Utilization of cholesterol-rich lipoproteins by perfused rat adrenals

Salman Azhar,[1] David Stewart, and Eve Reaven

Department of Medicine, Stanford University School of Medicine, and Geriatric Research, Education, and Clinical Center, Veterans Administration Medical Center, Palo Alto, CA 94304

Abstract This study describes high density lipoprotein (HDL) uptake in the rat adrenal using a newly developed nonrecycling perfusion technique to control both the quality and quantity of the supplied lipoprotein. The aim of the study was to quantify a nonendocytic (alternative) pathway in the delivery of HDL-cholesterol. All experiments were conducted using an acute lipoprotein-deficient rat model (24 h 4-aminopyrazolo-[3, 4-d]-pyrimidine, 4-APP) in which circulating levels of cholesterol were reduced by one half, but various adrenal gland measurements of cholesterol metabolism were unchanged. Both rat HDL (rHDL) and affinity-purified human HDL_3 ($hHDL_3$) were used throughout the study. Microscopic autoradiographs (ARGs) indicate that both ligands bind avidly and exclusively to cells of the adrenal fasciculata and reticularis zones. Despite differences in binding affinity, both ligands deliver approximately the same total cholesterol to the cell interior as estimated by double-labeled residualizing tags on HDL (i.e., ^{125}I-labeled dilactitol tyramine-[^{3}H]cholesteryl linoleyl ether (DTT-CLE) HDL). The internalized cholesterol can account for much of the corticosterone produced during the 90-min time frame; however, only a small fraction of this cholesterol could have been provided via the endocytic pathway. Data obtained with the use of ^{125}I-labeled DTT-[^{3}H]CLE-HDL show that only 8.0% (or 0.7%) of corticosterone produced with rHDL (or $hHDL_3$) could have come from cholesterol internalized as a component of intact HDL (i.e., via the endocytic pathway). These calculations strengthen the electron microscopy autoradiographic data that show that few exposed silver grains (representing the localization of the ^{125}I-isotope) are found within the cell cytoplasm. Thus, despite differences in the uptake characteristics of the two ligands, most of the HDL-cholesterol internalized and used for corticosterone production during adrenal perfusion apparently comes from a pathway in which intact HDL are not internalized. — **Azhar, S., D. Stewart, and E. Reaven.** Utilization of cholesterol-rich lipoproteins by perfused rat adrenals. *J. Lipid Res.* 1989. **30:** 1799–1810.

Supplementary key words HDL • HDL-cholesterol uptake • nonendocytic pathway • adrenal cholesterol uptake • corticosterone production • residualizing labels for HDL protein and cholesterol • autoradiography

Previous studies (1–9) suggest that tissues of the rat that require especially large amounts of exogenous lipoprotein-derived cholesterol (e.g., liver, adrenal, ovary) obtain much of this cholesterol from high density lipoproteins (HDL) by a cholesterol-uptake process that is an alternative to the classical endocytic pathway. In this alternative pathway, substantially more HDL-cholesterol than HDL apoprotein is taken up by cells (1–9). The pathway appears to operate equally well in vivo (2, 3, 6) and in vitro (1, 4, 5, 7–9).

In recent studies we have identified such a nonendocytic pathway for the uptake of lipoprotein-cholesterol in a rather specialized steroidogenic organ — the luteinized ovary of superovulated immature rats (5). What is of particular interest in the luteinized ovary is that not only HDL but also human low density lipoproteins (hLDL) appear to supply cholesterol by the alternative pathway (10–12). Since hLDL contain apolipoprotein B, one would anticipate that apoB,E receptor-mediated uptake of hLDL would be the major route by which cholesterol is obtained. Yet, in the luteinized ovary, hHDL and hLDL are bound similarly to the surface of the luteal cells (5, 10–12), and both ligands appear to supply cholesterol to the interior of the cell in large excess of interiorized apoprotein (10, 12). This cholesterol uptake parallels a dramatic increase in the progesterone output of the perfused ovary (5, 10–12). Our observations suggest that the luteinized ovary depends largely on a nonendocytic pathway for cholesterol uptake, regardless of the nature of the provided lipoprotein (10–12). This may be the case since this tissue appears to lack the well-developed apoB,E receptor system (12) found in many other tissues (13).

In an attempt to understand the general significance of the alternative pathway to lipoprotein cholesterol delivery,

Abbreviations: hLDL, human low density lipoprotein; $hHDL_3$, human high density lipoprotein$_3$; rHDL, rat high density lipoprotein; DTT, dilactitol tyramine; apoB, apolipoprotein B; apoE, apolipoprotein E; apoA-I, apolipoprotein A-I; apoA-IV, apolipoprotein A-IV; BSA, fatty acid-poor bovine serum albumin; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; 4-APP, 4-aminopyrazolo-[3,4-d]-pyrimidine; 17α-E_2, 17α-ethinyl estradiol; CLE, cholesteryl linoleyl ether; TCA, trichloroacetic acid; [^{125}I]DTT-[^{3}H]CLE-HDL, ^{125}I-labeled dilactitol tyramine and [^{3}H]cholesteryl linoleyl ether-labeled HDL.

[1]To whom reprint requests should be addressed at: GRECC (182-B), VA Medical Center, 3801 Miranda Avenue, Palo Alto, CA 94304.

we have now turned to the rat adrenal as a more conventional steroidogenic organ requiring high levels of exogenous lipoprotein cholesterol for function. In the current study we utilized many of the combined physiological, biochemical, and structural techniques used in earlier studies. As such, all experiments were conducted using a nonrecycling perfusion system to deliver ligand directly to the adrenal (14). With this technique, not only the amount of ligand delivered but also the quality of the ligand could be controlled; i.e., lipoprotein composition was not altered as a result of either recirculation in the body (15–17) or static incubation conditions in vitro. In addition, two different ligands were used in parallel experiments throughout the study. Both ligands were HDL. However, one was obtained from rat serum and contained, among other apoproteins, apoE known to have affinity for the tissue apoB,E receptor system (13, 18). It was presumed that this rat lipoprotein (rHDL) would be a good candidate for the adrenal endocytic pathway. The second ligand used was affinity-purified (apoE-free) HDL_3 obtained from human serum ($hHDL_3$) which contained only apolipoprotein A-I. This HDL was not a likely candidate for the apoB,E receptor pathway (13, 18) and its processing by the intact adrenal was to be compared with that of rHDL. For the most part, the perfused lipoproteins were chemically modified and used as residualizing (i.e., nonreleasable) ligands, as well as being doubly labeled with ^{125}I and [^{3}H]cholesteryl ether in respective protein and cholesteryl ester positions (2, 19). Also, electron microscopic autoradiography was used to confirm the interiorization of the labeled apoproteins into adrenal cells (5, 10). Finally, correction factors were applied to cholesterol uptake data (12) to account for the amount of intact ligand that was bound by the tissue but trapped extracellularly in a microvillar compartment of adrenal cortex cells (20).

It was anticipated that these combined techniques applied to acutely lipoprotein-deficient rats would permit an in-depth and novel mechanistic view of the HDL-cholesterol uptake process in the intact adrenal not possible with more conventional methods.

MATERIALS AND METHODS

Materials

^{125}Iodine (sodium iodide, sp act ~17.4 mCi/μg of iodine) was purchased from E. I. du Pont de Nemours & Co., Inc.; NEN Research Products, Boston, MA. [1α, 2α (n)-^{3}H]Cholesterol (sp act 40–60 Ci/mmol) was obtained from Research Products International Corp., Mount Prospect, IL. 4-Aminopyrazolo-[3, 4-d]-pyrimidine (4-APP) was the product of Aldrich Chemical Co., Milwaukee, WI. The following chemicals were supplied by Sigma Chemical Company: fatty acid-poor bovine serum albumin (BSA), galactose oxidase, lactose, tyramine, 17α-ethinyl estradiol (17α-E_2), and heparin-agarose. McCoy's 5a medium and antibiotics were purchased from GIBCO Laboratories, Grand Island, NY. All other chemicals used were of analytical grade.

Preparation of lipoproteins

Human (h) high density lipoprotein ($hHDL_3$) was isolated from fresh plasma of healthy male donors; rat (r) high density lipoprotein (rHDL) was obtained from male Sprague-Dawley rats (~3 months of age; Bantin and Kingman, Fremont, CA). In each case, 3 mM EDTA, 1 mM benzamidine, 1 mM phenylmethylsulfonylfluoride, and 10 U/ml of kallikrein inactivator (21) were added to the plasma, and lipoproteins were isolated by preparative ultracentrifugation (22) in a 60 Ti rotor (Beckman Instruments, Inc.; Palo Alto, CA) at 58,000 rpm; $hHDL_3$ was isolated between densities 1.125 and 1.210 g/ml (23). Each $hHDL_3$ fraction was recentrifuged for 18 h at the same density to minimize plasma protein contamination. Trace amounts of apoE present in $hHDL_3$ preparations were removed by heparin-agarose affinity chromatography (24); rHDL was isolated between densities of 1.080 and 1.210 g/ml (25) and used directly.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described previously (5, 10–12). $hHDL_3$ contained apoA-I as the major apoprotein and was devoid of apoE (**Fig. 1**). rHDL contained apoA-I, apoA-IV and apoE as major apoproteins with smaller amounts of apoCs (Fig. 1).

Iodination of lipoproteins. $hHDL_3$ and rHDL were iodinated using the iodine monochloride method of McFarlane (26) as modified for lipoproteins by Bilheimer, Eisenberg, and Levy (27). The average specific activity of $hHDL_3$ and rat HDL was in the range of 400–500 μCi/mg protein. More than 99% of the radioactivity was precipitated by 15% trichloroacetic acid and 2–3% $hHDL_3$ and 5–6% of rHDL were extractable with organic solvents (28, 29). Electrophoretic and autoradiographic procedures indicated that all apoproteins were uniformly labeled.

Preparation of [^{125}I] DTT-[^{3}H]CLE-HDL. These particles were prepared by two separate procedures. For the most part, labeling of HDL with [^{3}H]cholesteryl linoleyl ether (CLE) was carried out by a modification of the procedure of Sparks et al. (30). Briefly, 200 μCi of [^{3}H]CLE in toluene (100 μl) was added to 4 mg (in 400 μl) of rHDL or $hHDL_3$ in a glass tube at room temperature. The toluene was then evaporated under a gentle stream of N_2 for ~45 min. Two ml of rHDL or $hHDL_3$ (~20 mg) plus 1 ml of partially purified human plasma cholesteryl ester transfer protein (31, 32) were added and the mixture was incubated for 16–18 h at 37°C. After incubation and adjustment of the medium density with KBr (d 1.21 g/ml), the labeled lipoproteins were recovered by ultracentrifugation for 48 h at 58,000 rpm, dialyzed against NaCl (0.15 M)-Na_2 EDTA (0.4 mM) sodium

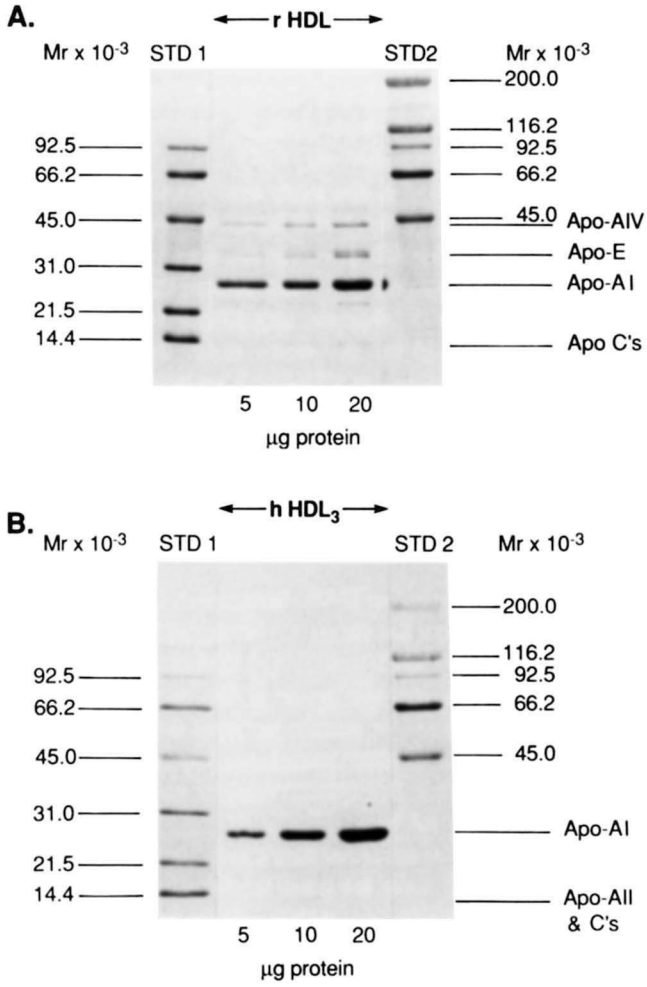

Fig. 1. SDS-PAGE pattern of rHDL (panel A) and $hHDL_3$ (Panel B) used for experiments. In both panels, lanes 1 and 5 show high and low molecular weight (M_r) standards. These are myosin (200,000), β galactosidase (116,000), phosphorylase b (92,500), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). In A, lanes 2–4 show rHDL containing increasing amounts of apoA-I, apoE, and apoA-IV with increasing concentrations of protein used for the gels. Trace amounts of apoC are also present. In B, lanes 2–4 show increasing amounts of apoA-I with increasing concentration of protein used.

phosphate, pH 7.4 (20 mM) and filtered (0.45 μm, pore size filter).

For comparison, labeling of HDL with [^{3}H]CLE was also carried out by the procedure of Hough and Zilversmit (32). In brief, egg phosphatidylcholine/[^{3}H]CLE liposomes were added to an HDL solution in PBS buffer. Lecithin:cholesterol acyltransferase (LCAT) inhibitor, 5,5'-dithiobis(2-nitrobenzoic acid) was also present (final concentration 1.5 mM). After incubation at 37°C for 20 h, [^{3}H]CLE-HDL was isolated by ultracentrifugation as described above.

The protein portion of the residualizing label was prepared by conjugation of [^{125}I]DTT with [^{3}H]CLE-HDL as described by Daugherty et al. (19). Briefly, a portion of DTT (10 nmol) in 0.5 M potassium phosphate buffer, pH 7.7, was added to a tube coated with 20 μg of Iodogen (Pierce Chemical Co., Rockford, IL) and containing 1 mCi of Na ^{125}I. After iodination at room temperature for 1 h, the reaction mixture was treated with 4 units of galactose oxidase to generate aldehyde residues. Subsequently, 2 mg of rHDL or $hHDL_3$ and 20 mM sodium cyanoborohydride were added and the incubation was continued for an additional 45 min at 37°C. The reaction mixture was made 0.1 M in ammonium bicarbonate and then dialyzed for 1 h at room temperature against 0.1 M ammonium bicarbonate. [^{125}I]DTT-[^{3}H]CLE-HDL was separated from free [^{125}I]DTT by gel filtration (Sephacryl S-300, column size 0.9 × 30 cm), followed by extensive dialysis against NaCl (0.15 M)-Na_2 EDTA (1 mM)-sodium phosphate, pH 7.4 (20 mM).

The residualizing function of these particles was tested on granulosa cells incubated in vitro for 24 h with the radiolabeled rHDL ± residualizing tags. Our studies showed that rHDL iodinated without residualizing label were degraded by granulosa cells and the degraded products were released into the medium. In contrast, less than 1% of internalized radioactivity of [^{125}I]DTT lipoproteins was released into the medium, while substantial amounts of the [^{125}I]DTT label accumulated with time within the cells. Also, particles tagged with the CLE label competed specifically for binding sites of native rHDL (data not shown; S. Azhar, L. Tsai, and E. Reaven, unpublished results).

Effect of 4-APP or 17α-E_2 treatment on the adrenal apoB,E receptor concentration. Membrane preparations (33) from adrenals of control, 4-APP-, and 17α-E_2-treated rats were used to compare and quantitate apoB,E receptor protein. In each case the tissues were homogenized in 10 volumes of 10 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM phenylmethylsulfonylfluoride (PMSF), and 4 μg/ml leupeptin; the homogenate was centrifuged (500 *g*) for 5 min, and the supernatant was centrifuged for 60 min at 100,000 *g*. The resulting pellet was washed once with buffer (6 ml/g tissue) to remove lipids, and the membrane pellets were frozen in liquid nitrogen and stored overnight at −90°C. The pellets were suspended (1 g tissue/ml) in 250 mM Tris-maleate, pH 6.0, 2 mM $CaCl_2$, 1 mM PMSF, and 4 μg/ml leupeptin, and sonicated 2 × 20 sec, followed by the addition of an equal volume of 0.32 M NaCl, 2 mM $CaCl_2$, 2% Triton X-100. This mixture was agitated for 10 min at 4°C and centrifuged at 100,000 *g* to remove undissolved material. The supernatant was used immediately for electrophoresis and ligand blotting of apoB,E receptor protein as described by Daniel et al. (34) except that 7% gels and the buffer system of Towbin et al. (35) were used. The transfer was carried out at 300 mA for 17 h, 4°C. After electrophoretic transfer, the nitrocellulose paper was incubated with gold-low density lipoprotein (Au-LDL) conjugates to detect apoB,E receptor protein (36). Scanning densitometry was used to quantitate receptor protein.

Adrenal perfusion

Male Sprague-Dawley rats (VAF-viral free, Charles River Co., Portage, MI) 180–200 g were used for all perfusion studies. The rats were maintained in filtered cages (Maxi-Miser Positive Individual Ventilated System, Thoren Caging System, Hazelton, PA), two rats per cage at 72°C on a 12-h dark/light cycle for approximately 1 week before use. The rats were used as untreated controls or given 4-aminopyrazolo-[3,4-d]-pyrimidine [4-APP 20 mg/kg body weight (10, 12)] for 1 day to reduce circulating levels of lipoproteins. On occasion, results from the acutely treated rats were compared with those from lipoprotein-deficient rats treated for 3 d with 4-APP or 5 days with 17α-ethinyl estradiol [5 mg/kg body weight 17α-E_2 (10)].

Adrenal perfusions were carried out as previously described from this laboratory (14). In general, the adrenals were perfused at room temperature with freshly prepared buffer (McCoy's 5a medium saturated with oxygen, enriched with 0.5% fatty acid-poor bovine serum albumin (BSA), 15 mM HEPES, 100 μg/ml streptomycin, and 100 U/ml penicillin. The buffer flush period was directly followed by perfusion of lipoproteins (2.2 ml/min) for 60–90 min. After ligand perfusion the adrenals were washed for 2 min and either excised for biochemical processing or perfusion-fixed with 2.5% glutaraldehyde (in 0.1 M cacodylate buffer at pH 7.3–7.4, 22°C).

Tissue techniques

Processing of the adrenals for morphological examination and autoradiography at the light and electron microscope level was done as previously described from this laboratory (5, 10–12). For light microscope autoradiographs (LM-ARG), slides were exposed in the dark for 4–8 days; for EM-ARGs, grids were exposed for 3–6 weeks. For quantification of exposed silver grains associated with z.fasciculata cells, this zone of the tissue was identified on the grids after which six-ten nucleated endocrine cells of good technical quality from three blocks of each experimental animal were photographed in their entirety (3,600 ×) without further selection. The resulting micrographs were photographically enlarged to 15,000×. At this point, the photos were not identified by experiment but, using a 3 × magnifying lens, all z.fasciculata cell-associated silver grains on the photos were encircled. Differently colored circles identified *a*) grains whose circles touched plasma membrane; *b*) grains whose circles were entirely within the cell cytoplasm but not apparently associated with organelles (e.g., endosomal-like vesicles, vacuoles, dense bodies, lysosomal-like bodies, Golgi complexes) thought to be associated with the endocytic/lysosomal pathway; and *c*) interiorized grains whose circles did touch some organelle of the endocytic pathway. If the encircled grain hit both the plasma membrane *a*) and an endocytic structure *c*), it was given one-half a value in both categories. Since all efforts to categorize grain distribution and subsequent grain counting were done before the photographs were identified as belonging to particular experiments, any mistakes in assignment of grains were believed to be cancelled out. Between 1000 and 2000 grains were counted for each experimental variable reflecting different levels of tissue labeling with isotopes, different exposure times, emulsion thicknesses, etc.

The ARGs prepared in this study were from adrenals perfused with 2–3 mCi ^{125}I-labeled lipoproteins (sp act 200–250 μCi/mg proteins; 200 μg/ml protein at 2.2 ml/min for 60 min). ARGs prepared with residualizing labels provided similar information (E. Reaven, unpublished observations) but were impractical for the entire study because of the poor coupling efficiency of [^{125}I]DTT to protein, and the resulting risks involved to personnel in obtaining large amounts (~25 mg protein/rat) of high specific activity ligand.

Lipoprotein binding and uptake studies

The various groups of HDL lipoproteins were used in adrenal perfusion studies either as ^{125}I-labeled ligands or as doubly-labeled ([^{125}I]DTT and [^{3}H]cholesteryl linoleyl ether) residualizing particles. For binding and uptake studies, the concentration of protein ranged from 20 to 500 μg/ml for 60–90 min. Competition experiments involved 20 μg/ml of ^{125}I-labeled HDL and 20-fold excess unlabeled HDL perfused for 60 min.

In experiments in which corticosterone secretion was assessed, the adrenals were perfused for 90 min with or without unlabeled hHDL or rHDL (500 μg/ml protein). Effluent collected from the renal vein during this period was frozen and subsequently assayed for corticosterone content (37).

Quantification of cholesterol internalization and determination of the ratio of cholesterol versus protein internalized under different experimental situations was carried out by a modification of a procedure previously used in this laboratory (12). In brief, adrenals were perfused with double-labeled residualizing HDL particles (50 μg protein/ml) for 90 min. Perfused and washed adrenals were homogenized, and trapped radioactivity was released by repeated freezing and thawing (5–7 ×) of the homogenate. A suitable aliquot of this homogenate was treated with trichloracetic acid to determine both insoluble (precipitable) and soluble ^{125}I radioactivity. A second aliquot was extracted with organic solvents (38) to determine ^{3}H radioactivity. The relative specific activities of the doubly-labeled particles permitted an estimate of the apoprotein:cholesterol ratio. Correction of the data (12) for HDL protein internalized by the cells [apart from HDL protein trapped extracellularly in microvillar channels (11, 20) or elsewhere] was based on the percent of total ^{125}I binding that was TCA-soluble[2] (representing de-

[2]Similar results were obtained using gel filtration techniques (12).

graded peptides accumulated in the cells with the residualizing label). This estimate of internalized protein permitted a calculation of the amount of internalized cholesterol which theoretically could have entered the cells as part of intact HDL (endocytic pathway). Any additional trapped [^{3}H]CLE-cholesterol must have entered the cells by another pathway, one in which lipoprotein apoproteins had not accompanied the cholesterol (alternative pathway).

Miscellaneous procedures

Cholesterol in plasma, as well as in the HDL fractions, was determined enzymatically according to the procedure of Nöel, Dupras and Filion (39). The protein content of lipoproteins was determined by a modification of the procedure of Lowry et al. (40) as described by Markwell et al. (41). Corticosterone was quantitated by radioimmunoassay using specific antiserum (37). [^{3}H]Cholesteryl linoleyl ether was synthesized using a modification (42) of the method of Stoll (43) as described previously (12). Dilactitol tyramine (DTT) was synthesized according to the procedure of Strobel, Baynes, and Thorpe (44). The procedure of Morton and Zilversmit (31) was used to partially purify human plasma cholesteryl ester transfer protein. Adrenal cholesteryl ester, and cholesterol content and microsomal HMG-CoA reductase activity were assayed as described by Popplewell and Azhar (45). The enzyme activity is expressed as pmol mevalonic acid formed · min $^{-1}$ · mg protein $^{-1}$.

RESULTS

Animal models

Table 1 provides pertinent cholesterol information on the acutely treated (1 day) lipoprotein-deficient rat used extensively in this study. Data from more standard models of lipoprotein-deficient rats are provided in Table 1 and Fig. 2 for comparison. The chronically treated animals show a more profound drop in circulating cholesterol levels and more adrenal metabolic changes (suggesting an increased need for cholesterol) than do animals treated for 1 day. However, severe patho-morphological changes associated with the microvillar surface of the adrenocortical cells (20) preclude the chronically treated rat as reasonable model for the current studies. In the acutely treated 4-APP model, adrenocortical cell morphology appears normal: ultrastructurally, one sees fewer lipoprotein particles associated with the microvillar surface of the adrenocortical cells of this model as compared to those of fed, control rats, but otherwise no changes are apparent (20). **Fig. 2** also shows that adrenal apoB, E receptor concentration is unchanged in the rats. For the purpose of the current studies, therefore, the acutely treated (1 day) 4-APP rat provides an experimental model with reduced circulating cholesterol levels, but little change in cholesterol-associated characteristics of the adrenal gland itself.

Adrenal uptake of ^{125}I-labeled HDL

Tissue specificity. **Fig. 3** is a light microscope autoradiograph of a 60-min rHDL-perfused adrenal illustrating the specific affinity of HDL-binding to cells of adrenal cortical zones which utilize exogenous HDL cholesterol for corticosterone production. Very few exposed silver grains (representing ^{125}I-labeled HDL protein) are associated with zona glomerulosa (ZG) cells of the perfused adrenal, whereas large numbers of grains outline the zona fasciculata cells (ZF) known to secrete corticosterone. The overdeveloped ARG of Fig. 3 permits us to see that *1*) the presence of exposed silver grains begins with the first layer of ZF cells; *2*) that most of the grains are associated with the periphery of the ZF cells, and not with their interior; and *3*) that the majority of the grains are associated with the sinusoidal surfaces, as opposed to the intercellular surfaces of the cells. Although not shown on this micrograph, HDL binding to zona reticularis cells is similar to that of ZF cells, but essentially no binding occurs with cells of the adrenal medulla. Adrenal perfusion with ^{125}I-labeled $hHDL_3$ rather than rHDL results in ARGs with a similar distribution of silver grains.

Cellular uptake. Autoradiograms at the electron microscope level confirm the impression given in Fig. 3 that the large majority of ZF cell binding of HDL occurs at the adrenocortical cell surface. In general, adrenals perfused wtih rHDL bind more isotope, and the resulting ARGs show

TABLE 1. Effect of 4-APP or 17α-E_2 treatment on cholesterol levels and HMG-CoA reductase activity in rats

	Plasma Cholesterol	Adrenal Cholesteryl Ester	Adrenal Free Cholesterol	Adrenal HMG-CoA Reductase
	mg/dl	*μg/mg tissue*		*pmol · min^{-1} · mg protein^{-1}*
Control	58.2 ± 2.8	38.5 ± 3.6	3.6 ± 0.2	18.0 ± 2.8
4-APP 1 day	34.9 ± 2.6	32.9 ± 0.6	3.6 ± 0.1	18.9 ± 4.2
4-APP 3 days	10.3 ± 0.7	6.7 ± 0.7	3.1 ± 0.1	353 ± 53
17α-E_2 5 days	3.5 ± 0.3	4.5 ± 0.8	3.4 ± 0.2	ND

Results are given as mean ± SE (n = 5); ND, not determined.

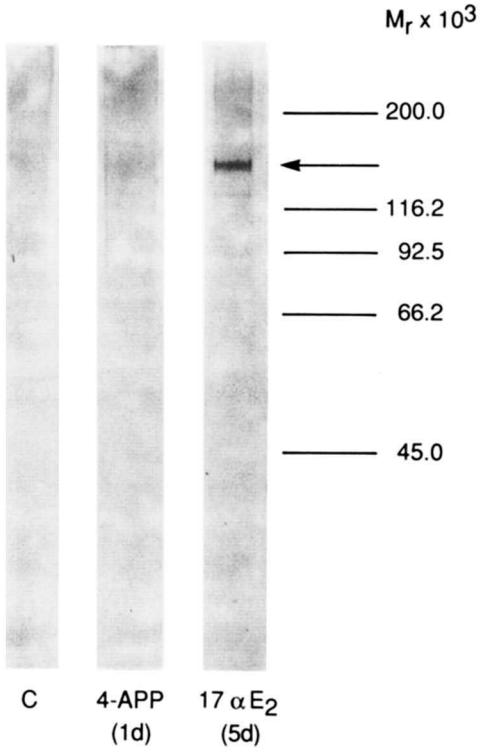

Fig. 2. Ligand blots of apoB,E receptor protein (~140 kDa). Crude membranes were prepared from adrenals of control, 4-APP (1 day)- and 17α-E_2 (5 days)-treated animals. The apoB,E receptor protein was identified by ligand blotting as described in Materials and Methods. In each case 100 μg of protein was applied to gel.

more exposed silver grains than do adrenals perfused with similar levels of $hHDL_3$. As a result, ARGs from $hHDL_3$-perfused adrenals are generally exposed in the dark for longer periods in an attempt to equalize the total number of grains.

Table 2 summarizes the EM-ARG data from ZF cells of a series of rats perfused with either rHDL or $hHDL_3$ for 60 min (2–3 mCi/rat; 200 μg protein/ml). With both ligands, most of the exposed grains are associated with the plasma membrane: with rHDL, this represents ~82% (or 18% internalized) of total ZF-bound grains; with $hHDL_3$ this represents ~88% (or 12% internalized) of total ZF-bound grains. Whether the increased interiorization of ^{125}I-labeled rHDL over ^{125}I-labeled $hHDL_3$ is a consequence of higher levels of lipid iodination in rHDL, (2–3% vs 6– 7%) or some other factor associated with rHDL is not yet clear. With both ligands, silver grain association with specific organelles of the endocytic-lysosomal pathway (i.e., endosomal-like vesicles, vacuoles, dense bodies, lysosomal-like bodies, or Golgi membranes) remains about the same (~4% of total binding).

In parallel experiments carried out with adrenals of 5 d 17α-E_2-treated rats, internalization of ^{125}I was still ~20% of total bound, but the association with specific endocytic organelles was often as high as 7% of total ZF grains.

Ligand specificity. **Fig. 4** shows that when adrenals are perfused with 20-fold excess (40 μg/ml protein) unlabeled rHDL or $hHDL_3$ plus the respective ^{125}I-labeled lipoproteins (20 μg/ml protein), binding is reduced by 80–85%.

Ligand concentration. Total uptake (i.e., total binding) of HDL by the perfused adrenal varies depending on the source of the ligand and its perfusate concentration. **Table 3** shows that at every ligand protein concentration used, ^{125}I-labeled rHDL binds to the adrenal more avidly than does ^{125}I-labeled $hHDL_3$. However, the extent of this difference varies with the final ligand mass. Thus, with a low protein mass (20 μg protien/ml), the delta difference in binding between rHDL and $hHDL_3$ is ~12-fold; at high concentrations of HDL protein (200–500 μg protein/ml), the difference in binding affinity between the ligands is ~4.7-fold.

Ligand removal. **Table 4** demonstrates the effect of prolonged washing on the removal of radioactive rHDL from the perfused adrenal. ^{125}I-labeled rHDL was perfused for 1 h at a concentration known to achieve submaximal binding (100 μg protein/ml, see also Table 3) and the adrenals were subsequently perfused with media alone for 30 or 90 min, after which the radioactivity associated with the adrenals was quantified and compared with standard 2-min washed samples. Radioactivity was reduced by ~50 percent after 30 min of wash, but no further reduction occurred at 90 min.

Adrenal uptake of [^{125}I]DTT-[^{3}H]CLE-HDL

Table 5 summarizes data obtained from adrenal perfusions carried out with residualizing particles prepared by the two separate methods described in Methods. In every respect the results using the two versions of the particles were similar. Panel A shows that total bound radioactivity of [^{125}I]DTT or [^{3}H]CLE is the same regardless of the particle used. The estimated amount of radioactivity trapped extracellularly (panel B) or accumulated within cells (panel C) is also similar. Although it is not possible to directly derive the [^{3}H]CLE figures for extracellular trapped cholesterol, it is theoretically the same as trapped protein since both labels are on the same particle.

In **Table 6**, similar data are shown comparing the effect of perfused rHDL and $hHDL_3$ residualizing particles prepared by the modified Sparks procedure (30); adrenals were perfused with 50 μg protein/ml rHDL or $hHDL_3$ double labeled as [^{125}I]DTT and [^{3}H]CLE residualizing ligands. Panel A shows that the total adrenal uptake of the [^{125}I]DTT tag with rHDL is several fold more effective than with $hHDL_3$. With both ligands, total [^{3}H]CLE (cholesterol) uptake is more than [^{125}I]DTT (protein) uptake, although the differential uptake between [^{3}H]CLE and [^{125}I]DTT was less with $rHDL_3$ (1.2 ×) than with $hHDL_3$ (9.4 ×). In panel B, we estimate the amount of this total radioactivity that is trapped extracellularly in microvillar channels (20) or elsewhere. The figure for [^{125}I]DTT represents total radioactivity (from A) minus the measured accumulated (i.e.,

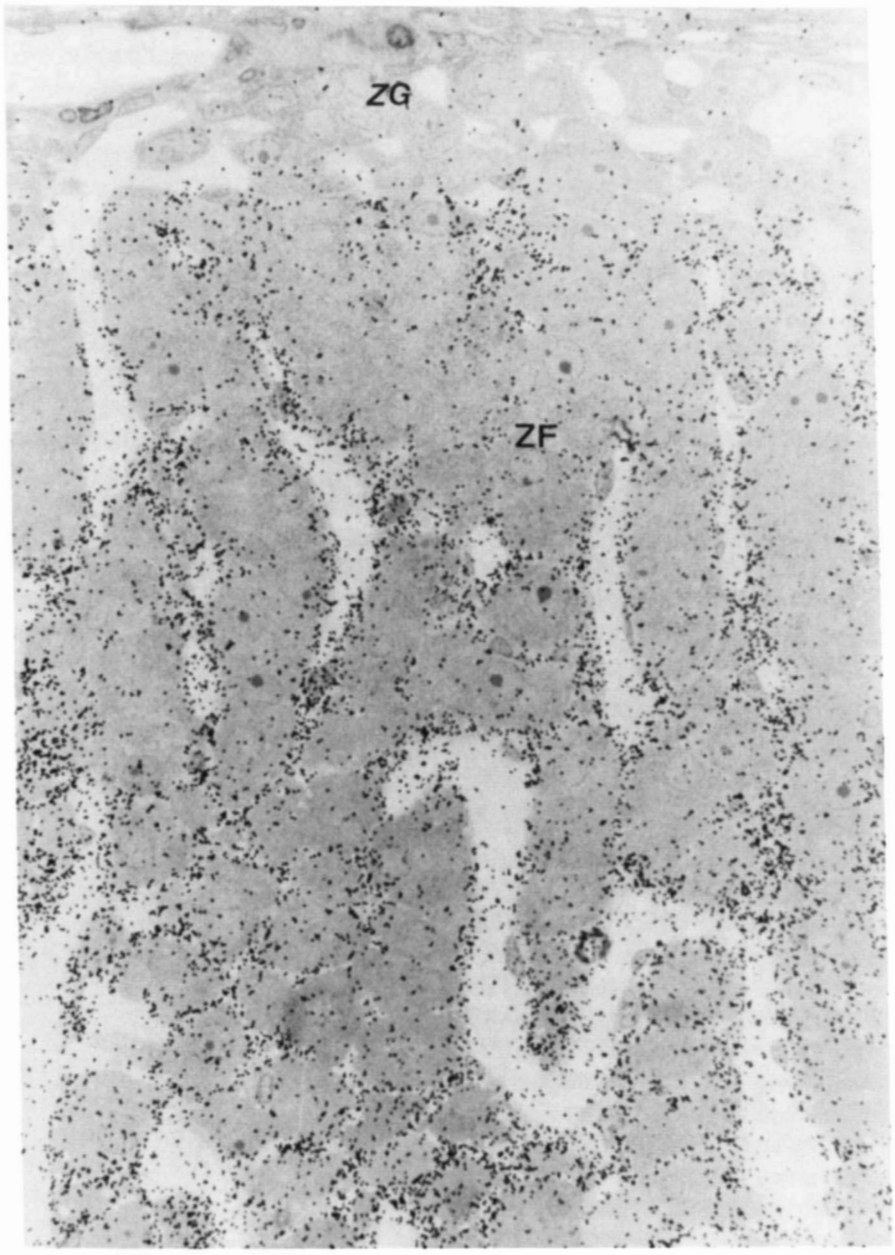

Fig. 3. Overexposed light microscope autoradiograph of adrenal cortex of a 4-APP-treated rat perfused for 60 min with rHDL. Z.glomerulosa (ZG) and z.fasciculata (ZF) are identified. The z.reticularis and medulla are not included in the photo. Exposed silver grains representing bound radioactive rHDL are primarily associated with the sinusoidal surfaces of ZF cells. Data from electron microscopic autoradiographs indicate that the thickness of this sinusoidal layer of grains is due to the binding of isotope to the extensive microvillar network lining the cells.

degraded, TCA-soluble) [^{125}I]DTT (panel C). When considered in this way, it can be seen that 96–97% of total accumulated [^{125}I]DTT is actually trapped extracellularly whether the perfused ligand is rHDL or $hHDL_3$. Panel C summarizes the direct TCA-soluble [^{125}I]DTT radioactivity (representing degraded HDL accumulated within the cells due to the residualizing tag) and [^{3}H]CLE radioactivity (representing total [^{3}H]CLE minus the theoretical amount trapped extracellularly).

These data show that more cholesterol than protein is internalized by cells of the perfused adrenal with both rHDL and $hHDL_3$. It appears that $hHDL_3$ is far more efficient (internalizing 190-fold more cholesterol than protein) than rHDL, which internalizes only 8.6-fold more cholesterol than protein. However, when the protein/cholesterol ratios of the original ligands are taken into consideration (protein: cholesterol rHDL = 1:1; $hHDL_3$ = 5:1), it turns out that the absolute cholesteryl ester internalized from the two dif-

TABLE 2. Localization of ^{125}I-labeled HDL with z. fasciculata cells (ZF) in 1 day 4-APP-treated rats

Ligand	Silver Grains Present within the ZF Cell Cytoplasm	Silver Grains Associated with Organelles of Endocytic/Lysosomal Pathway[a]
	% of total bound	
rHDL	18.0 ± 1.6	4.1 ± 0.6
$hHDL_3$	12.1 ± 1.7	4.4 ± 0.4

Data expressed as mean ± SE; n = 3 for each ligand.
[a]Endocytic/lysosomal organelles: endosomal-like vesicles and vacuoles, dense bodies, lysosomes, and Golgi membranes.

ferent ligands is comparable: i.e., 70 and 81 μg cholesterol, respectively, for rHDL and $hHDL_3$ (**Fig. 5**).

Corticosterone response

Table 7 describes the corticosterone response of adrenals perfused with or without HDL (500 μg protein/ml) for a 90-min time period. With rHDL or $hHDL_3$ as the ligand, corticosterone output was 6.9- or 4.5-fold increased over production rates when no lipoprotein was used.

DISCUSSION

The overall aim of this project was to describe lipoprotein-cholesterol uptake in a normal steroidogenic tissue using perfusion techniques to control both the quantity and quality of the supplied lipoprotein. Since the rat adrenal gland was the tissue of choice, it was necessary to develop a rat model that would permit binding of exogenously supplied lipoproteins to the adrenal tissue, but would at the same time allow normal uptake and metabolism of the bound particles. We believe the 24-h 4-APP-treated rat described in this report provides such a model; in the acute 4-APP animal, control circulating cholesterol levels are 50% reduced and endogenous lipoproteins on the surface of adrenocortical cells are diminished (20), but factors reflecting lipoprotein binding and adrenal cholesterol metabolism (adrenal cholesteryl ester levels, the activity of HMG-CoA reductase, and adrenal apoB,E receptor content) appear to be normal.

Also, to examine the mechanism by which lipoprotein uptake occurs in the adrenal, it was important to develop an adrenal system that would permit maximal lipoprotein uptake by the intact tissue without recycling or reuse of the ligand. The nonrecycling perfusion system described here provides such a model. HDL in the perfusate is exposed to the adrenal tissue only once; unbound HDL is discarded. The perfused HDL bind avidly (and specifically) to the adrenal cortex, but binding is mainly to the cells of the fasciculata and reticularis zones which are known to synthesize corticosterone. That this HDL binding is related to function is shown by the fact that perfusion with HDL (from both homologous or heterologous sources) is coupled to a 5- to 7-fold increase in corticosterone release in the same time interval.

Given this adrenal model system, it was possible to ask specific questions regarding HDL-protein and -cholesterol delivery to the adrenal cortex using both rat and human serum-derived HDL.

The initial questions concerned HDL binding. In every situation studied the adrenal tissue bound rHDL more avidly than $hHDL_3$. To a large extent this binding was concentration dependent. At the lowest protein concentration examined (20 μg protein/ml), ~12-fold more rHDL protein mass than $hHDL_3$ mass was bound per gram adrenal tissue; at high concentration of protein (e.g., 200 μg protein/ml) this differential in binding decreased and rHDL protein was bound ~4.5 times more than $hHDL_3$. The reason for this difference in affinity is not clear; it may relate to specific apoprotein or phospholipid differences between the HDL particles, or to physical characteristics such as size, or surface area of the different lipoproteins, which could influence particle uptake by the microvillar channels located on the surface of the steroidogenic cells (20).

To a large extent, the microvillar channels mentioned above influence our thinking on how HDL-cholesterol is actually delivered to the interior of the adrenal cells. Is HDL-cholesterol for steroidogenesis delivered to the adrenal

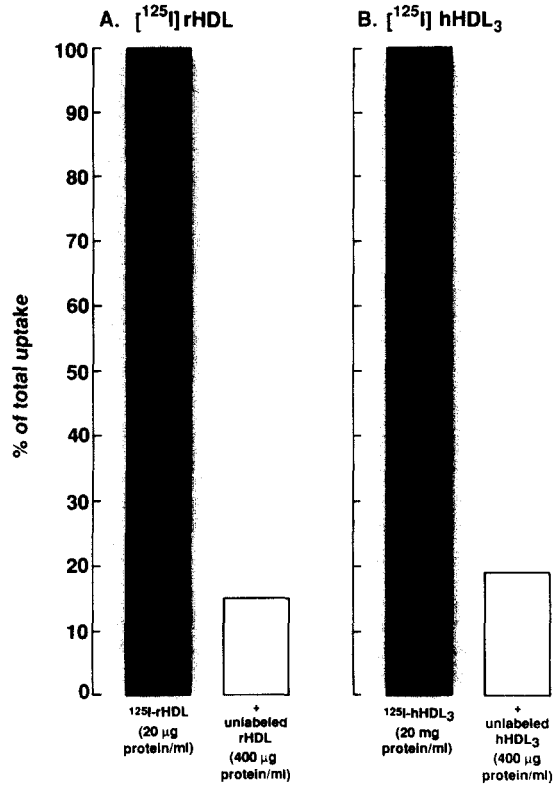

Fig. 4. Effect of simultaneous perfusion of 20-fold excess unlabeled HDL on ^{125}I-labeled rHDL and ^{125}I-labeled $hHDL_3$ uptake by rat adrenals.

TABLE 3. Uptake of ^{125}I-labeled rHDL and ^{125}I-labeled hHDL$_3$ by adrenals of 1 day 4-APP-treated rats: effect of ligand concentration

Concentration	Uptake		Ratio
	^{125}I-Labeled rHDL	^{125}I-Labeled hHDL$_3$	^{125}I-Labeled rHDL/^{125}I-Labeled hHDL$_3$
μg protein/ml	*μg/g tissue*		
20	109 ± 10	8.8 ± 1.0	12.4
200	286 ± 13	62 ± 6	4.6
500	334 ± 7	70 ± 3	4.8

Data are expressed as mean ± SE; n = six adrenals for each concentration of each ligand.

cells via the endocytic pathway where the intact particle is interiorized before cholesterol is released, or is HDL-cholesterol released at the cell surface as has been suggested by several workers (8, 9, 11, 12)? Does this delivery system vary depending on the source of the HDL particles? In the lipoprotein-perfused adrenal, the microvillar channels fill with lipoprotein particles, most of which are not removed by standard washing procedures (20); as a result, any assessment of internalized lipoprotein protein or cholesterol must be corrected by the amount of intact, extracellular, microvillar-trapped particles. In the present study we dealt with this issue in two ways. In the first, adrenals were perfused with rHDL or hHDL$_3$ equipped with isotope-labeled residualizing tags in the protein moiety of the lipoprotein. To provide confidence in data obtained with these altered lipoprotein particles, several controls were carried out. First, incubation with cells in culture known to internalize apoB/E-lipoproteins [granulosa cells (10)] showed that HDL particles labeled with [^{125}I]DTT by the modified Sparks method did, indeed, accumulate [^{125}I]DTT-labeled rHDL in culture. Secondly, in adrenal perfusion experiments, HDL particles labeled with residualizing tags by two separate procedures, provided identical results. Finally, EM ARGs showed that the cellular distribution of the [^{125}I]DTT-HDL in perfused adrenals was identical to that of native ^{125}I-labeled HDL. Overall, it appeared that the altered HDL functioned appropriately as nonreleasable ligands that could substitute for native ^{125}I-labeled HDL.

With the use of such ligands in adrenal perfusions of the 1 day 4-APP-treated rat, it became clear that only a minor fraction of the total adrenal-bound protein was actually internalized by cells whether rHDL (3% of total bound) or hHDL$_3$ (4.5% of total bound) was used as a ligand (Table 6). In absolute terms, 8 μg[^{125}I]DTT-rHDL versus 2 μg [^{125}I]DTT-hHDL$_3$ had been internalized, a very small amount of protein considering the total bound protein mass, but a 4-fold differential, nevertheless, between the two ligands.

Using EM autoradiography in a totally different approach, and using nonresidualizing isotope-labeled rHDL and hHDL, the same question was asked. The morphological answers matched the biochemical data in again indicating that only a small percentage of total cell-bound protein was internalized by z.fasciculata cells (represented by exposed silver grains). If one considered total grains within the cytoplasma, this fraction was 12–18% of total bound protein, but if one assumed that only the grains specifically associated with endocytic-lysosomal organelles were useful indicators of the endocytic pathway (Table 2), then the internalized fraction represented ~4% of the total, a number consistent with that found biochemically. It is our belief, however, that neither the biochemical or EM values should be rigidly adhered to. On the one hand, the biochemical data may underestimate the internalized protein if some internalized particles had not yet been degraded in the relatively short experimental period used, or if DTT-labeled particles are not as effectively degraded as native particles [though there is little evidence that this is the case from Daugherty et al. (19)]. Also, if significant retroendocytosis (46–48) had occurred, our method of correcting for TCA-soluble material (i.e., as an estimate of degraded, internalized HDL) would have obscured the results. The washing experiments of Table 4 suggest, however, that retroendocytosis does not play a major role, insofar as no loss in ^{125}I-labeled HDL occurs as the adrenals continue to be washed between 30 and 90 min. The EM data, on the other hand, could be falsely high for the rHDL values, since a substantial amount of lipid labeling (5–6%) occurs with this ligand. In general though, the same point is made by both types of experiments: very little of the total

TABLE 4. Uptake of ^{125}I-labeled rHDL by adrenals of 1 day 4-APP-treated rats: effect of washing with medium

Wash Time	^{125}I-Labeled rHDL Bound	Comparison with 2-Min Value
min	*μg/g tissue ± SE*	*%*
2	218 ± 17	100
30	118 ± 6	54
90	108 ± 22	50

Results are mean ± SE of four determinations. Ligand was ^{125}I-labeled rHDL (100 μg/ml for 60 min at 2.2 ml/min).

TABLE 5. Uptake and internalization of two different preparations of [^{125}I]DTT-[^{3}H]CLE-hHDL$_3$ by adrenal glands

	[^{125}I]DTT	[^{3}H]CLE
	μg protein/g tissue	
A. Total radioactivity		
Preparation 1	48.8 ± 2.6	516 ± 39
Preparation 2	44.2 ± 0.9	551 ± 41
B. Surface bound radioactivity[a]		
Preparation 1	46.5 ± 2.6	(46.5 ± 2.6)
Preparation 2	41.9 ± 0.9	(41.9 ± 0.9)
C. Internalized radioactivity[b]		
Preparation 1	2.27 ± 0.08	469 ± 36
Preparation 2	2.37 ± 0.32	509 ± 40

Four measurements (± SE) of adrenals for each ligand; hHDL$_3$ (50 mg/ml) was perfused for 90 min at 2.2 ml/min. Preparation 1 was prepared according to the procedure of Sparks et al. (30); preparation 2 was prepared according to the procedure of Hough and Zilversmit (32) as recently used by Pittman et al. (8).

[a]Data in B are derived by subtracting values for TCA-soluble radioactivity (see C) from total [^{125}I]DTT radioactivity (see A). Values for B represent trapped, extracellular (intact) [^{125}I]DTT-HDL. It is assumed that an equivalent amount of [^{3}H]CLE-HDL is also trapped (see values in parentheses).

[b]Data in C are obtained from accumulated TCA-soluble [^{125}I]DTT and accumulated [^{3}H]CLE radioactivity.

bound HDL-protein is found in the cytoplasm of the cell, suggesting that the apoB,E-mediated endocytic pathway is not primary in delivering HDL-cholesterol, even when apoE-containing ligands such as rHDL are used.

A more direct way to address the question of delivery of HDL-cholesterol is by assessment of the amount of cholesterol (or [^{3}H]CLE) that was accumulated by the tissue after perfusion with the same HDL particles used above. Once the total [^{3}H]cholesterol uptake data are corrected by the amount of cholesterol in the extracellular trapped particles, one sees that far more cholesterol than protein had been taken up by the adrenal tissues during the course of the experiments. Several interesting facts emerge: *1*) relatively more cholesterol (CLE) than protein is internalized by adrenal cells using either rHDL or hHDL$_3$ but the differential is far greater with hHDL$_3$ (~190-fold) than with rHDL (~8-fold); *2*) correcting for the known protein:cholesterol ratio of the starting ligands, (rHDL = 1:1; hHDL$_3$ = 5:1), one can obtain the absolute amount of cholesterol internalized (i.e, 70 μg CLE for rHDL and 81 μg CLE for hHDL$_3$). Thus, despite large differences in the relative uptake of protein versus cholesterol with the two lig-

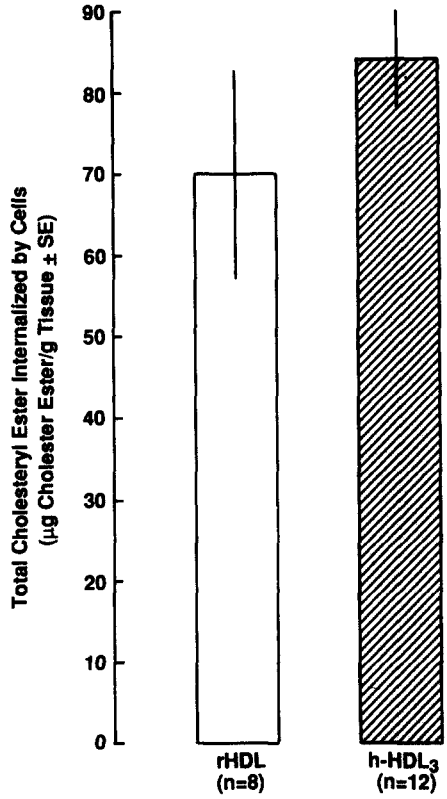

Fig. 5. Total HDL cholesteryl ester internalized by cells of the adrenal when ligand concentration used for perfusion is corrected for relative differences in protein/cholesterol ratios.

TABLE 6. Uptake and internalization of [^{125}I]DTT-[^{3}H]CLE rHDL$_3$ by adrenal glands of 1 day 4-APP-treated rats

	[^{125}I]DTT	[^{3}H]CLE
	μg protein/g tissue	
A. Total radioactivity		
rHDL	288 ± 13	346 ± 21
hHDL$_3$	48.2 ± 1.8	453 ± 29
B. Surface bound radioactivity[a]		
rHDL	280 ± 12	(280 ± 12)
hHDL$_3$	46.1 ± 1.9	(46.1 ± 1.9)
C. Internalized radioactivity[a]		
rHDL	8 ± 0.7	70 ± 13
hHDL$_3$	2.17 ± 0.10	407 ± 19

Twelve measurement (± SE) of adrenals for each ligand; ligand (50 mg/ml) were perfused for 90 min at 2.2 ml/min; preparation of ligands by procedure of Sparks et al. (30) as described in Methdos.

[a]Data in B and C are expressed as in Table 5.

TABLE 7. rHDL- and hHDL$_3$-supported corticosterone production by adrenal glands of 1 day 4-APP-treated rats

	Corticosterone	Fold Increase over Basal
	μg/g tissue/90 min	
Basal	17.1 ± 2.3	1
rHDL (500 μg protein/ml)	117.3 ± 18.2[a]	6.9
hHDL$_3$ (500 μg protein/ml)	71.3 ± 7.5[a]	4.5

Results are mean ± SE; n = five separate experiments with each variable.

[a]Corticosterone produced with lipoproteins is significantly ($P < 0.01$) increased over basal; difference in production with the use of rHDL versus hHDL$_3$ is not significant.

ands, the final amount of cholesterol delivered to the adrenal cells is comparable.

When one considers this second factor (i.e., that equivalent amounts of cholesterol are internalized from bound rHDL and $hHDL_3$), it is understandable why perfusion with both types of HDL ends up stimulating corticosterone production equally well.

Finally, it is possible to take the values for adrenal corticosterone production by rHDL and $hHDL_3$ during the course of the experiment and estimate how much of the required cholesterol could have been obtained via the endocytic pathway (i.e., though intact HDL) versus cholesterol obtained via an alternative pathway that does not require internalization of HDL-protein. Details of this calculation are presented in a footnote.[3] Based on the values obtained for internalized protein using rHDL, this calculation shows that between 8.0 and 11.9 percent of the required cholesterol could have been provided by rHDL taken in by the endocytic pathway, depending on whether one chooses to use the values obtained biochemically or from the EM data. With $hHDL_3$, less than 2% (0.6–2.0) of the required cholesteryl ester could have come from the ligand delivered by the endocytic pathway. Thus, in both cases, most of the cholesterol used for the corticosterone produced would have had to come from other sources, e.g., the alternative exogenous pathway in which intact HDL are not internalized by cells and/or endogenous stored cholesterol.

Overall then, the adrenal (from acute lipoprotein-deficient rats) is similar to the rat luteinized ovary in the manner in which it utilizes lipoprotein cholesterol for steroidogenesis (5, 10–12). In these tissues, most of the lipoprotein-derived cholesteryl ester appears to be directly transferred at the surface of the endocrine cells. Efforts to understand the nature of the lipoprotein–plasma membrane interaction are now underway.

Manuscript received 23 January 1989 and in revised form 14 June 1989.

[3]Note, all results are expressed as concentration per g tissue for 90 min; B, corticosterone; P, protein; C, cholesterol.

a. *Corticosterone production* (Table 7).
   rHDL = 117.3 − 17.1 = 100.4 μg B/g per 90 min
   $hHDL_3$ = 71.3 − 17.1 = 54.2 μg B/g per 90 min
b. *Total cholesterol internalized by cells* (Table 6).
   rHDL = 70 μg P/1 (P:C = 1:1) = 70 μg C ± 13
   $hHDL_3$ = 376 μg P/5 (P:C = 5:1) = 81.4 μg C ± 5.8
c. *Cholesteryl ester internalized via the endocytic pathway (i.e., via intact HDL)* (Table 6)
   rHDL = 8 μg P/1 (P:C = 1:1) = 8 μg C
   $hHDL_3$ = 2 μg P/5 (P:C = 5:1) = 0.4 μg C
d. *Maximal amount of HDL cholesteryl ester which could be supplied for B production (see a) by endocytic pathway (see c) assuming 100% conversion of cholesterol to B.*
   rHDL = 8 μg C/100.4 μg B × 100 = 8.0%
   $hHDL_3$ = 0.4 μg C/54.2 μg B × 100 = 0.7%

REFERENCES

1. Quarfordt, S., J. Hanks, R. S. Jones, and F. Shelburne. 1980. The uptake of high density lipoprotein cholesteryl ester in the perfused rat liver. *J. Biol. Chem.* **255:** 2934–2937.
2. Stein, Y., Y. Dabach, G. Hollander, G. Halperin, and O. Stein. 1983. Metabolism of HDL-cholesteryl ester in the rat, studies with a nonhydrolyzable analog, cholesteryl linoleyl ether. *Biochim. Biophys. Acta.* **752:** 98–105.
3. Glass, C., R. C. Pittman, D. B. Weinstein, and D. Steinberg. 1983. Dissociation of tissue uptake of cholesterol ester from that of apoprotein A-I of rat plasma high density lipoprotein: selective delivery of cholesterol ester to liver, adrenal, and gonad. *Proc. Natl. Acad. Sci. USA.* **80:** 5435–5439.
4. Leitersdorf, E., O. Stein, S. Eisenberg, and Y. Stein. 1984. Uptake of rat plasma HDL subfractions labeled with [^{3}H]cholesteryl linoleyl ether or with ^{125}I by cultured rat hepatocytes and adrenal cells. *Biochim. Biophys. Acta.* **796:** 72–82.
5. Reaven, E., Y-D.I. Chen, M. Spicher, and S. Azhar. 1984. Morphological eivdence that high density lipoproteins are not internalized by steroid-producing cells during in situ organ perfusion. *J. Clin. Invest.* **74:** 1384–1397.
6. Glass, C., R. C. Pittman, M. Civen, and D. Steinberg. 1985. Uptake of high-density lipoprotein-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.
7. Mackinnon, M., J. Savage, R. Wishart, and P. Barter. 1986. Metabolism of high density lipoproteins by the perfused rabbit liver. *J. Biol. Chem.* **261:** 2548–2552.
8. Pittman, R. C., T. P. Knecht, M. S. Rosenbaum, and C. A. Taylor, Jr. 1987. A nonendocytotic mechanism for the selective uptake of high density lipoprotein-associated cholesterol esters. *J. Biol. Chem.* **262:** 2443–2450.
9. Arbeeny, C. M., V. A. Rifici, and H. A. Eder. 1987. The uptake of the apoprotein and cholesteryl ester of high-density lipoproteins by the perfused rat liver. *Biochim. Biophys. Acta.* **917:** 9–17.
10. Reaven, E., Y-D.I. Chen, M. Spicher, S-F. Hwang, C. E. Mondon, and S. Azhar. 1986. Uptake of low density lipoproteins by rat tissues: special emphasis on the luteinized ovary. *J. Clin. Invest.* **77:** 1971–1984.
11. Reaven, E., J. Boyles, M. Spicher, and S. Azhar. 1988. Evidence for surface entrapment of cholesterol-rich lipoproteins in luteinized ovary. *Arteriosclerosis.* **8:** 298–309.
12. Azhar, S., A. Cooper, L. Tsai, W. Maffe, and E. Reaven. 1988. Characterization of apoB,E receptor function in the luteinized ovary. *J. Lipid Res.* **29:** 869–882.
13. Brown, M. S., and J. L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostatis. *Science.* **232:** 34–47.
14. Reaven, E., M. Kostrna, J. Ramachandran, and S. Azhar. 1988. Structure and function changes in rat adrenal glands during aging. *Am. J. Physiol.* **255:** E903–E911.

15. van't Hooft, F., and R. J. Havel. 1981. Metabolism of chromatographically separated rat serum lipoproteins specifically labeled with ^{125}I-apolipoprotein E. *J. Biol. Chem.* **256:** 3963–3968.
16. Eisenberg, S. 1984. High density lipoprotein metabolism. *J. Lipid Res.* **25:** 1017–1058.
17. Gavish, D., Y. Oschry, 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.
18. Mahley, R. W., and T. L. Innerarity. 1983. Lipoprotein receptors and cholesterol homeostasis. *Biochim. Biophys. Acta.* **737:** 197–222.
19. Daugherty, A., S. R. Thorpe, L. G. Lange, B. E. Sobel, and G. Schonfeld. 1985. Loci of catabolism of β-very low density lipoprotein in vivo delineated with a residualizing label, ^{125}I-dilactitol tyramine. *J. Biol. Chem.* **260:** 14564–14570.
20. Reaven, E., M. Spicher, and S. Azhar. Microvillar channels: a unique plasma membrane compartment for concentrating lipoproteins on the surface of rat adrenal cortical cells. *J. Lipid Res.* **30:** (Oct/89 issue).
21. Fless, G. M., C. A. Rolih, and A. M. Scanu. 1984. Heterogeneity of human plasma lipoprotein [a]: isolation and characterization of the lipoprotein subspecies and their apoproteins. *J. Biol. Chem.* **259:** 11470–11478.
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. Leikin, A. I., M. Mihovilovic, and A. M. Scanu. 1982. High density lipoproteins influence cholesterol homeostasis in cultured virus-transformed human lymphoblastoid cells: dependence on the lipoprotein concentration in the medium. *J. Biol. Chem.* **257:** 14280–14287.
24. Weisgraber, K. H., and R. W. Mahley. 1980. Subfractionation of human high density lipoproteins by heparin-Sepharose affinity chromatography. *J. Lipid Res.* **21:** 316–325.
25. Quarfordt, S. H., R. S. Jain, S. Robinson, and F. Shelburne. 1978. The heterogeneity of rat high density lipoproteins. *Biochem. Biophys. Res. Commun.* **83:** 786–793.
26. McFarlane, A. S. 1958. Efficient trace-labeling of proteins with iodine. *Nature (London)* **182:** 53–57.
27. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoproteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–221.
28. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and quantification. *Can. J. Biochem. Physiol.* **37:** 911–917.
29. Fielding, C. J. 1979. Validation of a procedure for exogenous labeling of lipoprotein triglyceride with radioactive triolein. *Biochim. Biophys. Acta.* **573:** 255–265.
30. Sparks, D. L., J. Frohlich, P. Cullis, and P. H. Pritchard. 1987. Cholesteryl ester transfer activity in plasma measured by using solid-phase-bound high-density lipoprotein. *Clin. Chem.* **33:** 390–393.
31. Morton, R. E., and D. B. Zilversmit. 1981. A plasma inhibitor of triglyceride and cholesteryl ester transfer activities. *J. Biol. Chem.* **256:** 11992–11995.
32. 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.
33. Schneider, W. J., U. Beisiegel, J. L. Goldstein, and M. S. Brown. 1982. Purification of the low density lipoprotein receptor, an acidic glycoprotein of 164,000 molecular weight. *J. Biol. Chem.* **257:** 2664–2673.
34. Daniel, T. O., W. J. Schneider, J. L. Goldstein, and M. S. Brown. 1983. Visualization of lipoprotein receptors by ligand blotting. *J. Biol. Chem.* **258:** 4606–4611.
35. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* **76:** 4350–4354.
36. Roach, P. D., M. Zollinger, and S-P. Nöel. 1987. Detection of the low density lipoprotein (LDL) receptor on nitrocellulose paper with colloidal gold-LDL conjugates. *J. Lipid Res.* **28:** 1515–1521.
37. Popplewell, P. Y., M. Tsubokawa, J. Ramachandran, and S. Azhar. 1986. Differential effects of aging on adrenocorticotropin receptors, adenosine 3',5'-monophosphate response, and corticosterone secretion in adrenocortical cells from Sprague-Dawley rats. *Endocrinology.* **119:** 2206–2213.
38. Dole, V. P. 1956. A relation between non-esterified fatty acids in plasma and the metabolism of glucose. *J. Clin. Invest.* **35:** 150–154.
39. Nöel, S-P., R. Dupras, and A-M. Filion. 1983. The activity of cholesterol ester hydrolase in the enzymatic determination of cholesterol: comparison of five enzymes obtained commercially. *Anal. Biochem.* **129:** 464–471.
40. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
41. Markwell, M. A. K., S. M. Hass, N. E. Tolbert, and L. L. Bieber. 1981. Protein determination in membrane and lipoprotein samples: manual and automated procedures. *Methods Enzymol.* **72:** 296–303.
42. Halperin, G., and S. Gatt. 1980. The synthesis of cholesteryl alkyl ethers. *Steroids.* **35:** 39–42.
43. Stoll, W. 1932. A new method of preparing cholesterol ethers. *Z. Physiol. Chem.* **207:** 147–151.
44. Strobel, J. L., J. W. Baynes, and S. R. Thorpe. 1985. ^{125}I-Glycoconjugate labels for identifying sites of protein catabolism in vivo: effect of structure and chemistry of coupling to protein on label entrapment in cells after protein degradation. *Arch. Biochem. Biophys.* **240:** 635–645.
45. Popplewell, P. Y., and S. Azhar. 1987. Effects of aging on cholesterol content and cholesterol metabolizing enzymes in the rat adrenal gland. *Endocrinology.* **121:** 64–73.
46. Ciechanover, A., A. L. Schwartz, A. Dautry-Varsat, and H. F. Lodish. 1983. Kinetics of internalization and recycling of transferrin and the transferrin receptor in a human hepatoma cell line. *J. Biol. Chem.* **258:** 9681–9689.
47. Nuñez, M-T., and J. Glass. 1983. The transferrin cycle and iron uptake in rabbit reticulocytes. Pulse studies using ^{59}Fe, ^{125}I-labeled transferrin. *J. Biol. Chem.* **258:** 9676–9680.
48. Schmitz, G., H. Robenek, V. Hohmann, and G. Assmann. 1985. Interaction of high density lipoproteins with cholesteryl ester-laden macrophages: biochemical and morphological characterization of cell surface receptor binding, endocytosis and resecretion of high density lipoproteins by macrophages. *EMBO J.* **4:** 613–622.