Characterization of apoB, E receptor function in the luteinized ovary

Salman Azhar, Allen Cooper, Louisa Tsai, William Maffe, and Eve Reaven[1]

Department of Medicine, Stanford University School of Medicine, Geriatric Research, Education and Clinical Center, Veterans Administration Medical Center, Palo Alto, CA 94305 and Research Institute, Palo Alto Medical Foundation, Palo Alto, CA 94301

Abstract Recent findings from this laboratory have led to the suggestion that the hormone-producing cells of the rat luteinized ovary in situ may obtain a large share of low density lipoprotein (LDL) cholesterol without actually internalizing the intact lipoprotein particles. We have shown that the lipoproteins are trapped at the surface of the luteal cells in a rich network of "microvillar channels" and have theorized that these channel membranes, with their large surface area for interacting with lipoprotein particles, may function in the cholesterol transfer process. In the current study, we try to establish what proportion of the human (h)LDL-cholesterol transfer in the in situ perfused tissue occurs by a classical apoB, E receptor-mediated process versus a surface extraction process. We examine the tissue for the presence of apoB, E receptors, and characterize the structural/functional interaction of hLDL with the apoB, E receptor utilizing a variety of modified hLDL particles as probes. Then, using nonmetabolizable radiolabels for both the protein and cholesteryl ester moieties of these LDL probes, we attempt to quantify the extent to which apoB, E receptors in the ovary contribute to the uptake of hLDL-cholesterol during steroidogenesis. Our experiments show that although the luteinized ovary contains apoB, E receptor protein, hLDL interacts with the tissue atypically. That is, despite modifications of LDL amino acid residues to prevent interaction with the apoB, E receptor, the modified ligands continue to contribute cholesterol for luteal cell internalization and/or steroidogenesis. We conclude, therefore, that in this tissue much of the LDL-cholesterol is not delivered by the apoB, E receptor pathway. — **Azhar, S., A. Cooper, L. Tsai, W. Maffe, and E. Reaven.** Characterization of apoB, E receptor function in the luteinized ovary. *J. Lipid Res.* 1988. **29:** 869–882.

Supplementary key words apoB, E receptors • hLDL-cholesterol transfer • rat luteinized ovary • steroidogenesis • cholesterol-rich lipoproteins

Previous studies from this laboratory utilizing the rat luteinized ovary for the uptake of cholesterol-rich lipoproteins have pointed to a nonconventional pathway as a major route for the transport of cholesterol into the cells (1–3). To date, our in situ perfusion studies suggest that both high and low density lipoproteins (HDL, LDL) bind primarily to the surface of ovarian luteal cells and release cholesterol for steroidogenesis without entering the cells as intact particles (1–3). Recent experiments using native rat and human lipoprotein particles for perfusion (which can be visualized as discrete particles with the electron microscope) have indicated that the lipoproteins have great affinity for binding to specialized plasma membrane sites between adjacent microvilli or between microvilli and adjacent segments of the cell surface (3). These narrow flat spaces between the opposed microvillar membranes have been referred to as microvillar channels (3): the lipoprotein particles crowd into these channels during the course of the 120-min perfusion study and within minutes after the onset of perfusion with the cholesterol-rich lipoproteins, one can measure an increase in ovarian progestin secretion. During the time frame of these studies, very few of the lipoprotein particles, whether HDL or LDL, appear to have undergone endocytic uptake by the luteal cells: i.e., intact particles are not found clustered in various vesicles or vacuoles of the cells and do not appear associated with coated pits, Golgi structures, or lysosomes (4, 5). Moreover, autoradiograms at the electron microscope level show that only a small fraction (less than 10%) of the bound HDL or LDL protein is internalized by the luteal cells during an 120-min perfusion period and a far smaller fraction (0.5 × 1.5%) can be found actually associated with organelles of the endocytic-lysosomal pathway (1, 2). These various findings have led us to suggest that the hormone-producing cells of the luteinized ovary may obtain a large share of their cholesterol from lipoproteins without actually internalizing the intact lipoprotein particles (1, 2). We have theorized that the microvillar channels, with their large capacity for trapping particles, play an important role in this process (3).

This hypothesis regarding the cell surface extraction of cholesterol does not preclude the possibility that luteal cells

Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; CHD, cyclohexadione; CLE, cholesterol linoleyl ether; 17α-E_2, 17α-ethinyl estradiol; TCA, trichloroacetic acid; Ac-, acetylated; Me-, reductively methylated.

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

also utilize lipoprotein cholesterol through a smaller, but perhaps more regulated, apoB, E receptor-mediated route (4–7). Although this idea was initially rejected by us (2), two facts have led to reexamining the issue. First, the use of a nonmetabolizable protein tag (8–10) has shown that approximately 8.5% of the bound protein is actually degraded (and accumulates) in luteal cells during the course of a 120-min perfusion with human LDL (hLDL) (3). Second, upon examining the luteal cell surface, one encounters specialized coated regions that appear to have direct continuity with the described microvillar channels (3). Although LDL have not actually been found in these unusual coated pits, the proximity of the pits to microvillar channel regions of high particle density suggests that they may relate to the membrane sites of lipoprotein interaction.

Accordingly, in the current study, we have taken a closer look at the functioning of the apoB, E receptor pathway in the luteinized ovary. Initially we examined the luteinized ovary for the presence of apoB, E receptors utilizing immunoblotting techniques with monospecific antibodies to rat apoB, E receptor protein. In subsequent studies, we characterized the structural and functional interaction of hLDL with the apoB, E receptor of the luteinized ovary utilizing a variety of amino acid residue-modified hLDL particles as probes. Finally, using nonmetabolizable (i.e., non-releasable) radiolabels for both the protein and cholesteryl ester moieties of hLDL and lysine-modified hLDL, we attempted to quantify the extent to which apoB, E receptors in the rat luteinized ovary contribute to the uptake of hLDL-cholesterol during steroidogenesis.

MATERIALS AND METHODS

Materials

^{125}Iodine (sodium iodide, sp act ~16.7 mCi/μg of iodine) was purchased from Amersham Corporation, Arlington Heights, IL. [1, 2-^{3}H(N)]Cholesterol (sp act 40–60 Ci/mmol) and [U-^{14}C]sucrose (sp act 350 mCi/mmol) were supplied by E.I. du Pont de Nemours & Co. (Inc.), NEN Research Products, Boston, MA, and ICN Radiochemicals, Irvine, CA, respectively. Acetic anhydride, formaldehyde (30–40%), and 4-amino-pyrazolo [3, 4-d] pyrimidine (4-APP) were the products of Aldrich Chemical Co., Milwaukee, WI. 1, 2-Cyclohexanedione and sodium borohydride were obtained from Pierce Chemical Co., Rockford, IL and Eastman Kodak Co., Rochester, NY, respectively. Medium 199 with Earles salts was purchased from KC Biological, Lenexa, KA. Other reagents used were of analytical grade.

Preparations, modifications and labeling of lipoproteins

Preparation of lipoproteins. Human (h) lipoproteins were isolated from fresh plasma of healthy male donors and rat (r) lipoproteins were obtained from male Sprague-Dawley rats (3–6 months of age; Bantin and Kingman, Fremont, CA). In each case, 3 mM EDTA, 1 mM benzamidine, 1 mM *p*-methylsulfonylfluoride (PMSF), and 10 U/ml of kallikrein inactivator (11) were added to the plasma, and lipoproteins were isolated by preparative ultracentrifugation (12) in a 60 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) at 58,000 rpm. Lipoprotein fractions (hLDL and $hHDL_3$) were isolated between densities of 1.02 and 1.050 g/ml (13) and 1.125 and 1.210 g/ml (14), respectively. Each fraction was recentrifuged for 18 hr at the appropriate densities to minimize plasma protein contamination. Rat (r) LDL was isolated between densities of 1.030 and 1.055 g/ml and was used without any further purification (15, 16). rHDL was isolated between densities of 1.080 and 1.210 g/ml (17).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described previously (1, 2). **Fig. 1** shows that hLDL contained apoB, and $hHDL_3$ contained apoA-I as major apoproteins; rLDL contained apoB with relatively small amounts of apoE and trace amounts of apoA-I: rHDL contained apoA-I, apoA-IV, and apoE as major apoproteins with smaller amounts of apoCs.

Iodination of lipoproteins. hLDL, $hHDL_3$ and rLDL were iodinated using the iodine monochloride method of McFarlane (18) as modified by Bilheimer, Eisenberg, and Levy (19). The specific activity of various preparations ranged from 600 to 900 cpm/ng of protein. Greater than 99% of the radioactivity was precipitated by 15% trichloroacetic acid and < 2.5% of hLDL, 2–3% $hHDL_3$, and 5–7% of rLDL was extractable with organic solvents (20, 21).

Preparation of [^{14}C]sucrose-labeled hLDL. Labeling of hLDL with [U-^{14}C]sucrose was carried out exactly as described in Pittman et al. (8, 9, 10, 22) using cyanuric chloride to activate sucrose for covalent coupling to protein under mild conditions. Briefly, a 0.714-μmol portion of [U-^{14}C]sucrose (250 μCi) was activated with cyanuric chloride (1.429 μmol), whereafter 20 mg of hLDL protein in 5 ml of buffer (0.15 M NaCl, 20 mM sodium phosphate, 1 mM EDTA buffer, pH 7.2) was added. After incubation at room temperature for 6–8 hr, the [^{14}C]sucrose-labeled LDL was separated from unreacted radioactive sucrose by dialysis twice against 0.15 M NaCl, 20 mM sodium phosphate, 1 mM EDTA buffer, pH 6.8; then against the same buffer at pH 7.0; and finally against 0.15 M NaCl, 0.3 mM EDTA, pH 7.0. The specific activity of the [^{14}C]sucrose was in the range of 3.8 to 5.6 μCi/mg of LDL protein.

^{14}C-Labeled degradation products of [^{14}C]sucrose-LDL were quantitated by either the trichloroacetic acid procedure (8, 22) or gel filtration (Sephadex G-50) column chromatography (8, 22).

Labeling of hLDL with [^{3}H]cholesteryl linoleyl ether. [^{3}H]Cholesteryl linoleyl ether was synthesized by a modification (23) of the procedure of Stoll (24). The purity of labeled cholesteryl linoleyl ether was checked by thin-layer chromatography using 5% ethyl acetate in chloroform

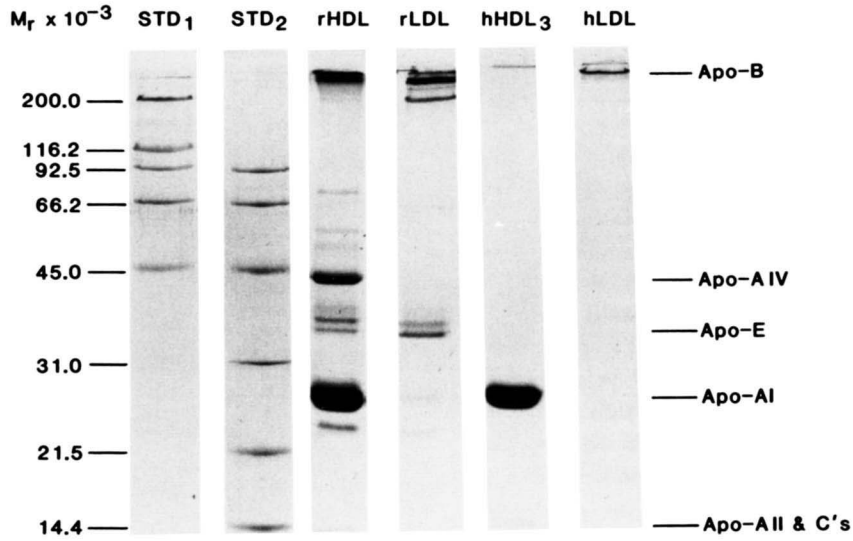

Fig. 1. SDS-PAGE pattern of lipoproteins used for experiments. Lanes 1 and 2 show high and low molecular weight (M_r) standards. These are myosin (200,000), β galactosidase (116,000), phosphorylase b (92,500), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). Lane 3 shows rHDL containing apoA-IV, apoE, apoA-I, and trace amounts of apoC; band at top of gel is denatured protein; lane 4 shows rLDL with apoB, apoE, and trace amounts of apoA-I; lane 5 shows $hHDL_3$ with apoA-I, and lane 6 shows hLDL with apoB.

or 3% diethyl ether in light petroleum (bp 30–60°C) as a solvent system. Greater than 98% of ^3H radioactivity comigrated with authentic cholesteryl linoleyl ether. Labeling of LDL with [^3H]cholesteryl linoleyl ether was carried out essentially according to the procedure of Stein, Halperin, and Stein (25) using the d < 1.21 g/ml fraction of human plasma as the source of cholesteryl ester transfer protein and $hHDL_3$ reconstituted with [^3H]cholesteryl linoleyl ether as cholesteryl linoleyl ether donor. Briefly, $hHDL_3$ was delipidated three times with n-heptane at −10°C for 30 min. The delipidated HDL_3 (5 mg/tube) in 1 ml of 10 mM Tris-HCl, pH 8.0, was reconstituted with [^3H]cholesteryl linoleyl ether (20 μCi) containing 300 μg of free cholesterol by sonication under N_2 at room temperature. The labeled (reconstituted) HDL_3 was reisolated by ultracentrifugation, dialyzed, and immediately used for the labeling of hLDL. The labeling of LDL (final volume 3–4 ml) was carried out by coincubation of 2–4 mg of hLDL with 1.5 mg/ml of [^3H]cholesteryl linoleyl ether–HDL_3 and 30 mg of protein/ml of d < 1.25 g/ml fraction of human plasma. Following incubation at 37°C for 18–24 hr, the labeled LDL was separated from donor HDL_3 by ultracentrifugation. [^3H]Cholesteryl linoleyl ether-labeled LDL ([^3H]cholesteryl linoleyl ether LDL) was dialyzed extensively against phosphate-buffered saline.

Chemical modification of lipoproteins. Reductive methylation (lysine residues) of [^{14}C]sucrose-hLDL, [^3H]cholesteryl linoleyl ether-hLDL, hLDL, $hHDL_3$ and rat (r) LDL was carried out according to the procedure described by Weisgraber, Innerarity, and Mahley (26). The extent of derivatization (methylation) of the lysine residues as determined by the colorimetric procedure (27) was in the range of 65–87%. Acetylation of hLDL and $hHDL_3$ was carried out according to the procedure of Basu et al. (28). The extent of acetylation was in the range of 70–80%. Cyclohexanedione treatment (arginine residues) was carried out essentially according to the procedure of Mahley et al. (29). The extent of derivatization was not quantitated. This procedure was shown to modify four of nine arginine residues in hLDL (29). The inability of modified LDL particles to compete for the higher affinity binding of ^{125}I-labeled hLDL to the apoB, E receptor was confirmed using human skin fibroblasts (see below).

Organ perfusions and in vivo studies

Superovulated immature rats [(± treatment with 4-aminopyrazolo [3, 4-d]pyrimidine, 4-APP (30, 31)] were prepared as previously described (2) and their luteinized ovaries were perfused in situ according to techniques used routinely in this laboratory (1, 2). The standard perfusion sequence involved a 2-min washout with oxygenated medium 199 (pH 7.4, 33–35°C) and a 2-min perfusion of radioactive ligand at 2.2 ml/min followed by 60–90 min of nonrecirculating (flow-through) ligand perfusion at 0.6 ml/min, and either a 2-min or 30-min wash with media alone (2.2 ml/min). In standard ligand uptake experiments, each animal was perfused with a specific ligand; in ligand competition experiments, each animal was simultaneously perfused with trace radiolabeled hLDL or $hHDL_3$ plus excess amounts of unlabeled "competing" ligands. Whenever

possible (i.e., whenever the ovarian tissue was not used for biochemical characterization), the ligand-perfused ovaries were finally perfusion-fixed for 10 min (with 2.5% glutaraldehyde) before being weighed and assayed for radioactivity (LKB Automatic Gamma Counter, 1275). The glutaraldehyde-hardened ovaries offered an independent demonstration of the technical success of each individual ligand perfusion experiment. Note also that reproducibility of ligand preparation and ovary uptake of the ligand by perfusion was very high: e.g., in four separate studies involving different batches of ^{125}I-labeled hLDL (a total of 20 separate ovary perfusions), variation in the mean uptake of ligand was less than 20%. Within specific studies, variation in hLDL uptake between animals was approximately 5%.

In one special set of experiments, especially long (6 hr) washout periods were used. In this case the ^{125}I-labeled hLDL (12 mg of protein; 380μCi) and wash were prepared with medium 199 + 0.5% BSA + 20% washed human red blood cells as routinely prepared in our laboratory (2). In another set of experiments, radiolabeled hLDL was injected intravenously as a bolus (200 μg of protein/ml; 6.5 μCi/ml), and ovaries were excised 1, 2, and 6 hr later (32, 33) and assayed for uptake of radioactivity.

Morphological studies

For morphology, the tissues were prepared as described in previous reports from this laboratory (1–3). Following perfusion with ligand (rHDL, rLDL, 100 μg of protein/ml for 90 min or ^{125}I-labeled hHDL, ^{125}I-labeled hLDL, 100–500 μg of protein/ml, 2–3 mCi/rat for 90 min), the ovary was washed for 2 min or 30 min wth media alone, then perfusion-fixed for 10 min with glutaraldehyde (2.5% in 0.1 M cacodylate buffer, pH 7.4), subsequently diced into 1- to 2-mm pieces and left overnight in fixative. The following day the samples were washed in 0.2 M cacodylate buffer, fixed for 1.5 hr in osmium tetroxide (1% in veronal acetate buffer with 2% sucrose), stained en bloc for 1.5 hr with uranyl acetate (2% aqueous), dehydrated, and embedded in epon-araldite plastic. Native or amino acid-modified rLDL or hLDL particles were visualized as discrete spheres ~250 Å in diameter. Autoradiograms were prepared as previously described (1, 2).

Lipoprotein binding studies

The binding, internalization, and degradation of ^{125}I-labeled hLDL by cultured human skin fibroblasts were determined by established procedures as described previously (34, 35). Degradation of ^{125}I-labeled hLDL was determined as the trichloroacetic acid-soluble noniodide radioactivity present in the medium. The amount of radioactivity associated with cell surface plus that present within the cell (internalized) was taken as total bound radioactivity.

^{125}I-labeled $hHDL_3$ binding to human fibroblasts was carried out according to Brinton et al. (36).

Competition experiments with modified lipoprotein particles

These experiments were carried out in several steps. Initially, hLDL or hHDL particles were acetylated (Ac-), reductively methylated (Me-), or cyclohexadione-treated (CHD) to alter lysine (26–28) and arginine (29) residues on the lipoproteins. Lipoproteins from heterologous (human) serum were used in preference to rat lipoproteins in these studies for ease in obtaining particles with a clearly defined apolipoprotein composition. Indeed, the SDS-PAGE pattern of Fig. 1 shows that the hLDL that was utilized contained only apoB, and that the $hHDL_3$ that was utilized contained only apoA-I. Also, previous studies have shown that human serum-derived lipoproteins bind to rat luteinized ovaries (1, 2, 37, 38), support steroidogenesis (1, 2, 37, 38), and are found in surface microvillar channels (3), as are LDL and HDL from homologous serum. That the chemically altered lipoproteins were, in fact, functionally altered, was monitored by fibroblast binding.

Following this characterization, each group of modified lipoproteins was used in perfusion experiments involving the luteinized ovary. In each case, perfusion with the ligand was carried out for 60 min (32–35°C) and involved the simultaneous use of 15μg of protein (^{125}I-labeled native hLDL or hHDL per ml) + 300 μg of protein (unlabeled native hLDL, hHDL or variously modified hLDL and hHDL) per ml. In the special case of hLDL and methylated hLDL, experiments were also carried out using 30, 90, 150, and 300 μg of unlabeled protein/ml. Following perfusion with ligand, each ovary was washed for 2 min with media alone, excised, and assayed for ^{125}I. In order to insure optimal uptake of the altered particles, lipoprotein-depleted luteinized rats (pretreated with 4-APP) were used for all the ligand competition studies. The ability of excess native particles, or excess modified lipoprotein particles, to compete with the binding of their own native ^{125}I-labeled particles was assessed by the uptake of radioactivity per ovary (corrected for ovary wet weight). In addition, cross-competition experiments were carried out where excess unmodified or modified particles of one lipoprotein species (e.g., hLDL) were used to compete for binding with radiolabeled native particles of another lipoprotein species (e.g., hHDL).

Additionally, each ligand used in the competition experiments was evaluated by its ability to supply cholesterol to the perfused luteinized ovary for steroidogenesis. In these experiments, luteinized ovaries of 4-APP-treated rats were perfused for 60 min with 500 μg of hLDL or 500 μg of hHDL protein/ml [lipoprotein concentrations previously shown to maximally stimulate steroidogenesis in the system (1, 2)]. Samples of perfusate effluent were collected from the vena cava in 4-min intervals, frozen, and subsequently assayed for the amount of total progestins produced [progesterone + 20 α-hydroxypregnen-4-ene-3-one (2)].

Quantitation of protein internalization by LDL receptor-dependent and receptor-independent pathways in the luteinized ovary

For these experiments [^{14}C]sucrose-coupled hLDL ([^{14}C]sucrose-hLDL) was prepared as described by Pittman et al. (8, 10, 22). Aliquots of the sucrose lipoproteins were subsequently methylated ([^{14}C]sucrose-Me-hLDL) and the biological function of both types of modified particles was evaluated with cultured human fibroblasts. The ligands were used in separate perfusion experiments with luteinized ovaries (50 μg of protein/ml; 0.2 μCi/ml for 90 min) followed by a 30-min wash period with medium alone. The perfused and washed ovaries were subsequently homogenized; membrane-bound counts were released by freezing and thawing (2×), and evaluated for total radioactivity by liquid scintillation counting. Degraded, tissue-bound [^{14}C]sucrose residues were also assayed using both trichloracetic acid or gel filtration methods (8, 22). It is worthy of note that preliminary experiments showed that various methods for extracting membrane-trapped radioactivity gave comparable results (e.g., homogenates 2× frozen-thawed, or treated with 1% Triton X-100 for 1–2 hr, or treated with 1 N NaOH for 30 min, or sonicated (Sonifier, cell disruptor ~350, Heat Systems-Ultrasomes, Inc.) in 20-sec intervals for 3 min gave mean (± SEM) degradation values when assayed with trichloracetic acid of 120 (± 14), 116 (± 11), 129 (± 15), and 117 (± 14) ng of [^{14}C]sucrose-hLDL/100 mg of tissue, respectively, as compared to 107 (± 10 ng) obtained with untreated homogenates). Overall, trichloracetic acid values were found to be 20–25% lower than numbers obtained with gel filtration methods.

Results were expressed using values for either total (ovary-bound) LDL protein or degraded (i.e., TCA-soluble or gel filtration-recovered) LDL protein residues. In either case, estimates for LDL taken up by the receptor-independent pathway were based on values for accumulated [^{14}C]sucrose-Me-hLDL, whereas estimates for LDL taken up by the receptor-dependent pathway were based on the calculated difference between total [^{14}C]sucrose-hLDL uptake and [^{14}C]sucrose-Me-hLDL uptake (9, 32, 33).

Quantitation of cholesterol internalization by LDL receptor-dependent and receptor-independent pathways in the luteinized ovary

[^{3}H]Cholesterol linoleyl ether-labeled hLDL particles ([^{3}H]-CLE-hLDL) were reconstituted as described by Stein et al (25). Aliquots of these particles were subsequently methylated ([^{3}H]-CLE-Me-hLDL) and binding and uptake characteristics of both types of cholesterol-reconstituted particles were tested on cultured fibroblasts and compared with the binding characteristics of an equal mass of [^{14}C]sucrose-coupled hLDL particles. Subsequently, [^{3}H]-CLE-hLDL and [^{3}H]-CLE-Me-hLDL were separately perfused into luteinized ovaries under conditions identical to that used for perfusion of the sucrose-coupled particles (i.e., 50 μg of protein/ml; 0.47 μCi/ml for 90 min followed by a 30-min wash with medium). Perfused and washed ovaries were homogenized, total lipids were extracted with chloroform–methanol (20, 21), and [^{3}H]cholesteryl linoleyl ether was measured by liquid scintillation spectrometry. These values were converted to ng of LDL-protein uptake per tissue weight using the specific activity of cholesteryl linoleyl ether LDL. Estimates for the amount of cholesterol taken up by a receptor-independent process were based on the tissue uptake of [^{3}H]-CLE-Me-hLDL, whereas estimates for cholesterol taken up by a receptor-dependent process were based on total tissue uptake of [^{3}H]-CLE-hLDL minus [^{3}H]-CLE-Me-hLDL.

Identification of luteinized ovary apoB, E receptors

Membrane preparations of luteinized ovary were compared with membrane preparations from liver and adrenals of control and 17 α-ethinyl estradiol-primed (39, 40), male rats (180–200 g) for the presence of apoB, E receptor protein.

In each case the tissues were homogenized in five volumes of Tris–HCl buffer, pH 8, containing 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM PMSF, centrifuged (800 *g*) for 10 min, and the supernatant was centrifuged for 60 min at 100,000 *g*. The resulting pellet was washed with buffer to remove lipids and the membranes were used for immunoblotting of apoB, E receptor protein as described by Cooper, Nutik, and Chen (41).

Miscellaneous procedures

Cholesterol in plasma, as well as in the LDL and HDL fractions, was determined enzymatically according to the procedure of Noël, Dupras and Filion (42). The protein content of lipoproteins was determined by a modification of the procedure of Lowry et al. (43) as described by Markwell et al. (44). Progesterone (45) and its metabolite, 20 α-hydroxypregn-4-ene-3-one (46), were quantitated by radioimmunoassay using specific antiserum.

RESULTS

Throughout this report, ligand "uptake" is meant to indicate total tissue-associated ligand and does not imply extracellular or intracellular localization of ligand unless specifically stated.

Localization of LDL

Fig. 2 shows that perfused Me-rLDL are concentrated on the surface of luteal cells and especially fill the flat narrow spaces formed by closely opposed microvillar plasma membranes (microvillar channels). These are the same extracellular sites that trap native (nonmethylated)

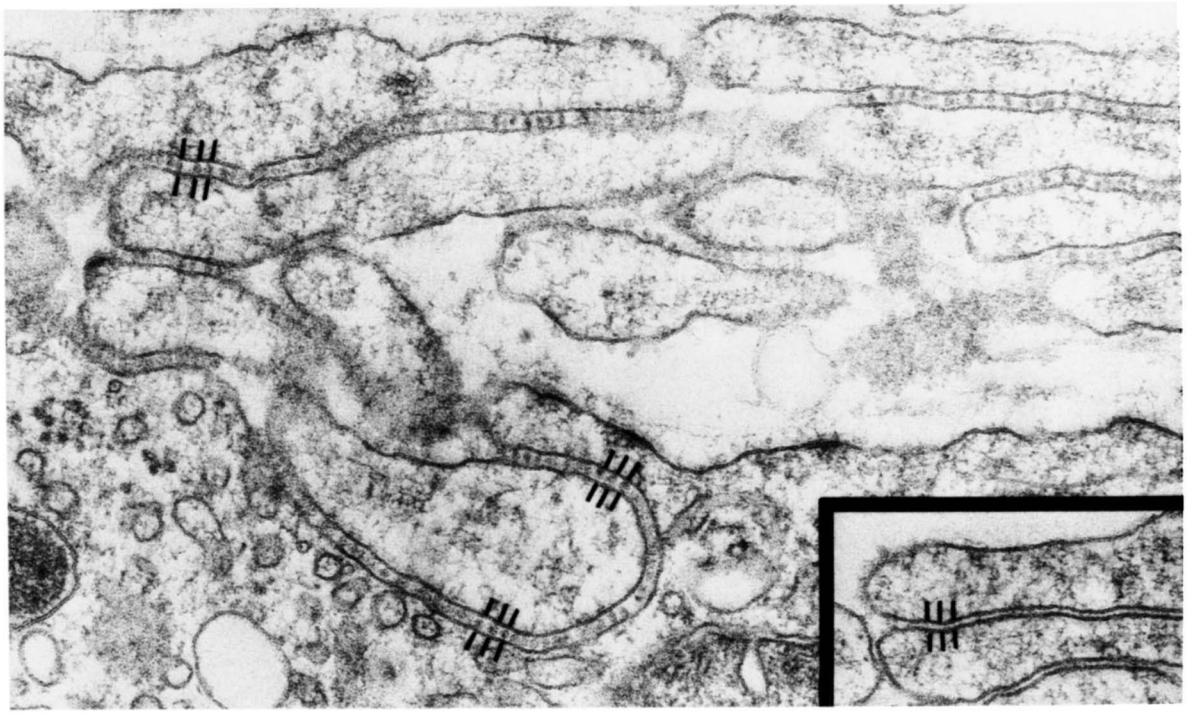

Fig. 2. Representative view of the surface of a luteal cell from a luteinized ovary perfused with Me-hLDL (100 μg of protein/ml) for 90 min and washed for 2 min. The micrograph shows that Me-hLDL are lodged in most of the extracellular channel spaces formed between closely opposed plasma membranes. In these channels the lipoproteins can be seen as individual discrete particles. Note that few Me-hLDL are found on free plasma membrane surfaces of the cell. The width of the channels (as shown by the marker bars) depends on the material within the channels. For example, the insert to Fig. 2 is from the same cell (taken at the same magnification) but shows the appearance and the reduced width of a microvillar channel (see marker bars) when it is not filled with lipoproteins.

r- or hLDL following perfusion (3); indeed, it is not possible to morphologically distinguish between tissues that have been perfused with native versus Me-LDL. Channels filled with lipoproteins can also be identified by measuring the distance between the opposed plasma membranes (3); for example, the average width of the Me-LDL-filled channels of Fig. 2 is 270 Å, whereas the width of channels without lipoproteins (Fig. 2 insert) averages 120 Å (3).

Although direct visualization of lipoprotein particles is possible in the surface microvillar channels where large numbers of particles are trapped, lipoprotein particles within cells are not easily identified. Therefore, to estimate the level of internalization of Me-LDL by the luteinized ovary following perfusion, we turned again to autoradiography (1, 2). In **Table 1** we see that the distribution of exposed grains from perfused ^{125}I-labeled Me-hLDL is essentially like that of perfused ^{125}I-labeled hLDL, i.e., <10% of total (luteal cell-bound) grains is found over the cytoplasm of the cells and <1% of total luteal cell bound grains is associated with endocytic-lysosomal structures of the LDL pathway regardless of the ligand used (Table 1).

Identification of apoB, E receptors

Fig. 3 shows autoradiographs of immunoblots identifying apoB, E receptor protein (135 kDa band) in membranes of luteinized ovary, liver, or adrenal tissue. The immunoblots show that all the membranes tested, including those obtained from the luteinized ovary, have the 135 kDa band as well as other lower molecular weight forms of the receptor protein. Although quantitative comparisons between the tissue types were not attempted (since the relative proportion of plasma memnbrane protein per total membrane protein is not known for each of the preparations used), on a per gram basis, the luteinized ovary had an amount of receptor protein similar to normal liver, and considerably less than that of adrenals or liver from 17α-ethinyl estradiol-treated rats.

TABLE 1. Luteal cell localization of perfused ^{125}I-labeled lipoproteins

Ligand	Grains Located inside Cells	Grains Located within Structures of Endocytic Lysosomal Pathway
	% of total	
hLDL	5.0 ± 1.0	0.4 ± 0.1
Me-hLDL	6.8 ± 1.1	0.8 ± 0.2

Results are from two perfusion experiments using each ligand preparation; a total of 950 and 784 grains were counted for hLDL and Me-hLDL, respectively. Ligand concentration was 500 μg of protein/ml (4–5 mCi/rat).

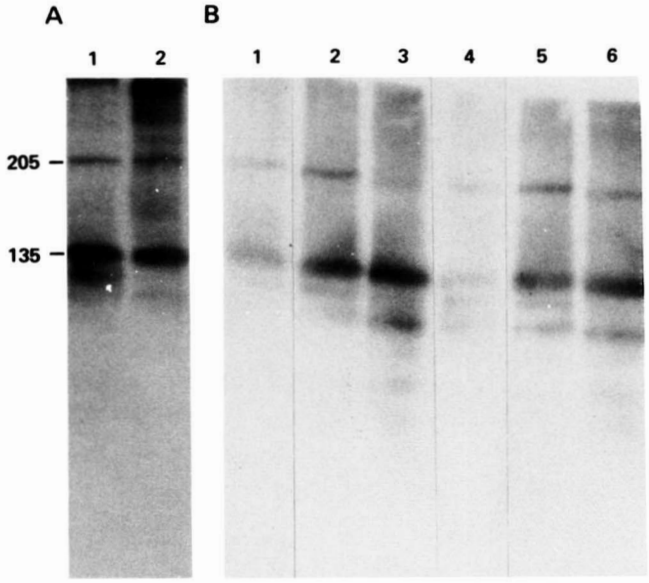

Fig. 3. Immunoblots (autoradiographs) of apoB, E receptor protein (135 kDa). Crude membranes were prepared from luteinized ovary, liver, and adrenal from control and 17α-ethinyl estradiol (17α-E_2)-treated animals. The LDL receptor was identified by immunoblotting as described in Methods. In panel A, normal liver (lane 1) and luteinized ovary (lane 2) at a concentration of 300 μg of protein per lane are compared. In panel B, in a separate experiment, luteinized ovary (lanes 1 and 4), adrenal from 17α-E_2-treated rat (lanes 2 and 5), and liver from a 17α-E_2-treated rat (lanes 3 and 6) are shown. In lanes 1–3,300 μg of protein was applied to the gel, and in lanes 4–6, 200 μg of protein was applied.

Characterization of apoB, E receptor

Competition experiments with modified particles. Fibroblast Binding. The biological activity of various amino acid-modified lipoprotein particles, assessed on fibroblast cultures, is shown in **Fig. 4.** As anticipated, native unlabeled hLDL (Fig. 4A) displaced native labeled hLDL binding to fibroblasts in a concentration-dependent fashion reaching maximal levels at ~100 μg of protein/ml at which point ^{125}I-labeled hLDL binding was only 15% of control levels. Incubation of fibroblasts with CHD-, Me-, and Ac-hLDL particles, on the other hand, did not displace the binding of native ^{125}I-labeled hLDL, even at high concentrations of the unlabeled particles.

In contrast, CHD-, Me-, and Ac-modified $hHDL_3$ particles are effective in competing for ^{125}I-labeled hHDL binding to fibroblasts (Fig. 4B).

Ovary binding. The uptake of amino acid residue-modified LDL by the intact, 4-APP- luteinized ovary was quite different from that observed in fibroblast cultures. For example, when such ovaries were perfused with equal amounts of native ^{125}I-labeled hLDL, or ^{125}I-labeled Me-hLDL, the tissue uptake of the two ligands was similar **(Table 2).** Moreover, in competition experiments, when luteinized ovaries were perfused with 20-fold excess amounts of unlabeled hLDL or various modified lipoproteins along with the ^{125}I-labeled hLDL trace, it appeared that excess unlabeled hLDL, hHDL, or Me-hLDL, CHD-hLDL, or Ac-hLDL were all very effective in reducing ^{125}I-labeled hLDL binding; i.e., all the tested unlabeled lipoproteins, whether native particles or amino acid-modified particles, competed effectively for ~80–85% of the hLDL binding sites **(Fig. 5A).** Likewise, 20-fold excess quantities of unlabeled native, HDL_3 (or hLDL), or various preparations of amino acid-modified HDL_3 or hLDL particles perfused with trace levels of ^{125}I-labeled HDL_3 competed effectively for ~65–75% of HDL_3 binding sites (Fig. 5B).

Despite this similarity in binding of the various ligands, a dose–response curve for one particular amino acid-modified particle (Me-hLDL shown in **Fig. 6**), suggests that some dissociation between the tissue binding of the altered particle and mature particle may exist when very low concentrations of ligand are used; thus, the ability of Me-hLDL at both 30 and 90 μg/ml to compete for ^{125}I-labeled hLDL may be slightly less than that of native particles.

Ovary binding of hLDL differed from fibroblast binding of hLDL in still another respect, that having to do with removal of surface binding with washing. Our current study shows that even after 6 hr of wash (with RBC + BSA-enriched medium 199), ovary uptake of hLDL was still 60% of control values (obtained from ovaries of an animal perfused for just 90 min with hLDL in the same enriched medium). Note also, that ovaries of animals injected in vivo with hLDL showed no loss of hLDL uptake even 6 hr after the injection of the labeled particles.

Progestin secretion. **Table 3** shows the stimulatory effect of perfused native and modified human serum-derived lipoproteins on the amount of progestins secreted during perfusion. When perfused at high concentrations (i.e., 500 μg/ml) all the lipoproteins used, regardless of species or amino acid modification, were equally effective in stimulating progestin secretion.

Quantitation of apoB, E receptor-dependent uptake of hLDL-derived protein and cholesterol

Fibroblast binding. Fibroblast binding experiments summarized in **Fig. 7** show that further modification of hLDL particles by coupling to [^{14}C]sucrose, or reconstitution with [^{3}H]cholesteryl linoleyl ether (CLE), did not alter the binding characteristics of the particles to fibroblasts; i.e., all non-methylated lipoproteins continued to compete effectively for ^{125}I-labeled hLDL binding to fibroblasts and all methylated lipoproteins were relatively ineffective.

Ovary binding. Uptake of sucrose-coupled hLDL. When luteinized ovaries are perfused with [^{14}C]sucrose-hLDL or [^{14}C]sucrose-Me-hLDL for 90 min (and subsequently washed for 30 min with buffer), then homogenized and counted, the values indicate that receptor-dependent uptake of hLDL protein represents ~25% of total [^{14}C]sucrose-hLDL binding **(Fig. 8,** panel A).

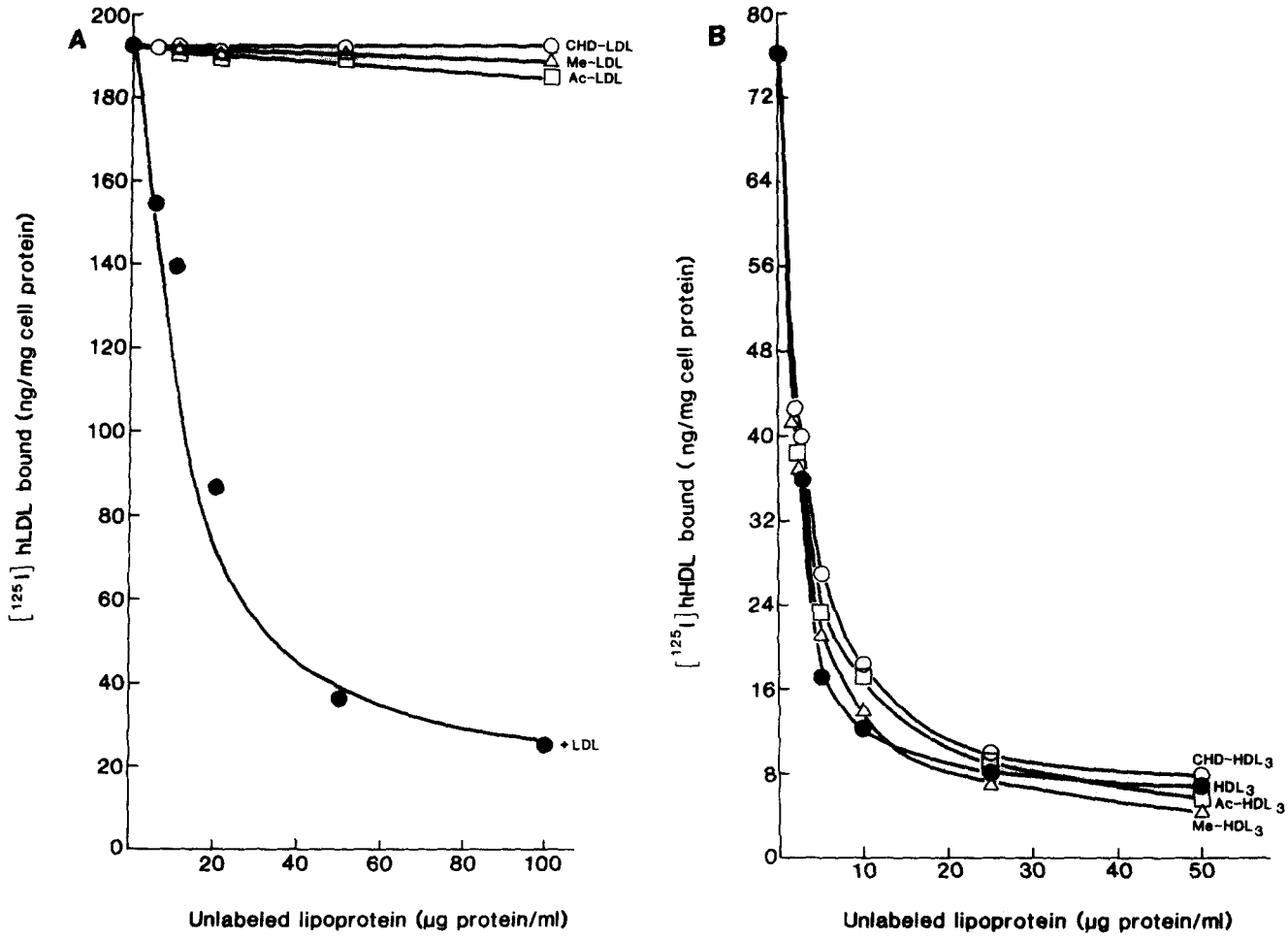

Fig. 4. Effect of amino acid modifications of hLDL and $hHDL_3$ on binding of the ligands to h-fibroblasts in culture. Fig. 4A represents the binding of native particles (hLDL or acetylated (-Ac), methylated (-Me), or cyclohexanedione (-CHD)-modified hLDL. Fig. 4B represents the binding of native $hHDL_3$, or acetylated (-Ac), methylated (-Me), or cyclohexanedione (-CHD)-modified $hHDL_3$; n = three experiments.

However, Fig. 8, panel B indicates that only a fraction of the accumulated [^{14}C]sucrose-labeled protein of either the bound hLDL or Me-hLDL is TCA-soluble (and therefore can be identified as having been truly internalized by the cells and degraded). The major difference between the amount of [^{14}C]sucrose-coupled hLDL or Me-hLDL accumulated by the ovary in panel A and the TCA-soluble residues accumulated by the ovary in panel B probably can be accounted for by the LDL bound extracellularly in microvillar channels (see Fig. 2), i.e., lipoprotein particles not released from microvillar channels with washing. Calculations of receptor-dependent uptake of hLDL using the figures obtained with the TCA-soluble residues are quite different from the uncorrected values of panel A and suggest that only 5% (69/1300 or Bc/Aa) of total bound [^{14}C]sucrose-hLDL is actually internalized by the cell using a receptor-dependent pathway. It should be noted that the TCA data assumes that all the material taken up was, in fact, degraded during the time course of the study. When gel filtration techniques were used to quantify degraded protein, results were 20–25% higher than with TCA; calculations based on these data can be found in the legend to Fig. 8. Thus, only a small amount of protein appears to have been internalized by the cells. However, of this small amount, approximately 60% probably entered by way of the apoB, E receptor pathway (i.e., the difference between

TABLE 2. Uptake of ^{125}I-labeled hLDL and ^{125}I-labeled Me-hLDL by luteinized ovaries of 4-APP-treated rats

	Uptake
	ng/100 mg tissue ± SEM
^{125}I-Labeled hLDL	5734 ± 648
^{125}I-Labeled Me-hLDL	5784 ± 703

The concentration of ligand used was 125 μg of protein/ml; specific activity 73 μCi/ml. Results are mean ± SEM from six separate perfusions using each ligand.

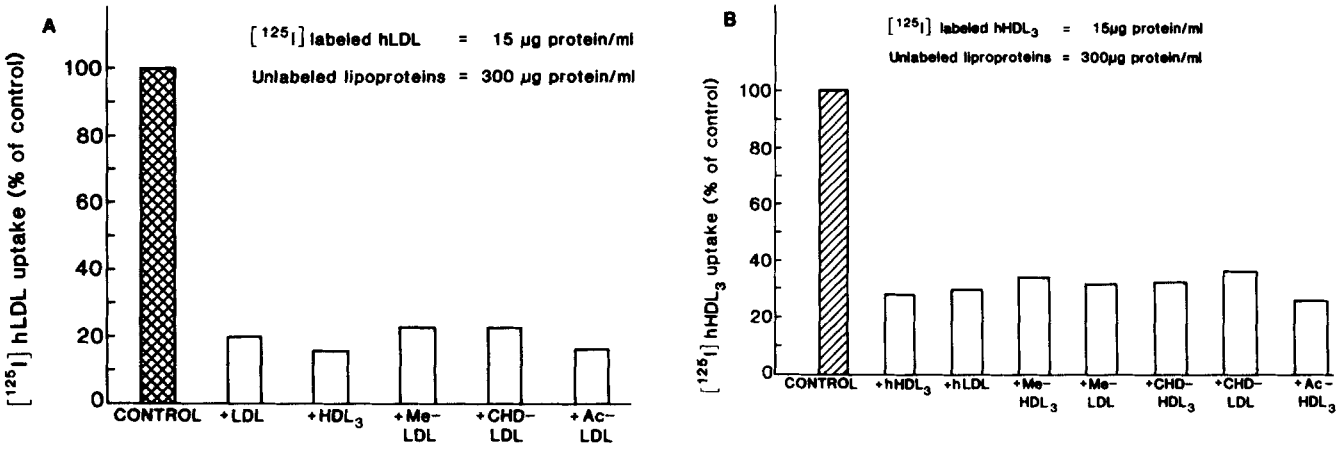

Fig. 5. Effect of various lipoprotein preparations on uptake of ^{125}I-labeled hLDL (Fig. 5A) and ^{125}I-labeled hHDL (Fig. 5B) by luteinized ovaries of 4APP-treated rats. In each case, the uptake of the radiolabeled ligand by itself is shown as a control (100% uptake). Competition for this ligand by other unlabeled native or amino acid residue-modified particles (in 20-fold excess amounts) is shown by the clear bars. Me(methylation); CHD (cyclohexanedione treatment); Ac (acetylation). When expressed as mean (± SEM), uptake of ^{125}I-labeled hLDL (5A) per 100 mg of ovarian tissue, the values are 3038 (± 186), 600 (± 63), 471 (± 30), 704 (± 74), 697 (± 116), and 526 (± 57) for ^{125}I-labeled LDL (alone) or excess LDL, HDL_3, Me-LDL, CHD-LDL, and Ac-LDL, respectively. When expressed for uptake of ^{125}I-labeled $hHDL_3$ (5B) per 100 mg of ovarian tissue, the values are 946 (± 31), 251 (± 17), 274 (± 39), 318 (± 37), 306 (± 31), 316 (± 29), 338 (± 46), and 249 (± 27) for ^{125}I-labeled HDL_3 (alone) or excess $hHDL_3$, hLDL, Me-HDL_3, Me-LDL, CHD-HDL_3, CHD-LDL, and Ac-HDL_3, respectively. Results are a mean of four to eight separate experiments for each ligand situation.

degraded total [^{14}C]sucrose-hLDL uptake, panel B, a, and [^{14}C]sucrose-Me-hLDL uptake, panel B, b.

Uptake of cholesterol-labeled hLDL. Similar conditions exist for interpreting the data of **Fig. 9.** Here, hLDL particles were reconstituted with [^{3}H]cholesteryl linoleyl ether ([^{3}H]CLE) and both [^{3}H]CLE-hLDL and [^{3}H]CLE-Me-hLDL were used for perfusions in the luteinized ovary. After extraction of the tissue, the accumulated non-metabolizable radioactive cholesterol ether was measured and recalculated for ng of LDL protein. These data appear in Fig. 9 and suggest that hLDL-cholesterol entering the cells by a receptor-dependent pathway (the difference in uptake of [^{3}H]CLE-hLDL and [^{3}H]CLE-Me hLDL) represents only 16% of the total accumulated cholesterol.

As in the case with the sucrose-coupled particles (Fig. 8), a large proportion of the tissue-accumulated cholesterol may be accounted for by extracellularly trapped [^{3}H]CLE-hLDL particles. With cholesterol-labeled particles, adjustments for internalization (using TCA or gel filtration techniques to estimate LDL degradation) are not possible. However, under the assumption that the [^{14}C]sucrose-coupled hLDL and [^{3}H]CLE-hLDL particles bind equally well (see Fig. 7), certain corrections can be made. **Table 4,** column 1 shows the amount of hLDL protein that was internalized (TCA-soluble, degraded) and accumulated by the tissue during the 2-hr perfusion experiment (90 min with ligand plus a 30-min wash), i.e., information taken from Fig. 8, panel B. Based on this protein content, and assuming that this protein entered the cell as part of intact LDL particles, one can calculate the amount of cholesterol that must have entered the cell as part of the LDL particle (Table 4, column 2). It is then possible to compare this theoretical figure for cholesterol uptake with the actual measured cholesterol found in the tissue (Table 4, column 3). It appears from these calculations that the total

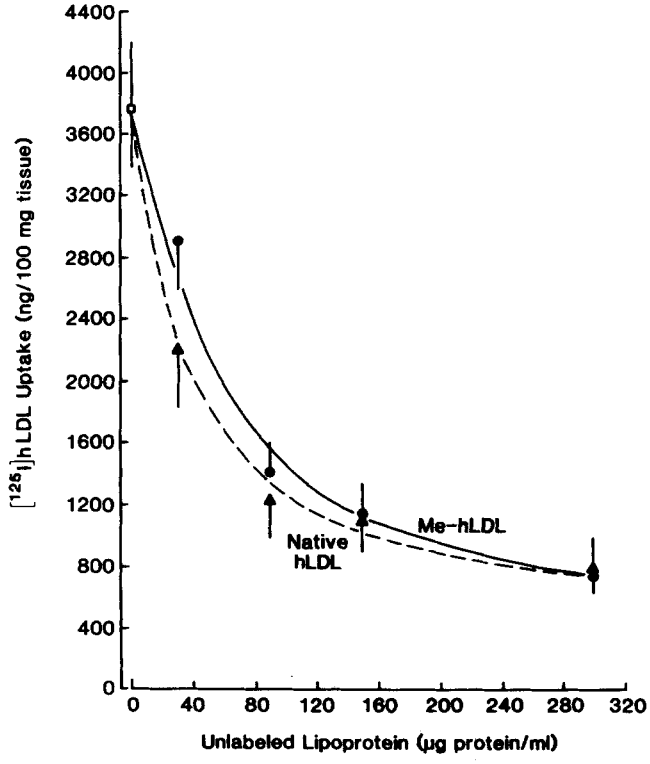

Fig. 6. Effect of concentration of unlabeled Me-hLDL versus unlabeled native hLDL on ^{125}I-labeled hLDL uptake by the luteinized ovary. Results are the mean (± SEM) of two perfusion experiments for each ligand at each concentration.

TABLE 3. Effect of hLDL, Me-hLDL, and $hHDL_3$ on progestin secretion by the perfused luteinized ovaries of 4APP-treated rats

	Progestins Secreted
	ng/min ± SEM
hCG alone	7.5 ± 0.6
hLDL + hCG	25.1 ± 3.1
Me-hLDL + hCG	22.3 ± 2.2
$hHDL_3$ + hCG	21.8 ± 1.8

The concentrations of lipoprotein and hCG used were 500 μg/ml and 1 μg/ml, respectively. Results are mean ± SEM from three separate perfusions using each ligand.

amount of cholesterol internalized by the cells is ~7 times the theoretical amount of cholesterol internalized during the perfusion experiments.

DISCUSSION

The results of this study can be viewed in terms of the luteal cell's intricate system of extracellular microvillar channels. These channels not only create a large increase in luteal cell surface, but they represent a mechanism by which lipoprotein particles can be kept in intimate contact with the plasma membrane of the cells for prolonged periods of time; i.e., normal washing procedures will not release the particles from this space (3). It has been shown that a variety of cholesterol-rich lipoproteins can be lodged in the channels. In freshly isolated ovaries we have observed filamentous-like structures within the channels (1), which we now believe may be remnants of endogenous HDL. In the microvillar channels of perfused ovaries, we find whatever lipoprotein has been perfused, i.e., h or rHDL and LDL, as well as amino acid-modified forms of these particles.

Given this picture of the luteal cell surface, what can be said about the nature of the ovarian luteal cell apoB, E receptor system? Certain observations seem straightforward. Competition experiments using various amino acid-modified LDL suggest that modification of arginine or lysine residues on hLDL molecules minimally interferes with hLDL uptake by the luteinized ovary. That is, excess amounts of amino acid-modified particles are effective in competing for ^{125}I-labeled hLDL removal. This observation suggests that apoB, E receptors are not substantially involved in binding of hLDL to the luteinized ovary. The finding that $hHDL_3$ (which contains neither apoB nor apoE) also competes successfully for hLDL removal, simply reinforces this idea of a nonconventional luteal cell surface lipoprotein binding site. At this juncture, it is not known whether these various lipoproteins are specifically "bound" to the luteal cell surface or merely trapped in the microvillar channel space. The fact remains, however, that the perfused lipoproteins (irregardless of their mechanism of interaction with the plasma membrane sites and irregardless of the characteristics of the specific lipoproteins) contribute to a 25-fold increase in progestin response in the luteinized ovary. Thus, despite unusual removal features for cholesterol-rich lipoproteins such as hLDL, when these ligands do interact with the surface of luteal cells, they appear able to release cholesterol for use in steroidogenesis.

Although these findings with the luteinized ovary demonstrate atypical binding of lipoproteins, it is clear that a classical form of the apoB, E receptor protein does exist in this tissue. Our immunoblotting techniques cannot differentiate which cells of the ovarian tissue contain the apoB, E receptors, but they do indicate that crude membrane preparations from rat luteinized ovary, like membranes from other rat tissues (39–41), contain a 135 kDa protein that interacts with monospecific anti-rat apoB, E receptor protein. Quantitation of the relative amount of this luteal tissue apoB, E receptor in different rat tissues awaits the use of purified plasma membrane preparations, but crude estimates based on the gel radioautographs as well as immunocytostaining of tissues (J. Boyles and E. Reaven, unpublished observations) suggest that membranes from the luteinized ovary

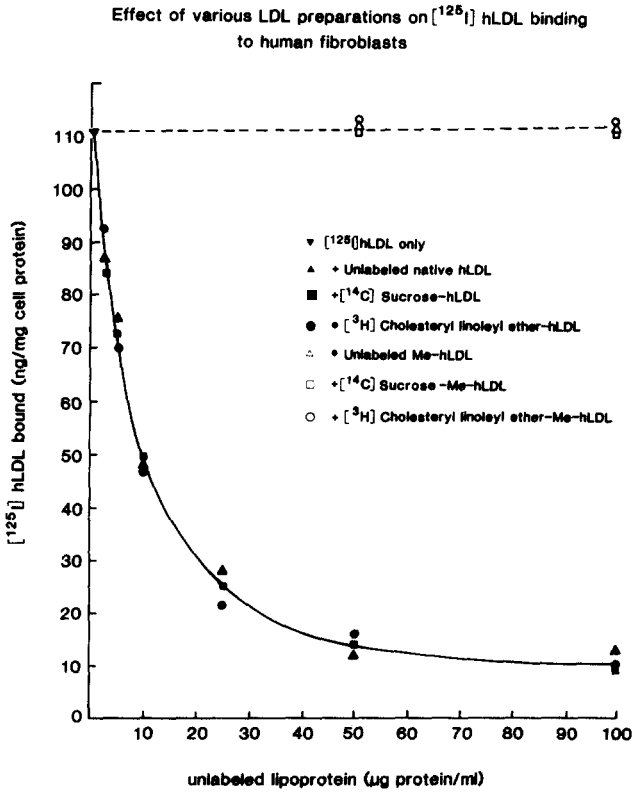

Fig. 7. Effect of various LDL preparations on ^{125}I-labeled hLDL binding to h-fibroblasts (2-hr incubations) showing that coupling with [^{14}C]sucrose or reconstitution with [^{3}H]cholesteryl linoleyl ether does not affect the binding of hLDL. Methylation (Me) of the same particles (clear symbols) prevents binding and competition for the ^{125}I-labeled ligand; n = three experiments.

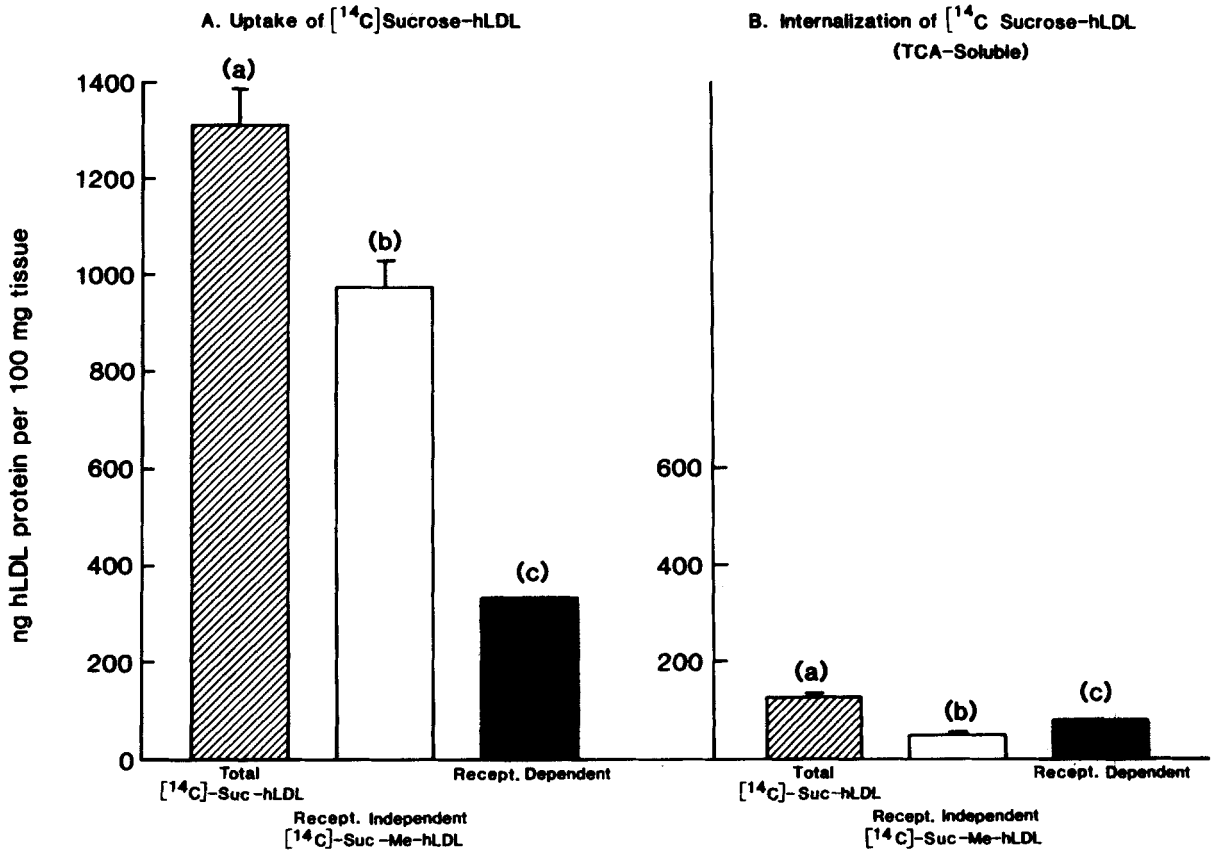

Fig. 8. Luteinized ovary uptake and internalization of nonmetabolizable ([^{14}C]sucrose-coupled) hLDL. Perfusions with this ligand (50 μg/ml) were continuous for 90 min followed by 30 min of wash. Panel A shows total, receptor-independent, and receptor-dependent uptake of hLDL utilizing methylated and nonmethylated forms of the ligand. Panel B shows the trichloroacetic acid (TCA)-soluble counts from the same ovary fractions to distinguish intracellular peptides (degraded, accumulated product) from extracellular (nondegraded) intact particles possibly trapped in microvillar channels. Results are mean (± SEM) from three or four perfusion experiments using each ligand preparation. Values for receptor-dependent uptake are derived from mean values for (a) and (b). Note, if gel filtration data are used instead of TCA data in panel B, a = 137 ± 8, b = 54 ± 3, and c = 83 ng of hLDL protein/100 mg of tissue.

contain somewhat more receptor protein than control liver, but far less receptor protein than membranes of up-regulated (i.e., 17α-ethinyl estradiol-treated) liver or adrenal.

More relevant to our interest is the extent to which the specific apoB, E receptor pathway is expressed in the luteinized ovary. This has been examined in the current study utilizing indirect means to compare the relative amount of LDL-protein versus LDL-cholesterol which accumulates in the luteinized ovary via specific apoB, E receptors during standard perfusion experiments. Such indirect methodology was necessary because of the extensive extracellular trapping of lipoproteins by the luteal cells, and our inability by even extended (i.e., 6 hr) washes to remove more than 40% of the bound material. The experiments involved nonmetabolizable forms of radiolabeled hLDL protein and hLDL-cholesterol. With ^{14}C-labeled hLDL, it was possible to eliminate the extracellular contribution of LDL protein (introduced by proteins trapped in microvillar channels) by determining the extent of total bound protein that was degraded and, therefore, internalized by the cells. Also, by using Me-hLDL for perfusion, we were able to differentiate between LDL-protein that entered the cells via apoB, E receptor-dependent and receptor-independent pathways (7–10, 32, 33). Our results indicate that ~5% of total bound hLDL-protein is internalized by luteal cells via a receptor-dependent pathway. Since the time period used in our experiments is relatively short (total perfusion time = 2 hr), not all internalized LDL protein would likely be degraded (8–10) during the time course of the study. Thus, one might argue that this is a minimum estimate. Were this a substantial problem, we believe autoradiographic studies, carried out at the electron microscope level on hLDL-perfused ovaries treated identically to these (2), would reveal a relatively high number of exposed silver grains (representing radioactive protein) associated with the cytoplasm of the cells. As it is, such techniques reveal that less than 10% of the luteal cell-bound grains are associated with cytoplasm—and only 1% of the luteal cell-bound grains appear specifically associated with structures thought

TABLE 4. Comparison of protein and cholesterol internalization by receptor-dependent and receptor-independent pathways in luteinized ovaries

Pathway	Protein Internalized[a]	Theoretical Amount of Cholesterol Internalized[b]	Cholesterol Actually Internalized[c]
		ng/100 mg tissue	
Receptor dependent[d]	69	125	77
Receptor independent	43 ± 2	77 ± 4	1296 ± 78
Total (dependent + independent)	112 ± 7	202 ± 13	1373 ± 122

[a]Values based on accumulation of protein degradation (TCA-soluble) products ([^{14}C]sucrose-hLDL), Fig. 8.
[b]Assuming hLDL enters the cell as intact particles (protein:cholesterol = 1:1.8).
[c]Calculated from uptake data using [^{3}H]CLE-hLDL and [^{14}C]sucrose-hLDL particles: e.g., total (dependent + independent) uptake = 2070 (Fig. 9, a) − 1307 (Fig. 8A, a) = 763 ng of LDL protein ÷ 112 ng (column 1, above) = 6.8 × 202 ng (column 2, above) = 1373 ng/100 mg tissue (column 3, above).
[d]Only mean values are given here since the numbers are derived from other data.

to be associated with the classical endocytic pathway (i.e., vacuoles, vesicles, Golgi complexes, dense bodies, or lysosomes). From the current experiments utilizing [^{14}C]sucrose-Me hLDL, it is interesting that, of the total protein actually believed to be internalized, at least 60% appears to have entered the cells via a receptor-regulated pathway, a figure showing some concordance with that observed in other rat tissues (8–10). Thus, we conclude from these experiments that although only a small fraction of the bound hLDL is internalized by the cells of the luteinized ovary, that which is taken up is transported through the classical pathway for LDL particles. Confirmation of these data awaits a more direct protocol to accurately measure extracellular as well as intracellular ligand accumulation.

Another question we should address is whether the estimated small fraction of internalized LDL protein is sufficient to account for the amount of progestins produced. Our calculation, based on the progestins secreted by luteinized ovaries perfused under identical conditions, suggests that it is not enough and, even assuming the unlikely possibility of 100% conversion of cholesterol for steroidogenesis (38, 47), could at best supply only 16–20% of the amount needed.[2]

Given this situation, we attempted in the final experiments of this study to quantify the amount of hLDL-cholesterol that is actually internalized by the luteinized ovary during the perfusion experiments. In these experiments we prepared hLDL using a nonmetabolizable form of cholesteryl ester (cholesteryl linoleyl ether) and used the particles in perfusion studies with the luteinized ovary. Since the reconstituted cholesterol hLDL particle bound as well to cells as did the sucrose-coupled hLDL particle (Fig. 6), we felt it reasonable to use the level of internalized (i.e., TCA-soluble) protein associated with the perfused luteinized ovary (Fig. 8, Table 4) as a measure of internalized intact particles, and to calculate the amount of cholesterol that would have entered the ovary cells as part of such intact lipoprotein particles. Such considerations were necessary

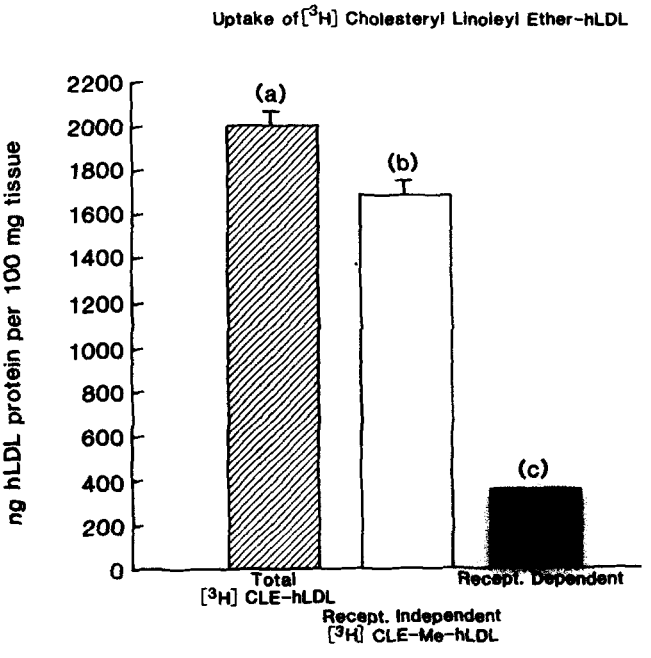

Fig. 9. Luteinized ovary uptake of a nonmetabolizable ([^{3}H]cholesteryl linoleyl ether) component of hLDL. Perfusions were carried out as in Fig. 8. Ligand concentration was 50 μg/ml. The figure provides information on total, receptor-dependent, and receptor-independent uptake and accumulation of the ligand. With this label we cannot distinguish between the cholesterol that is in intact lipoproteins (possibly trapped extracellularly in microvillar channels, see text) from cholesterol that has been internalized by the cell. Results are mean ± SEM of four perfusion experiments using each ligand preparation. The value for (c) is derived from mean values of (a) and (b).

[2]This calculation is based on the observation that under the perfusion conditions of these experiments (50 μg hLDL/ml for 90 min), luteinized ovaries produce ~750 ng protestins per 90 min per 100 mg tissue (2, 3). Based on data from Fig. 8, the specific uptake of [^{14}C]sucrose-LDL = 117 ng cholesterol (i.e., amount internalized = 69 ng per 90 min per 100 mg tissue × 1.8). Thus, even if 100% of the delivered cholesterol could be utilized for steroidogenesis, the receptor-mediated endocytic pathway could supply only 16% of that needed (117/750): i.e., ~84% would have to be supplied by a non-endocytic pathway. Likewise, if gel filtration data were used for this calculation (see legend to Fig. 8), one could estimate that ~20% of the cholesterol needed (149/750) could be supplied by the receptor-mediated endocytic pathway and 80% would have to be supplied by a non-endocytic route.

to eliminate any contribution of trapped extracellular particles to an estimate of total cholesterol uptake. The theoretical values in column B of Table 4 show these figures. As it turns out, ~7 times more cholesterol was found to be associated with the luteinized ovary than the theoretical values predicted. Based on the use of reconstituted methylated hLDL particles (i.e., [^{3}H]CLE-MU-hLDL), the great majority (~90%) of this cholesterol entered the cells via a receptor-independent pathway, a figure consistent with that calculated as necessary for progestin production (see footnote 2).

In conclusion, the experiments of this study suggest that, although apoB, E receptors do exist and may function in delivering lipoprotein cholesterol to the luteinized ovary via a receptor-mediated system, most of the cholesterol is delivered by a receptor-independent pathway. Although these results are atypical for LDL (4, 5), a number of recent studies in the rat in which HDL is the ligand have also reached the same conclusion (48–51), i.e., that a large part of the internalized HDL-cholesterol comes via a receptor-independent pathway. Our working hypothesis is that in the rat luteinized ovary the alternative cholesterol pathway is somehow related to a microvillar channel system, where large numbers of cholesterol-rich lipoproteins can interact with the cell plasma membrane. Although the nature of this membrane–lipoprotein interaction is not yet defined, our data show that a variety of cholesterol-rich lipoproteins (including hLDL, hHDL, and lipoproteins with modified amino acid residues) function in the microvillar channels with similar efficiency. To what extent these data are unique for the luteinized ovary remains to be determined.

Supported by a grant from the National Institute of Health (HL-33881).

Manuscript received 11 June 1987 and in revised form 10 November 1987.

REFERENCES

1. Reaven, E., Y-D.I. Chen, M. Spicher, and S. Azhar. 1983. Morphological evidence that high density lipoproteins are not internalized by steroid-producing cells during in situ organ perfusion. *J. Clin. Invest.* **74:** 1384–1397.
2. 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.
3. Reaven, E., J. Boyles, M. Spicher, and S. Azhar. 1988. Evidence for surface entrapment of cholesterol-rich lipoproteins in the luteinized ovary. *Arteriosclerosis.* In press.
4. Brown, M. S., R. G. W. Anderson, and J. L. Goldstein. 1983. Recycling receptors: the round trip itinerary of migrant membrane proteins. *Cell.* **32:** 663–667.
5. Brown, M. S., and J. L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostatis. *Science.* **232:** 34–47.
6. Bersot, T. P., R. W. Mahley, M. S. Brown, and J. L. Goldstein. 1976. Interaction of swine lipoproteins with the low density lipoprotein receptor in human fibroblasts. *J. Biol. Chem.* **251:** 2395–2398.
7. Mahley, R. W., and T. L. Innerarity. 1983. Lipoprotein receptors and cholesterol homeostatis. *Biochim. Biophys. Acta.* **737:** 197–222.
8. Pittman, R. C., S. R. Green, A. D. Attie, and D. Steinberg. 1979. Radiolabeled sucrose covalently linked to protein. A device for quantifying degradation of plasma proteins catabolized by lysosomal mechanisms. *J. Biol. Chem.* **254:** 6876–6879.
9. Carew, T. E., R. C. Pittman, and D. Steinberg. 1982. Tissue sites of degradation of native and reductively methylated [^{14}C]sucrose-labeled low density lipoprotein in rats. *J. Biol. Chem.* **257:** 8001–8008.
10. Pittman, R. C., A. D. Attie, T. E. Carew, and D. Steinberg. 1982. Tissue sites of catabolism of rat and human low density lipoproteins in rats. *Biochim. Biophys. Acta.* **710:** 7–14.
11. 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.
12. 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.
13. Innerarity, T. L., and R. W. Mahley. 1978. Enhanced binding by cultured human fibroblasts of apoE-containing lipoproteins as compared with low density lipoproteins. *Biochemistry.* **17:** 1440–1447.
14. Leikin, A. I., M. Mihovilovic, and A. M. Scanu. 1982. High density lipoproteins influence cholesterol homeostatis in cultured virus-transformed human lymphoblastoid cells: dependence on the lipoprotein concentration in the medium. *J. Biol. Chem.* **257:** 14280–14287.
15. Fielding, C. J., I. Vlodavsky, P. E. Fielding, and D. Gospodarowicz. 1979. Characteristic of chylomicron binding and lipid uptake by endothelial cells in culture. *J. Biol. Chem.* **254:** 8861–8868.
16. Quarfordt, S. H., R. S. Jain, L. Jakoi, S. Robinson, and F. Shelburne. 1978. The heterogeneity of rat high density lipoproteins. *Biochem. Biophys. Res. Commun.* **83:** 786–793.
17. Andersen, J. M., and J. M. Dietschy. 1981. Kinetic parameters of the lipoprotein transport systems in the adrenal gland of the rat determined in vivo. *J. Biol. Chem.* **256:** 7362–7370.
18. McFarlane, A. S. 1958. Efficient trace-labeling of proteins with iodine. *Nature (London)* **182:** 53–57.
19. 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.
20. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
21. Fielding, C. J. 1979. Validation of a procedure for exogenous isotopic labeling of lipoprotein triglyceride with radioactive triolein. *Biochim. Biophys. Acta.* **573:** 255–265.
22. Pittman, R. C., A. D. Attie, T. E. Carew, and D. Steinberg. 1979. Tissue sites of degradation of low density lipoproteins: application of a method for determining the fate of plasma proteins. *Proc. Natl. Aad. Sci. USA.* **76:** 5345–5349.
23. Halperin, G., and S. Gatt. 1980. The synthesis of cholesteryl alkyl ethers. *Steroids.* **35:** 39–42.
24. Stoll, W. 1932. A new method of preparing cholesterol ethers. *Z. Phys. Chem.* **207:** 147–151.

25. Stein, Y., G. Halperin, and O. Stein. 1981. The fate of cholesteryl linoleyl ether and cholesteryl linoleate in the intact rat after injection of biologically labeled human low density lipoprotein. *Biochim. Biophys. Acta.* **663:** 569–574.
26. Weisgraber, K. H., T. L. Innerarity, and R. W. Mahley. 1978. Role of lysine residues of plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. *J. Biol. Chem.* **253:** 9053–9062.
27. Haseeb, A.F.S.A. 1966. Determination of free amino-groups in proteins by trinitrobenzesulfonic acid. *Anal. Biochem.* **14:** 328–336.
28. Basu, S. K., J. L. Goldstein, R. G. W. Anderson, and M. S. Brown. 1976. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc. Natl. Acad. Sci. USA.* **73:** 3178–3182.
29. Mahley, R. W., T. L. Innerarity, R. E. Pitas, K. H. Weisgraber, J. H. Brown, and E. Gross. 1977. Inhibition of lipoprotein binding to cell surface receptors of fibroblasts following selective modification of arginyl residues in arginine-rich and B apoproteins. *J. Biol. Chem.* **252:** 7279–7287.
30. Anderson, J. M., and J. M. Dietschy. 1978. Relative importance of high and low density lipoproteins in the regulation of cholesterol synthesis in the adrenal gland, ovary, and testis of the rat. *J. Biol. Chem.* **253:** 9024–9032.
31. Christie, M. H., J. F. Strauss III, and G. L. Flickinger. 1979. Effect of reduced blood cholesterol on sterol and steroid metabolism by rat luteal tissue. *Endocrinology.* **105:** 92–98.
32. Spady, D. K., S. D. Turley, and J. M. Dietschy. 1985. Receptor-independent low density lipoprotein transport in the rat in vivo: quantitation, characterization, and metabolic consequences. *J. Clin. Invest.* **76:** 1113–1122.
33. Spady, D. K., J. B. Meddings, and J. M. Dietschy. 1986. Kinetic constants for receptor-dependent and receptor-independent low density lipoprotein transport in the tissues of the rat and hamster. *J. Clin. Invest.* **77:** 1474–1481.
34. Bierman, E. L., O. Stein, and Y. Stein. 1974. Lipoprotein uptake and metabolism by rat aortic smooth muscle cells in tissue culture. *Cir. Res.* **35:** 136–150.
35. Goldstein, J. L., and M. S. Brown. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts. *J. Biol. Chem.* **249:** 5153–5162.
36. Brinton, E. A., J. F. Oram, C-H. Chen, J. J. Albers, and E. L. Bierman. 1986. Binding of high density lipoprotein to cultured fibroblasts after chemical alteration of apoprotein amino acid residues. *J. Biol. Chem.* **261:** 495–503.
37. Gwynne, J. T., and J. F. Strauss III. 1982. The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. *Endocr. Rev.* **3:** 299–329.
38. Paavola, L., and J. F. Strauss III. 1983. Uptake of lipoproteins by in situ perfused rat ovaries: identification of binding sites for high density lipoproteins. *J. Cell Biol.* **97:** 593–607.
39. Kovanen, P. T., M. S. Brown, and J. L. Goldstein. 1979. Increased binding of low density lipoprotein to liver membranes from rats treated with 17α-ethinyl estradiol. *J. Biol. Chem.* **254:** 11367–11373.
40. Havel, R. J., and J. L. Goldstein. 1980. The estradiol-stimulated lipoprotein receptor of rat liver: a binding site that mediates the uptake of rat lipoproteins containing apoproteins B and E. *J. Biol. Chem.* **255:** 10464–10471.
41. Cooper, A. D., R. Nutik, and J. Chen. 1987. Characterization of the estrogen-induced lipoprotein receptor of rat liver. *J. Lipid Res.* **28:** 59–68.
42. 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.
43. 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.
44. 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.
45. Abraham, G. E., R. Garza, and F. S. Manlimos. 1977. Radioimmunoassay of steroids. *In* Handbook of Radioimmunoassay. G. E. Abraham, editor. Marcel Dekker, New York. 591–656.
46. Orezyk, G. P., M. Hichens, G. Aeth, and H. R. Behrman. 1979. Progesterone and 20α-hydroxyprogesterone. *In* Methods of Hormone Radioimmunoassay. 2nd edition. B. M. Jafe and H. R. Behrman, editors. Academic Press, New York. 701–708.
47. Wilks, J. W., G. B. Fuller, and W. Hansel. 1970. Role of cholesterol as a progestin precursor in rat, rabbit and bovine luteal tissue. *Endocrinology.* **87:** 581–587.
48. 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.
49. Lietersdorf, E., O. Stein, S. Eisenberg, and Y. Stein. 1984. Uptake of rat plasma 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.
50. Glass, C., R. C. Pittman, M. Given, 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.
51. Pittman, R. C., T. P. Knecht, M. Rosenbaum, and C. L. Taylor, Jr. 1987. A nonendocytotic mechanism for the selective uptake of high density lipoprotein-associated cholesterol esters. *J. Biol. Chem.* **262:** 2443–2450.