (1, 2). At least under the extreme conditions used in the present study, cholesterol deprivation results in up-regulation of selective uptake by some extrahepatic tissues. Thus, at very low levels of plasma cholesterol, HDL cholesteryl esters are directed away from the liver to other sites. In normal animals, there appears to be enough cholesterol available to keep the selective uptake pathway down-regulated in all tissues except the liver and steroidogenic tissues.

Cultured human cells in vitro exhibit a pattern of selective uptake much like that observed in rat cells (7). It is therefore possible that this pathway plays some role in cholesterol homeostasis and reverse cholesterol transport in man in vivo as it does in the rat.

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