Characterization of high density lipoprotein binding activity in rat adrenocortical cells

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Abstract Rat adrenocortical cells take up high density lipoprotein cholesterol for use as steroidogenic substrate. To better understand this unique uptake process, we have first characterized HDL binding. Infusion of human ^{125}I-labeled HDL into rats pretreated with 4-APP demonstrated that the adrenal and ovary accumulate HDL in a saturable fashion in vivo. Subsequent studies using isolated rat adrenocortical cells demonstrated that cellular uptake of HDL is comprised of two events. One event is characterized by reversible membrane binding and is complete by 60 min ($t_{1/2}$ = 20 min). The second event is marked by irreversible apoprotein accumulation which continues for at least 3 hr. Reversibly bound material exhibits the same apoprotein distribution as unincubated HDL. Irreversible accumulation could not be attributed to internalization or lysosomal accumulation inasmuch as it also occurred with partially purified plasma membranes and was not enhanced by addition of chloroquine. Reversible binding of human HDL_3 exhibited a saturable dependence on concentration (K_d = 27 μg protein/ml; N = 3.0×10^6 sites/cell) similar to that previously reported for rat liver, ovary, and testis. Cell accumulation of HDL decreased by over 80% at 4°C compared to 37°C, did not require calcium, and was not diminished by prior cell treatment with trypsin or pronase. These results indicate that rat adrenocortical cells possess plasma membrane recognition sites for HDL with different properties than those of the LDL receptor. Moreover, adrenal accumulation of HDL apoproteins does not lead to secondary lysosome formation.—**Gwynne, J. T., T. Hughes, and B. Hess.** Characterization of high density lipoprotein binding activity in rat adrenocortical cells. *J. Lipid Res.* 1984. **25:** 1059–1071.

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Circulating serum lipoproteins provide cholesterol substrate for steroidogenesis in the adrenal and ovaries of most species including man and the rat (1–3). In the fasting state, two major lipoprotein classes, high density (HDL) and low density (LDL) lipoproteins, carry greater than 90% of circulating cholesterol. The relative amount of each lipoprotein class varies among species, with HDL predominant in rat (4) and LDL predominant in man (5). The biological activity of lipoprotein particles is determined by their constituent apoproteins and lipids rather than by their density (6, 7).

One mechanism by which adrenal cells have been shown to accumulate lipoprotein cholesterol is receptor-mediated endocytosis (3). This process, which is identical to that described by Brown and Goldstein in cultured human fibroblasts (8), is initiated by binding to the "LDL receptor." The properties of the "LDL receptor" have been well characterized (9–12). Cultured mouse Y-1 adrenal tumor cells (13), long-term cultures of bovine adrenal cortical cells (14, 15), human fetal adrenal cells (16), and rabbit adrenocortical cells (17) have been shown to accumulate LDL but not HDL cholesterol by "LDL receptor"-mediated endocytosis. LDL cholesterol accumulated in this fashion provides substrate for enhanced steroidogenesis.

Considerable evidence now indicates that the adrenal glands (18–22), testes (23), and ovaries of rats (24, 25) and mice (26) and possibly bovine ovaries as well (27) possess a distinct mechanism for accumulating HDL cholesterol. Addition of human or rat HDL to ACTH-stimulated rat adrenocortical cells results in a saturable, concentration-dependent enhancement of steroid hormone production (19). The increase in steroid hormone production greatly exceeds the amount of cholesterol released by cell-associated HDL protein degradation, suggesting that lysosomal degradation of the HDL particle is not required for utilization of HDL cholesterol as steroidogenic substrate. Furthermore, Glass et al. (22) have shown that rat adrenal accumulation of HDL cholesterol ethers, a nondegradable analog of cholesteryl esters, greatly exceeds accumulation of HDL apoproteins covalently linked to nondegradable tyramine-cellobiose.

Our preliminary studies (28) have shown that rat adrenocortical cells possess distinctive saturable recognition sites for high density lipoproteins. Christie,

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; 4-APP, 4-aminopyrazolopyrimidine; MEM, minimum Eagle's medium; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid.

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Gwynne, and Strauss (29) and Strauss, MacGregor, and Gwynne (30) have demonstrated HDL binding sites in rat ovarian cells and have shown that binding is hormonally regulated, while Chen, Kraemer, and Reaven (31) have reported that rat testes membranes bind HDL in a hormonally dependent fashion. The in vivo studies of Kovanen et al. (26) in the mouse likewise suggest that cellular uptake of HDL cholesterol may be mediated by a receptor distinct from that responsible for uptake of LDL cholesterol. In order to better understand the mechanism responsible for cellular uptake of HDL cholesterol, we have first sought to further characterize the interaction between HDL and rat adrenocortical cells.

Our studies indicate that HDL receptor activity differs from the LDL receptor in specificity, kinetics, temperature dependence, effects of divalent cations, and susceptibility to enzymatic attack. The kinetics of HDL binding suggest that only a small portion of bound HDL apoprotein becomes internalized. Moreover, internalization does not necessarily lead to HDL apoprotein degradation.

METHODS

Preparation and characteristics of lipoproteins

Serum for preparation of human HDL_3 ($1.125 < d < 1.210$ g/ml) and LDL ($1.019 < d < 1.055$ g/ml) was collected in EDTA (1 mg/ml) from normal fasting males and females. We have frequently used human as well as rat HDL in our studies since rat HDL contains large amounts of apoE (32), an apoprotein which mediates binding to the LDL receptor (33), while human HDL, particularly that of density greater than 1.085 g/ml, contains practically no apoE. Moreover, our earlier observations (28) have shown little difference in affinity between human and rat HDL. Thus, human HDL, while behaving similarly to rat HDL, can be prepared in a form which will not interact with the LDL receptor. The lipoproteins were separated by sequential density ultracentrifugation according to the method of Havel, Eder, and Bragdon (5). Centrifugation was performed for 24 hr at 4°C and 50,000 rpm in a Beckman 60 Ti centrifuge head, except at density 1.21 g/ml where centrifugation was continued for 48 hr. Lipoproteins were recovered by tube slicing and recentrifuged once for 24 hr at the upper density limit. Purified lipoproteins gave a single band on agarose electrophoresis (34), and were free of contaminating lipoproteins and serum albumin as judged by Ochterlony immunodiffusion. To assess purity and further characterize the isolated lipoproteins, a qualitative estimation of apoprotein complement was obtained by polyacrylamide slab gel electrophoresis (40 to 100 μ protein per lane) in 0.1% SDS according to Rudolph and Krueger (35). By these techniques human HDL was free of apoB and apoE while human LDL was free of HDL apoproteins. The apoE content of three different preparations of HDL used in these experiments, kindly determined by radioimmunoassay by Dr. Conrad Blum, was less than 0.3% of the protein by weight. Assuming a molecular weight of 110,000 for total HDL apoprotein and 33,000 for apoE (6), less than 1% of the HDL particles would be expected to possess apoE. Moreover, HDL used in these studies was ineffective at concentrations of up to 300 μg of protein/ml in enhancing steroid production by cultured mouse Y-1 adrenal cells which accumulate steroidogenic substrate cholesterol by LDL receptor-mediated endocytosis (13). The mean ratios of cholesterol to protein for human HDL and human LDL were 0.5 and 2.3, respectively. HDL was iodinated as previously described (28). Iodinated lipoproteins were dialyzed immediately prior to use.

Preparation and characteristics of cell suspension and primary cultures

Untreated retired female breeder Sprague-Dawley rats (Zivic-Miller) (250–350 g), maintained in a light-controlled environment and fed standard laboratory chow, were killed by pentobarbital (30 mg i.p.) between 8:00 and 9:00 AM, at the beginning of the 12-hr light period. Primary suspensions of adrenocortical cells were prepared from decapsulated glands using collagenase, hyaulronidase digestion, and mechanical dissociation as previously described (19). Where indicated, cells were also prepared in a similar fashion without additional difficulty from rats injected intraperitoneally daily for 3 days with 4-aminopyrazolopyrimidine (4-APP) (2 mg/100 g body weight) (19). Freshly prepared cells were suspended in lipoprotein-poor media A (Gibco MEM #320-2570, 10% lipoprotein-poor fetal calf serum, 1% penicillin-streptomycin) and used immediately, or in complete media B (MEM, 10% fetal calf serum, 1% penicillin-streptomycin) and plated in sterile plastic culture dishes (Falcon #3002) in the absence of ACTH.

Analytical methods

Lipoprotein binding was determined by incubating fresh cell suspensions with ^{125}I-labeled HDL at 37°C in a shaking water bath (approximately 12 cycles/min). Under these conditions cells remained intact, viable, and responsive to ACTH as shown by their ability to maintain linear rates of steroid output for up to 8 hr. Bound and free ^{125}I-labeled HDL were separated by centrifugation of 200-μl aliquots of the incubation mixture for 2 min in a Beckman #152 microfuge over a 200-μl layer of phosphate-buffered saline (pH 7.4). The supernatant was aspirated and the tip of the centrifuge tube containing the cell pellet was cut from the tube

and counted in a Beckman Model 300 gamma counter with an efficiency of approximately 70%. These conditions were chosen on the basis of preliminary experiments that indicated that cell pelleting was complete by 2 min, but that contamination of the tip of the centrifuge tube by diffusion of unbound ^{125}I-labeled HDL was virtually absent. Unless otherwise indicated, specific binding, the difference between the amount bound in the presence and absence of excess unlabeled lipoprotein, is reported. Similarly, we have used the expression "nonspecific" binding to indicate tissue accumulation of tracer which is not prevented by concurrent exposure to excess unlabeled ligand.

Protein was measured by the method of Lowry et al. (36). DNA was measured using mithramycin by the fluorescent technique of Hill and Whatley (37). The statistical significance of differences in the mean was determined using Student's *t*-test. Unless otherwise indicated, lipoprotein concentrations are reported as lipoprotein protein per unit volume.

Materials

Carrier-free ^{125}I was obtained from Amersham Corp., Arlington Heights, IL and 4-APP was from Aldrich, Milwaukee, WI. Goat anti-human albumin and anti-human LDL were obtained from Antibodies Inc., Davis, CA and rabbit anti-human HDL was from Behring Diagnostics, Summerville, NJ. All other materials were reagent grade or better.

RESULTS

Binding in vivo

Experiments were performed to determine if saturable binding of human HDL previously observed in vitro (28) also occurs in vivo. Rats, pretreated with 4-APP to lower circulating lipoprotein concentration were anesthetized with pentobarbital, and the femoral vein was cannulated. Human ^{125}I-labeled HDL in 0.15 M NaCl alone or with a 10-fold excess of unlabeled HDL was infused continuously for 25 min. Two hours after the start of the infusion the animals were killed with pentobarbital (40 mg i.p.) and the vascular bed was perfused via the left ventricle with 50 ml of normal saline. The adrenals, ovaries, and fragments of other organs were excised, blotted, weighed, and counted. The adrenals and ovaries from rats perfused both with and without unlabeled HDL bound more ^{125}I-labeled HDL than did nonsteroid-producing tissues such as kidney, heart, and adipose tissue (**Fig. 1**). Moreover, the amount of ^{125}I-labeled HDL associated with the adrenal was significantly reduced by concurrent administration of unlabeled HDL

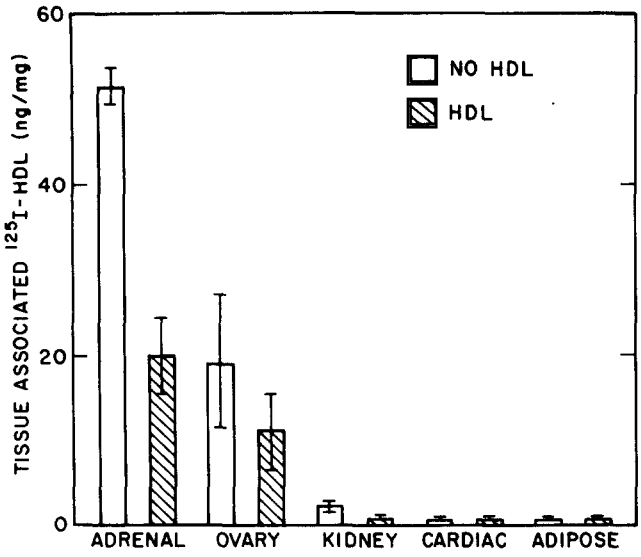

Fig. 1. In vivo binding of ^{125}I-labeled HDL. Adult Sprague-Dawley rats (250–300 g) treated with 4-APP (2 mg/100 g i.p. daily for 3 days) were anesthetized with pentobarbital (30 mg i.p.) and the femoral vein was cannulated prior to infusion of human ^{125}I-labeled HDL (0.15 mg, 133 cpm/ng) in 0.5 ml of normal saline alone (no HDL) or with unlabeled HDL (1.5 mg). Infusion was continued for 25 min. Ninety-five min later, the animals were killed, the inferior vena cava was incised, and the carcass was perfused through the left ventricle with 50 ml of normal saline. The organs were excised, trimmed, blotted, weighed, and counted. The amount of bound ^{125}I-labeled HDL was related to the tissue net weight. The mean and range of duplicate determinations of duplicate infusions are shown.

(Student's *t* test; $P < 0.05$) suggesting that the rat adrenal cortex binds HDL in vivo in a saturable fashion similar to that previously observed in vitro (28). Similar findings have been reported by Andersen and Dietschy (20) in the rat and Kovanen et al. (26) in the mouse. Concurrent administration of unlabeled HDL also decreased ovarian binding. The association of large amounts of ^{125}I-labeled HDL with the ovary is consistent with the studies of Strauss, MacGregor, and Gwynne (30) who have reported the presence of specific HDL binding sites in luteinized rat ovaries. Although of essential importance, in vivo studies such as these require large quantities of lipoproteins, they may be subject to ambiguous interpretation because of uncontrolled hormonal and metabolic factors, and they are difficult to quantitate because circulating lipoproteins undergo intravascular modifications (6, 7). To better understand the properties and function of the adrenal HDL receptor, we have therefore used isolated adrenocortical cells.

Specific ^{125}I-labeled HDL binding increases linearly with increasing cell concentration. When freshly isolated cell suspensions prepared from untreated rats at the end of the dark period were incubated with ^{125}I-labeled HDL (14 μg/ml) for 20 min in the presence and absence of unlabeled HDL (540 μg/ml), the amount of ^{125}I-labeled HDL specifically bound increased linearly with

increasing cell concentration up to the maximum concentration examined, 2.0×10^6 cells per ml. Subsequent studies have employed cells suspended at a concentration of between 1.0 and 2.0×10^6 cells/ml. At this cell density and using concentrations of human ^{125}I-labeled HDL from 5 to 15 μg/ml, conditions frequently employed in this study, up to 9% of the added ^{125}I-labeled HDL is specifically bound.

To determine the time course of association, fresh suspensions of rat adrenocortical cells were incubated at 37°C with human ^{125}I-labeled HDL for 5 hr in the presence and absence of excess unlabeled HDL (**Fig. 2**). At regular intervals, free and cell-associated ^{125}I-labeled HDL were separated by centrifugation. In agreement with our preliminary observations (28), the amount of cell-associated ^{125}I increased rapidly during the first 60 min of incubation but much more slowly thereafter. The time at which maximum accumulation of ^{125}I occurred varied greatly among experiments. In twelve different preparations of fresh adrenal cell suspensions, maximum accumulation had occurred by 3 hr in five, and by 8 hr in three. In the remaining four experiments a plateau value had not been reached when the experiment was terminated between 4 and 5 hr.

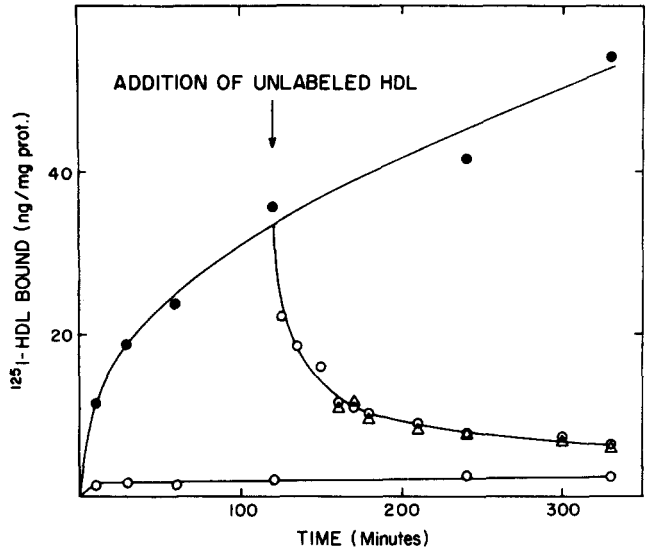

Fig. 2. Rates of association and dissociation of ^{125}I-labeled HDL with suspension and rat adrenocortical cells. Fresh suspensions of adrenocortical cells (1.8×10^6 cells/ml in 9 ml) of media A prepared from glands excised from adult male Sprague-Dawley rats were incubated with ^{125}I-labeled HDL (1 μg/ml, sp act 340 cpm/ng) in the presence (○) or absence (●) of excess unlabeled HDL (650 μg/ml). At 2 hr, excess unlabeled HDL (100 μg/ml) was added to 6 ml of the cell suspension previously incubated with ^{125}I-labeled HDL alone (○). Cells from a second 6-ml aliquot were recovered by centrifugation (1100 *g* for 6 min) and resuspended in ten times the initial volume (△). The amount of ^{125}I-labeled HDL bound was determined by counting the cell pellets recovered by centrifugation of duplicate 200-μl aliquots of duplicate incubations and related to cell protein. The range of determinations was always less than 10% of the mean.

The slow cellular accumulation of ^{125}I beyond 60 min could not be attributed to "nonspecific" adsorption inasmuch as concurrent addition of excess unlabeled HDL prevented both the initial (0 to 60 min) rapid and second (beyond 60 min) slower process of association. Moreover, binding was performed in the presence of 10% lipoprotein-poor fetal calf serum indicating that nonlipoprotein serum proteins do not compete with HDL for receptor site occupancy. To determine if exchange of labeled lipids could account for the increasing accumulation beyond 60 min, cells incubated with ^{125}I-labeled HDL for 90 and 180 min were recovered by centrifugation and the counts extractable with chloroform–methanol 2:1 were determined. In one typical experiment, the total number of cell-associated counts increased 11% during this interval. At 90 min, 6.1% of the total counts were extractable. This increased slightly to 9.0% at 180 min. This exchange of labeled lipid accounted for less than 30% of the total increase while "nonspecific" binding accounted for less than 16%. Thus, greater than 50% of the increase could be attributed to specific cellular uptake of iodinated protein.

The rate of cell accumulation of ^{125}I from 60 to 180 min could be approximated as a linear function of time. In five experiments in which cells prepared from untreated rats were incubated with human ^{125}I-labeled HDL (5–10 μg/ml), the mean rate of association was 1.13 ± 0.51 ng of ^{125}I-labeled HDL mg of cell protein per minute (range 0.68 to 1.91). The mean correlation coefficient was 0.95, indicating that over this time period accumulation was indeed linear with time. When the rate of accumulation beyond 60 min was extrapolated to zero time and subtracted from total cell accumulation, the resulting accumulation plateaued in less than 60 min. The rate constant, for rapid accumulation, was 20.1 ± 5.5 minutes. As will be shown below, this is in remarkable agreement with the rate constant for accumulation of reversibly bound material.

We next sought to determine if binding was reversible. Addition of excess unlabeled HDL to cells preincubated for 120 min with human ^{125}I-labeled HDL resulted in rapid dissociation (Fig. 2, ○) of only a portion of the cell-associated ^{125}I. The remainder was released much more slowly. The initial rate of dissociation was too rapid to determine using current techniques but appeared complete by 5 min. Dissociation occurred with equal rapidity and to the same extent when labeled ligand was dissociated in the absence of added excess unlabeled HDL (Fig. 2, △). To remove the labeled ligand, in the absence of excess unlabeled HDL, cells pre-incubated with ^{125}I-labeled HDL were recovered by centrifugation and resuspended in ten times their initial volume of lipoprotein free media. The coincident dissociation curves observed in the presence and absence

of excess unlabeled HDL indicate that receptor cooperativity (38) does not account for the observed dissociation kinetics.

The amount of cell-associated ^{125}I that could be rapidly released by addition of unlabeled HDL was next determined as a function of time. At various intervals, up to 2 hr, excess unlabeled HDL was added to suspensions of rat adrenocortical cells preincubated with ^{125}I-labeled HDL in the absence of unlabeled HDL (**Fig. 3**). The incubation was continued for 10 min to allow complete release of the dissociable material and free and bound ^{125}I-labeled HDL were separated by centrifugation. Control incubations in the presence of unlabeled HDL were also performed. The total amount of cell-associated material and the amount remaining cell-associated 10 min after addition of unlabeled HDL are shown in curve A and curve B, respectively (Fig. 3). The amount of reversibly bound material, the difference between total and nondisplaceably bound ^{125}I-labeled HDL (dotted line, Fig. 3) increased rapidly until a plateau value was reached at approximately 60 min. In contrast, the material that was not rapidly dissociable increased much more slowly but continued to increase well beyond 60 min. Qualitatively identical results were obtained when fresh suspensions of rat adrenocortical cells were incubated with iodinated rat HDL (data not shown). However, the amount of displaceable rat HDL accumulated by the cells reached a maximum in less than 30 min, rather than 60 min as with human HDL.

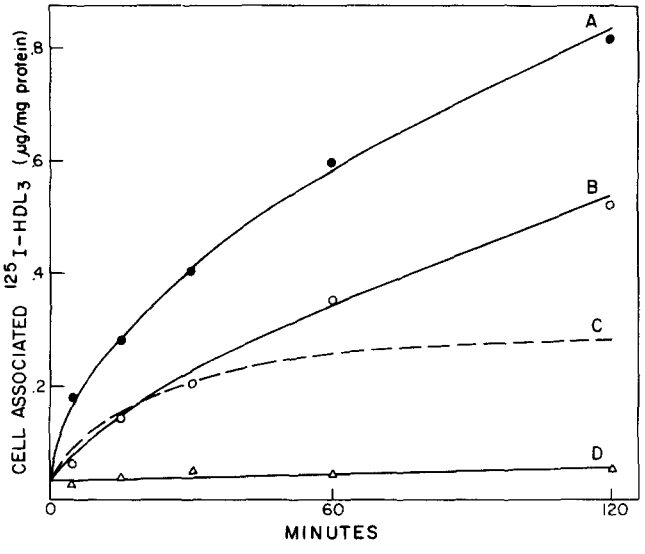

Fig. 3. Reversibly and nonreversibly bound ^{125}I-labeled HDL as a function of time. Fresh suspensions of adrenocortical cells (1.22 × 10^6 cells/ml) prepared from adult female Sprague-Dawley rats were incubated with ^{125}I-labeled HDL (1.1 μg/ml, 89 cpm/ng) in the absence (curve A) and presence (curve D) of excess unlabeled HDL (500 μg/ml). At the indicated times, duplicate aliquots (0.2 ml) were removed and bound ^{125}I-labeled HDL was determined. In addition, duplicate aliquots (0.2 ml) were added to 0.2 ml of HDL (1 mg/ml), incubated for 5 min at 37°C, and bound ^{125}I-labeled HDL was determined (curve B). Curve C was calculated as the difference between curves A and B.

One phenomenon that could account for the observed cellular accumulation of nondisplaceable ^{125}I-labeled HDL is endocytosis of surface bound material. To explore this possibility, binding by broken cell preparation was examined (**Fig. 4**). Rat adrenal glands were excised, trimmed, decapsulated, and homogenized by ten strokes of a loose-fitting sintered glass homogenizer. Nuclei and unbroken cells were removed by low speed centrifugation (800 *g*, 10 min). A partially purified membrane fraction also containing numerous intracellular organelles was derived from the supernatant by subsequent centrifugation (10,500 *g*, 20 min). The pelleted material was resuspended (130 μg of protein/ml) and incubated with ^{125}I-labeled HDL (4 μg/ml) in the presence and absence of a 100-fold excess of unlabeled HDL. Bound and free ^{125}I-labeled HDL were separated by centrifugation. At regular intervals total binding was determined and excess unlabeled HDL was added to incubation not previously exposed to unlabeled HDL. The rate of decrease in bound ^{125}I-labeled HDL following addition of unlabeled HDL was also followed with time (Fig. 4). As with intact cells, addition of excess unlabeled HDL to cell homogenates preincubated with ^{125}I-labeled HDL resulted in rapid displacement of only a portion of the specifically bound material. The remaining bound iodinated material was released much more slowly. Thus, cell integrity is not required for accumulation of nondissociable ^{125}I-labeled HDL, suggesting that the nondissociable material has not been internalized.

To determine if lysosomal processing is responsible for nondissociable cell accumulation of ^{125}I-labeled HDL, the effects of chloroquine on cellular accumulation of ^{125}I-labeled HDL were examined. In the case of LDL (8, 12) a portion of the internalized lipoprotein particles undergoes lysosomal degradation. Inhibition of lysosomal hydrolysis by chloroquine in cultured human fibroblasts leads to decreased LDL degradation and enhanced cellular accumulation. Although our previous studies have shown that lysosomal degradation of HDL is not necessary for utilization of HDL cholesterol as steroidogenesis substrate, they do not exclude the occurrence of internalization and lysosomal degradation of HDL. Iodinated human HDL was therefore incubated with primary cultures of rat adrenal cells in the presence and absence of varying concentrations of chloroquine, an inhibitor of lysosomal hydrolysis (**Fig. 5**). Addition of chloroquine produced a dose-dependent inhibition of steroidogenesis, but had no effect on ^{125}I-labeled HDL degradation. Moreover, addition of chloroquine did not enhance cellular accumulation of iodinated HDL at any dose up to 500 μg/ml (data not shown). In three other

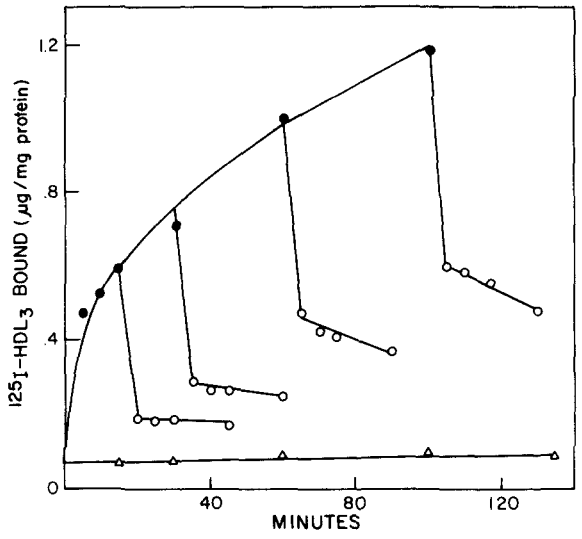

Fig. 4. Time course of binding and dissociation of ^{125}I-labeled HDL to partially purified rat adrenal plasma membranes. Crude plasma membrane suspensions prepared by differential centrifugation from homogenates (six strokes of a loose-fitting sintered glass homogenizer) of decapsulated adrenal glands excised from untreated adult female Sprague-Dawley rats (130 μg protein/ml) were incubated in MEM plus 10% lipoprotein-poor fetal calf serum with human ^{125}I-labeled HDL (4 μg/ml; sp act 257 cpm/ng) in the absence (●) and presence (△) of unlabeled HDL (500 μg/ml). At the indicated times, triplicate 0.2-ml aliquots were removed from duplicate incubations and bound ^{125}I-labeled HDL was separated by centrifugation. In addition, at 5, 10, 15, and 30 min following addition of unlabeled HDL (500 μg/ml), bound ^{125}I-labeled HDL was measured after centrifugation of duplicate 0.2-ml aliquots.

experiments of up to 5 hr duration, mean total cellular accumulation of ^{125}I-labeled HDL was no different in the presence than in the absence of chloroquine (50–100 μM). To be sure that the apoproteins of HDL could undergo lysosomal proteolysis, we examined the degradation of ^{125}I-labeled HDL by adrenal homogenates at acid pH (**Fig. 6**). Maximum degradation occurred at pH 4.5 and was more than 10 times greater than that occurring at pH 7.4. The HDL apoproteins do not therefore appear resistant to lysosomal degradation. Inhibition of lysosomal function does not lead either to inhibition of HDL degradation or to enhanced cellular accumulation of HDL. Thus, if endocytosis of HDL occurs, it is not necessarily coupled to lysosomal apoprotein degradation. Additional studies are underway to determine the subcellular location of the nonreversibly bound iodinated material.

To characterize the apoprotein complement of the reversibly and nonreversibly bound iodinated material, fresh cell suspensions were preincubated with human ^{125}I-labeled HDL (5 μg/ml) for 4.5 hr and recovered by sedimentation. The reversibly bound material was released from resuspended cells by incubation with a one hundred-fold excess unlabeled HDL for 10 min. The released iodinated material and that bound to the cells were solubilized by SDS (2%). The apoprotein composition was analyzed by polyacrylamide gel electrophoresis and compared to unincubated HDL and media. Each sample exhibited only three bands which comigrated with apoA-I, apoA-II, and C-peptides. No higher nor lower molecular weight bands were observed. It should be noted that apoE-containing material could not be identified by this technique in any of these fractions.

Our preliminary studies (28) have shown that cellular accumulation of human and rat ^{125}I-labeled HDL occurs by a saturable process since addition of excess unlabeled lipoprotein prevents accumulation of labeled material. In order to determine the affinity and number of putative binding sites, fresh suspensions of adrenal cells obtained at the beginning of the light cycle from untreated rats were incubated for 40–60 min with increasing amounts of human ^{125}I-labeled HDL in the presence and absence of excess unlabeled HDL (1.05 mg/ml). The duration of incubation was chosen to allow near completion of the reversible phase of cell accumulation while minimizing the contribution of the nonreversibly bound counts. Results typical of five such experiments

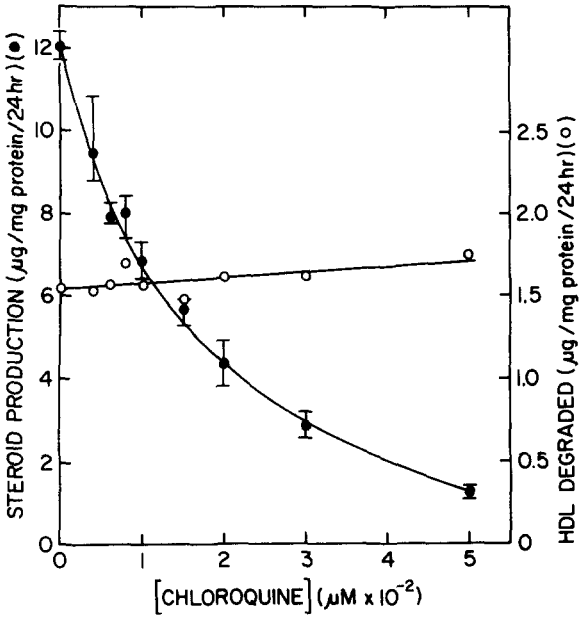

Fig. 5. Effects of chloroquine and steroidogenesis and ^{125}I-labeled HDL degradation. Fresh suspensions of rat adrenocortical cells were prepared from decapsulated glands excised from adult female Sprague-Dawley rats treated for 3 days with 4-APP (4 mg i.p.) and incubated for 20 hr with ^{125}I-labeled HDL (24 μg/ml; sp act 122 cpm/ng) in the presence and absence of unlabeled HDL (260 μg) and various concentrations of chloroquine. Bound and free ^{125}I-labeled HDL were separated by centrifugation and specific binding was determined as the difference between binding in the presence and absence of unlabeled HDL. Specific degradation was determined as the difference between the number of counts remaining in solution after TCA treatment (30 min, 4°C) and centrifugation of media obtained from incubation with and without cells.

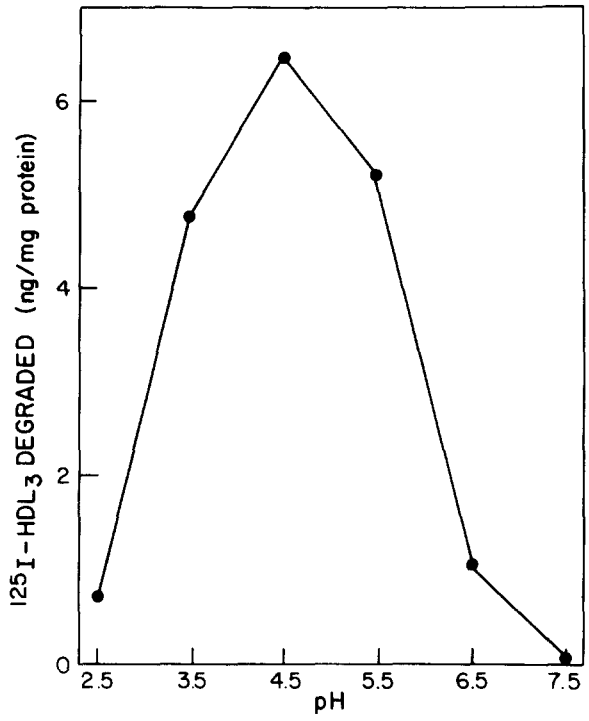

Fig. 6. Degradation of ^{125}I-labeled HDL by rat adrenal homogenates at acid pH. Adrenal glands were excised from adult female Sprague-Dawley rats, trimmed, and suspended in either 0.05 M sodium phosphate (pH 6.5 and 7.5) or 0.05 M sodium acetate, and homogenized for 10 sec with a Polytron. The pH was adjusted by addition of concentrated acid or base and ^{125}I-labeled HDL (10 μg/ml, sp act 200 cpm/ng) was added. Following incubation (90 min, 37°C), the particulate material was removed by centrifugation (10,000 *g*, 10 min); the supernatant was treated with TCA (10%, 30 min, 4°C) after addition of bovine serum albumin (final concentration 0.5%). The amount of ^{125}I-labeled HDL degraded was determined by dividing the number of soluble counts, after removal of the TCA-precipitable material by centrifugation (10,000 *g*, 20 min), by the specific activity of added ^{125}I-labeled HDL.

are shown in **Fig. 7.** "Nonspecific" binding increased linearly with increasing lipoprotein concentration over the entire range examined. When the specifically bound ^{125}I-labeled HDL, which under these conditions is greater than 90% reversibly bound, was determined as the difference between total and nonspecific binding, it had failed to plateau even at concentrations as high as 200 μg protein/ml. These data were analyzed graphically using double reciprocals (Lineweaver-Burk) (**Fig. 8A**) and according to Scatchard (Fig. 8B). When fitted to a single straight line, the mean (n = 6) affinity constant derived from double reciprocal analysis was (K_d = 37 ± 12 μg/ml), (mean correlation coefficient = 0.98) and from Scatchard analysis were (K_d = 56 ± 16 μg/ml), (mean correlation coefficient = −0.91). The number of sites per cell was 5.63 ± 4.1 × 10^5 and 7.45 ± 5.2 × 10^5, respectively. Klotz (39) has recently pointed out the tendency of Scatchard analysis to underestimate binding capacity if insufficiently high ligand concentrations are examined. Thus, the estimates of binding capacity should be considered as minimal estimates. Moreover, subsequent studies have shown that the number of binding sites is regulated by ACTH.

The number and affinity of lipoprotein binding sites were also determined by incubating cell suspensions with a fixed amount of iodinated HDL in the presence of increasing concentrations of unlabeled HDL (**Fig. 9A**) (29, 30). At each concentration of added unlabeled material, a new specific activity was calculated and the amount of total cell-associated material was determined from total cell-associated counts and the revised specific activity (Fig. 9B). These results are typical of four such experiments. This type of "competitive binding" experiment is technically much easier to perform, since it facilitates measurement of binding at high lipoprotein concentrations and requires fewer cells. The results of these studies using a fixed amount of labeled material were identical to those obtained using increasing amounts of iodinated HDL. The data were analyzed graphically using double reciprocals (K_d = 27 ± 23 μg/ml, N = 3.2 ± 2.0 × 10^6 sites/cell) or Scatchard (K_d = 47 ± 40 μg/ml, N = 1.01 ± 1.02 × 10^6 sites/cell) employing a one-site model. Similar studies have been performed using rat HDL.

To determine if the putative HDL receptor has properties similar to those of the LDL receptor of cultured fibroblasts and bovine adrenocortical cells, we examined the effect of temperature, calcium, and protease treatment on HDL binding. When cell suspensions

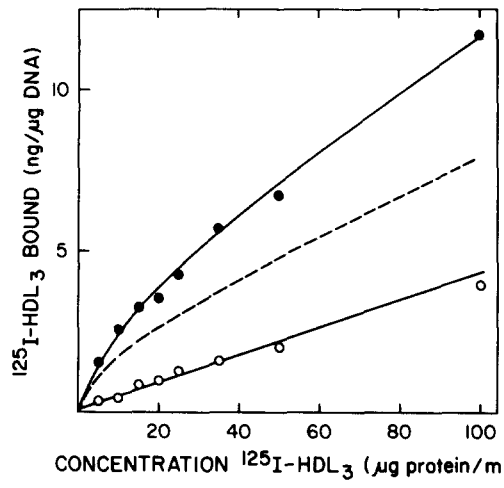

Fig. 7. Concentration dependence of total and nonspecific ^{125}I-labeled HDL binding. Fresh suspensions of rat adrenal cells (23 μg DNA/ml) from decapsulated glands excised from adult female Sprague-Dawley rats were incubated (40 min, 37°C) with increasing concentrations of ^{125}I-labeled HDL (sp act 90 cpm/ng) in the presence (○) and absence (●) of unlabeled HDL (1.05 mg/ml). Bound and free ^{125}I-labeled HDL were separated by centrifugation. Specific binding (– – –) was calculated as the difference between total and nonspecific binding.

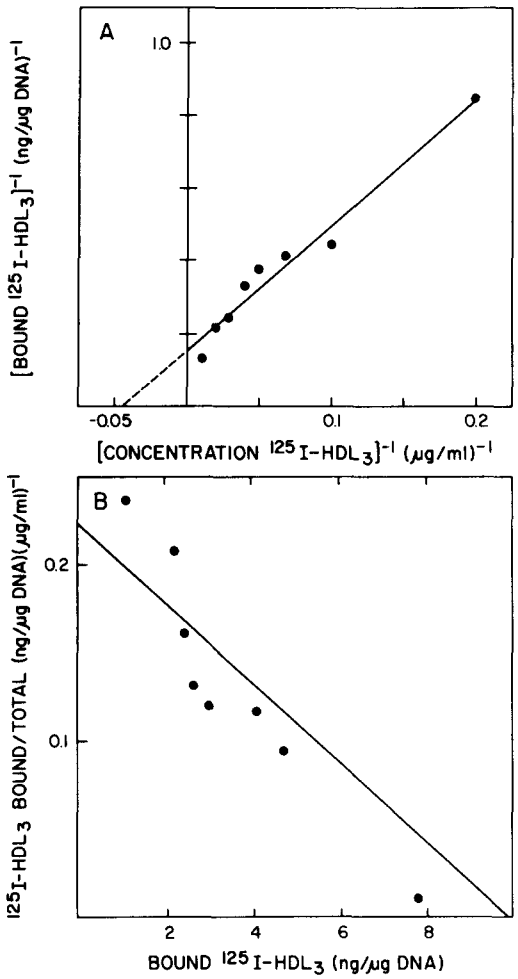

Fig. 8. Graphical analysis of data in Fig. 7 by A, double reciprosals (cc = 0.98) and B, Scatchard analysis (cc = −0.80).

were incubated with ^{125}I-labeled HDL at 37°C, 25°C, and 4°C (**Fig. 10**), the amount of ^{125}I-labeled HDL accumulated by the cells decreased with decreasing temperature at all times examined. The decline is due not only to a decreased rate of association but also to a decreased number of interactions since binding at 4° and 25° plateaus much lower than at 37°C. Both the rapid and slow processes of accumulation were inhibited. In order to determine if dissociation could be retarded at low temperatures, cells (3.3×10^5 cells/ml) were preincubated at 37°C with ^{125}I-labeled HDL (20 μg/ml) for 2 hr and then transferred to 4°C. After 5 min at 4°C, excess unlabeled HDL (290 μg/ml) was added. Cells transferred to 4°C but not receiving unlabeled HDL and cells maintained at 37° with and without added unlabeled HDL served as controls. The rates of dissociation following addition of unlabeled HDL at 37°C and 4°C were identical. No dissociation occurred from control cells maintained in the presence of ^{125}I-labeled HDL without addition of unlabeled HDL either at 4° or 37°C.

To determine if calcium is required for binding of HDL, cells (2.4×10^6/ml in phosphate-buffered saline, pH 7.4, plus 0.5% bovine serum albumin) were incubated with ^{125}I-labeled HDL in the presence and absence of EDTA (1 mM). Cells incubated with EDTA bound much more (147 ng/mg cell protein) than did cells incubated in the absence of EDTA (96 ng/mg cell protein). That added EDTA effectively chelated available calcium was confirmed by abolition of ACTH-stimulated steroidogenesis in control cell incubations (40). Addition of calcium at concentrations up to 10 mM in the absence of EDTA to cells (2.5×10^6 cells/ml in MEM plus 10% lipoprotein-poor human serum) incubated with ^{125}I-labeled HDL (0.5 μg/ml) had no effect on accumulation. Finally, we have examined the effects of cell exposure to trypsin or pronase on the ability of cells to bind HDL. Suspensions of adrenocortical cells freshly prepared from 4-APP-treated rats were preincubated in

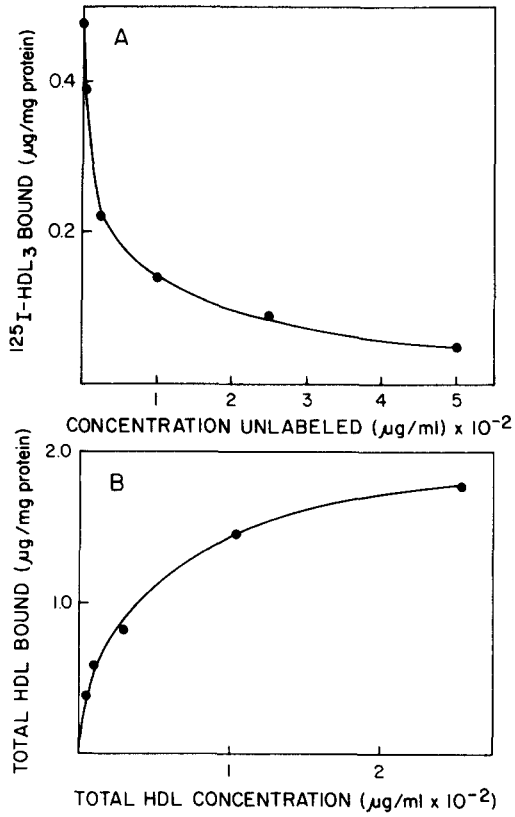

Fig. 9. Effects of increasing unlabeled HDL on binding of ^{125}I-labeled HDL by rat adrenal cells. A, Fresh suspensions of rat adrenal cells (0.47×10^6 cells/ml) prepared from glands excised from adult female Sprague-Dawley rats pretreated for 3 days with 4-APP (2 mg/100 g, i.p.) were incubated (2 hr, 37°C) with ^{125}I-labeled HDL (5.4 μg/ml, sp act 209 cpm/ng) in the presence of increasing concentrations of unlabeled HDL. Bound and free ^{125}I-labeled HDL were separated by centrifugation. B, Total bound HDL was calculated from the data in A according to the text after subtracting nonspecifically bound HDL determined as the amount bound in the presence of a 100-fold excess of unlabeled HDL.

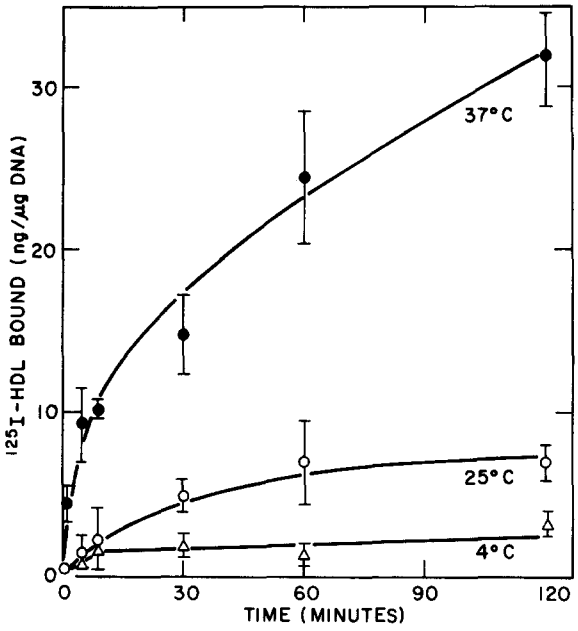

Fig. 10. Effects of temperature on time course of ^{125}I-labeled HDL binding. Duplicate suspensions of fresh adrenal cells prepared from adult female Sprague-Dawley rats were incubated with ^{125}I-labeled HDL (10 μg/ml, sp act 255 cpm/ng) in the presence and absence of a 15-fold excess of unlabeled HDL at the temperature indicated. At regular intervals, duplicate 0.2-ml aliquots were removed and bound and free ^{125}I-labeled HDL was separated by centrifugation.

the presence and absence of trypsin (2.5 mg/ml) or pronase (2 μg/ml) for 1 hr at 37°C. In each case, treated and control cells were recovered by centrifugation, washed once with Eagle's minimum media (MEM), resuspended in MEM plus 10% lipoprotein-poor fetal calf serum, and incubated (30–90 min, 37°C) with ^{125}I-labeled HDL (0.7 to 10 μg/ml) in the presence and absence of at least a 10-fold excess of unlabeled HDL. To insure that the digestive enzymes were active, the amount of TCA-soluble ^{125}I produced by incubating ^{125}I-labeled HDL with enzyme in the absence of cells was determined. In four separate experiments employing several different concentrations of ^{125}I-labeled HDL and durations of incubation, no difference in the amount of ^{125}I-labeled HDL specifically bound could be detected between cells preincubated with and without either degradative enzyme.

DISCUSSION

The first objective of these studies was to determine if saturable cellular uptake of ^{125}I-labeled HDL previously observed in vitro also occurs in vivo. After pretreating the animals with 4-APP to lower endogenous lipoprotein levels, the ovary and adrenal were found to accumulate much more ^{125}I-labeled HDL than any other tissues examined, suggesting that HDL may perform a unique specific function in these tissues. Concurrent administration of unlabeled HDL significantly reduced adrenal ^{125}I-labeled HDL accumulation. Thus in vivo accumulation like uptake in vitro is saturable. Similar findings have been reported by Andersen and Dietschy (20, 21) and Glass et al. (22, 41) in the rat, and Kovanen et al. in the mouse (26). Our findings in the ovary support the earlier findings of Strauss and colleagues (29, 30) which also suggest that in vivo accumulation of ^{125}I-labeled HDL is saturable. The failure of specific ovarian uptake to reach statistical significance in this study may be due to the small number of observations and the uncertain state of gonadotropin stimulation in older female rats.

Since intravascular lipoprotein modification makes interpretation of in vivo lipoprotein action difficult, we subsequently examined HDL binding by isolated cells. All binding studies were performed in a range of cell concentrations where HDL binding was linearly related to cell number. Because of the relative low affinity of the HDL interaction and the difficulty in obtaining large numbers of rat adrenal cells, most binding studies were performed under conditions where only 1–3% of the added labeled HDL was bound. At higher cell concentrations, as much as 9% of the added labeled HDL was specifically bound. This precludes apoE as the sole determinant for receptor recognition since it comprised less than 0.3% by weight of the added HDL apoprotein. Moreover, cyclohexanedione modification, previously shown to block apoE-mediated binding to the LDL receptor (42), does not alter HDL binding to the adrenal (Gwynne, J. T., T. Hughes, and B. Hess, unpublished data).

The kinetics of association and dissociation in vitro suggest that adrenal uptake of ^{125}I-labeled HDL is comprised of at least two events. One event is reversible binding to a saturable number of sites. A portion of the cell-associated ^{125}I-labeled HDL can be rapidly dissociated either by addition of excess unlabeled HDL or by lowering the concentration of unbound ligand. When characterized by SDS-PAGE, the reversibly bound material exhibits the same distribution of ^{125}I among apoproteins as unincubated HDL. Thus the reversibly bound species is not a subpopulation of added ligand selected on the basis of apoprotein complement. Moreover, no modification in the complement of apoprotein results from reversible binding. Reversible binding progressed rapidly and reached a maximum by 60 min. Our previous studies have shown that addition of HDL to cholesterol-depleted rat adrenocortical cells in the presence of ACTH causes a progressive increase in the rate of steroidogenesis for the first 60 min. After the first 60 min the maximal rate of enhanced steroidogenesis is

achieved (19). Thus, as the amount of reversibly bound ^{125}I-labeled HDL increases, so does the rate of steroidogenesis, suggesting that reversible HDL binding is at least initially responsible for increased delivery of steroidogenic substrate cholesterol.

Our results indicate that the second event resulting in cellular uptake of ^{125}I-labeled HDL is characterized by loss of reversibility, since neither addition of excess unlabeled material nor thorough washing releases ^{125}I accumulated by this process. This second event begins immediately upon addition of ^{125} I-labeled initially. It progresses more rapidly and subsequently continues more slowly, but at nearly a linear rate well beyond 3 hr. This slow irreversible accumulation of ^{125}I results from a limited number of interactions since addition of excess unlabeled HDL blocks this phenomenon. Moreover the slow and protracted rate of irreversible association suggests that it is not due to adsorption alone, since adsorption is diffusion-controlled and rapidly complete. The process of adsorption may exhibit characteristics identical to those of receptor binding (43). Adsorption is generally characterized by interactions at a large number of low affinity sites. Adsorption of some radiolabeled ligands, such as insulin (44) and LDL (45), to nonphysiologic surfaces, such as talc or glass, may exhibit properties including affinity and ligand specificity identical to those which characterize physiologically meaningful interactions. Such nonphysiologic surfaces may possess properties such as charge which are similar to the physiologic receptor. Thus the demonstration of a true receptor requires that binding be related to a physiologic response. In the case of rat adrenocortical cells, HDL binding is associated with enhanced steroidogenesis, although the mechanisms linking binding and cholesterol uptake are unknown. In contrast, human or rat ^{125}I-labeled HDL does not associate either reversibly or irreversibly to human or rat red blood cells (data not shown).

The properties of HDL responsible for receptor recognition and, therefore, the receptor specificity remain unknown. Our preliminary results (28) have shown partial competition of LDL for ^{125}I-labeled HDL binding, suggesting that either apoB and apoA-I share common determinants or a nonprotein; perhaps a glycolipid constituent found in both LDL and HDL determines binding. These possibilities are currently under investigation in our laboratory. Both reversible and irreversible accumulation of ^{125}I-labeled HDL exhibits specificity for lipoproteins since our studies were performed in the presence of 10% lipoprotein-poor fetal calf serum which did not block HDL binding.

Several mechanisms that could explain the loss of reversibility were examined. First, the increase in lipid extractable counts was directly measured and could not account for the increase. Second, the lack of high molecular weight bands on SDS-PAGE indicates that covalent association of the HDL apoprotein with a cellular constituent, such as has been reported for insulin (46), is not occurring. Third, loss of reversibility does not appear due to lysosomal accumulation since chloroquine inhibition of lysosomal degradation does not enhance accumulation. Moreover, it seems unlikely that irreversible association can be attributed to sequestration at any intracellular site since it progresses despite cell disruption. It should be noted that while our findings indicate secondary lysosome formation is not necessary for either enhanced steroidogenesis or irreversible apoHDL accumulation, they do not indicate that lysosomal degradation of HDL does not occur since chloroquine may impede not only lysosomal function but also secondary lysosome formation (47). The ability of chloroquine to inhibit adrenal steroidogenesis has been previously observed (48) although the mechanism remains unknown. Chloroquine inhibition occurs even when intracellular cholesterol stores are the source of the steroidogenic substrate. Thus the mechanism of inhibition does not involve exclusively the lipoprotein pathways but probably some more general intracellular mechanism.

At least three possible explanations for irreversible cell accumulation remain. Loss of reversibility in binding could result from *i*) incorporation of HDL apoproteins into the plasma membrane; *ii*) enhanced receptor affinity induced by binding; or *iii*) formation of membrane vesicles with apoprotein trapped within the interior. Several macromolecular ligands have been shown to be internalized by hepatocytes within membrane bound vesicles which do not form secondary lysosomes (49, 50). The relationship of reversible binding to irreversible apoprotein accumulation is not known. Since reversible binding initially progresses more rapidly than irreversible accumulation, reversible binding may lead to irreversible uptake. Alternatively, reversible binding and irreversible accumulation could occur by interactions at distinct sites.

The apparent affinity of the reversible receptor for HDL has been determined. Specific binding was measured under pseudo-equilibrium conditions at 40 min, a time at which reversible binding is nearly complete but nonreversible binding is minimal. Binding approached saturation at about 200 μg/ml. Two techniques, direct and competitive, were employed to measure binding at varying HDL concentrations. Both techniques yielded similar results indicating that the tracer behaves the same as uniodinated HDL. When analyzed graphically, human HDL exhibited a K_d of 37 $\pm$ 12 μg/ml. This is in close agreement with the reported dissociation constants for HDL binding in other systems

including human ^{125}I-labeled HDL binding to isolated rat ovarian cells reported by Christie et al. (K_d = 19.8 μg of protein/ml) (29); rat ^{125}I-labeled HDL binding to rat testicular membranes reported by Chen et al. (K_d = 32 μg of protein/ml) (31); human ^{125}I-labeled HDL binding to isolated rat hepatocytes ($K_a = 8.2 \times 10^6 \cdot M^{-1}$); approx. ($K_d$ = 20 μg of protein/ml) and human ^{125}I-labeled HDL binding to hepatic membranes (K_d = 10.5 μg of protein/ml) reported by Ose et al. (51) and Chacko (52), respectively.

These values are considerably less than the concentration of HDL required to achieve half-maximal enhancement of steroidogenesis in the adrenal in vitro (19). Several possibilities could explain the discrepancy. First, cholesterol uptake may result not only from reversible binding but also from the second irreversible event as well. The dependence of the latter event on HDL concentration is not yet known. Secondly, all receptor interactions might not result in cholesterol utilization for steroidogenesis. In fact, we have shown previously that cellular cholesterol accumulation occurs concurrently with HDL enhancement of steroidogenesis (19). The concurrent occurrence of these two events mediated by the same receptor would produce a rightward shift of the dose-response curve for steroidogenesis compared to that for binding.

Finally, we have compared the properties of the putative HDL binding sites to the properties of the LDL receptor and find that they differ not only in specificity and kinetics but in at least three additional ways as well. First, HDL binding does not require calcium. Second, maximal HDL binding decreases with decreasing temperature, although this may be due to temperature-induced changes in HDL structure rather than changes in temperature-induced changes in receptor activity. Third, the HDL receptor is not sensitive to trypsin digestion.

In summary, the results of these studies further confirm that the interaction of HDL with steroidogenic tissues occurs by a mechanism distinct from the mechanism responsible for LDL uptake. The receptor differs in specificity, proteolytic sensitivity, and in the effects of temperature and calcium. Moreover, the kinetics of binding and dissociation also differ and, together with studies in broken cell preparation of the effects of lysosomatotropic agents, indicate that formation of a secondary lysosome is not essential for cellular utilization of HDL cholesterol. Moreover, many properties of adrenal HDL binding activity are exhibited by HDL binding activity in other tissues, suggesting that these receptors are similar if not identical.

If HDL cholesterol is not released by secondary liposome formation, by what mechanism is HDL cholesterol made available for use as steroidogenic substrate? Three likely hypotheses are that endocytosis occurs and results either in *1*) changes in vesicle conditions causing release of HDL-cholesterol analogous to the acid-induced release of iron from transferrin captured within hepatic endocytotic vessels (53) or 2) delivery of HDL to an intracellular organelle such as the GERL apparatus where lipoprotein dissembly could occur but would not result in apoprotein degradation. Such a process would be consistent with the known polarity of the GERL apparatus (54) and could be a reversal of the lipoprotein assembly mechanism (55). Finally, cholesterol could be taken by a specific enzyme or transport mediated process which would not require internalization of the lipoprotein particle but could account for the nonreversible phase of lipoprotein binding.

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