Uptake of Lipoproteins by In Situ Perfused Rat Ovaries: Identification of Binding Sites for High Density Lipoproteins

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ABSTRACT We have examined the uptake and distribution of ^{125}I-labeled human high density lipoprotein, apolipoprotein E-free ($hHDL_3$), ^{125}I-rat high density lipoprotein (HDL), and human HDL (hHDL) reconstituted with [^{3}H]cholesteryl linoleate after their in situ vascular perfusion to ovaries of gonadotropin-primed immature rats on days 6–9 post human chorionic gonadotropin (hCG)-injection. Some rats were treated with 4-aminopyrazolopyrimidine to reduce plasma lipoproteins and ovarian cholesteryl ester stores. Perfused ovaries were analyzed biochemically and autoradiographically, and progestin content of the ovarian effluent was quantified. Infusion of ovine luteinizing hormone and hHDL increased ovarian progestin secretion severalfold, indicating that the perfused ovary was functional. After perfusion with HDL reconstituted with [^{3}H]cholesteryl linoleate, radioactive progestin appeared in the effluent; thus, sterol carried by exogenous HDL was converted to steroid. At 37°C, uptake of ^{125}I-$hHDL_3$ was greatest after 15 min of perfusion with label. This was decreased by 80% when the perfusion was carried out at 4°C and by 70–95% when excess unlabeled hHDL, but not human low density lipoprotein (hLDL), was included in the perfusate with ^{125}I-hHDL. Aminopyrazolopyrimidine treatment enhanced ^{125}I-hHDL uptake twofold. After perfusion for 15 min with ^{125}I-$hHDL_3$, radioactivity in the ovary was high for 3–30 min of HDL-free wash, then declined 75% by 30–60 min. With light and electron microscope autoradiography, ^{125}I-$hHDL_3$ was localized to corpora lutea, both along luteal cell surfaces and over their cytoplasm. The plasma membrane grains appeared to be associated with segments that lacked bristle coats. Perfusion with ^{125}I-rat HDL produced a similar pattern of labeling. In ovaries perfused with ^{125}I-BSA, silver grains were concentrated over macrophage-like cells but were sparse over luteal cells. We conclude that the in situ perfused rat ovary takes up ^{125}I-$hHDL_3$ by a temperature-dependent, lipoprotein-specific process, and that this lipoprotein is accumulated by luteal cells.

Highly luteinized ovaries of rats appear to use exogenous cholesterol in the form of high density lipoproteins (HDL)[1] as a primary source of sterol for the biosynthesis of steroid hormones (2, 23, 30, 33). HDL is taken up by steroid-secreting cells of the rat through a saturable process that seems to differ from the well characterized pathway for the uptake of low density lipoproteins (LDL) (3, 7, 19). Additional data supporting this view come from work of Kita et al. (18). They have shown that antibodies to the bovine adrenal LDL receptor do not block the uptake of ^{125}I-HDL by mouse adrenal gland in vivo, whereas they inhibit the uptake of ^{125}I-LDL by that tissue. Moreover, the sites involved in the uptake of HDL and LDL differ in several ways, including their requirements for divalent cations and sensitivity to proteases, and in the ability of heparin to displace the bound ligand (9, 10). The sites with which HDL associates thus appear to be distinctly different from those for LDL.

However, the identity of the cells that bear the putative HDL binding sites remains in question. Schuler et al. (30)

[1] *Abbreviations used in this paper:* APP, 4-aminopyrazolapyrimidine; HDL, high density lipoprotein; hCG, human chorionic gonadotropin; hHDL, human HDL; $hHDL_3$, apolipoprotein E-free hHDL; hLDL, human LDL; KRB, Krebs-Ringer-bicarbonate buffer; LDL, low density lipoprotein; LH, luteinizing hormone; and TCA, trichloroacetic acid.

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have suggested that in rat ovaries HDL is accumulated by parenchymal cells. On the other hand, Kovanen et al. (19), in studies of adrenal cortex, have raised the possibility that binding sites for HDL need not reside on adrenocortical cells; e.g., HDL may be taken up and processed by capillary endothelial cells. It is particularly important to identify which specific structures and cells in the ovary take up HDL because this organ has several compartments which make steroids, i.e., follicles, corpora lutea, and interstitial cells. Moreover, each contains several types of cells.

Most of the information available on the uptake and metabolism of lipoproteins, including HDL, by the ovary and other tissues has emerged from studies of dispersed or cultured cells or from work on whole animals. Each of these approaches, however, has certain limitations. For example, when cells are removed from their normal environment and placed in culture, they may express phenotypes not normally expressed in situ. As a consequence, even though in vitro studies have provided much valuable information on lipoprotein metabolism, it is not yet clear whether similar processes are used in the intact organ. There are also a number of problems in studies using whole animals, where labeled lipoproteins are injected into the systemic circulation. They include (*a*) metabolism of exogenous lipoproteins by other organs before they reach the tissue of interest; (*b*) exchange of lipoprotein components in the circulation; and (*c*) dilution of labeled lipoproteins by endogenous lipoproteins.

Clearly, there is a strong need to examine lipoprotein metabolism as it occurs in the intact organ and to identify by morphological means those compartments and cells that take up HDL. To this end, we studied the distribution and metabolism of ^{125}I-labeled human and rat HDL after administration of these lipoproteins to ovaries of rats via in situ vascular perfusion. This approach offers several advantages over other systems previously employed. First, it permits the responses of ovarian cells to be studied under conditions that mimic the normal physiological situation. During in situ perfusion the ovary remains relatively undisturbed and, importantly, its constituent cells maintain their structural relationships with each other and the surrounding tissues. Moreover, the exogenous lipoproteins must cross the vascular wall to gain access to ovarian cells, as would occur in the normal situation. Secondly, lipoproteins are delivered directly to the ovary, thus avoiding the complications accompanying systemic administration.

Our main probe in these studies has been ^{125}I-labeled human HDL_3 ($hHDL_3$). Subclasses of human HDL contain various amounts of apolipoproteins A-I, A-II, Cs, and E (12). Since apolipoprotein E is recognized by binding sites for LDL (21), we used human HDL that was essentially free of apolipoprotein E to insure that we were examining the HDL and not the LDL pathway.

MATERIALS AND METHODS

Animals: Highly luteinized ovaries were produced in immature CD-strain rats 21 d old (Charles River Breeding Laboratories, Inc., Wilmington, MA) by priming the rats with 50 IU of pregnant mares serum gonadotropin (Organon Inc., Oss, The Netherlands), followed 60 h later with 25 IU of human chorionic gonadotropin (hCG, Sigma Chemical Co., St. Louis, MO). Rats were used on days 6–9 after hCG injection, when HDL uptake is maximal (33). Some gonadotropin-primed rats received intraperitoneal injections of 4-aminopyrazolopyrimidine (APP, Aldrich Chemical Co., Inc., Milwaukee, WI), 10 mg/kg body wt, dissolved in sodium phosphate buffer, pH 3, for three successive days prior to use. This treatment lowers plasma cholesterol from 50 mg/dl to <15 mg/dl within 24 h and reduces ovarian cholesteryl ester stores (31).

Isolation and Labeling of Lipoproteins and Albumin: Human LDL (density = 1.019–1.063 g/ml) and human and rat HDL (density = 1.125–1.21 g/ml) were isolated from blood of normal human donors or rats according to Havel et al. (14). $hHDL_3$ prepared in this manner is essentially free of apolipoprotein E (10).

The lipoproteins were labeled with ^{125}I by the iodine monochloride technique of Bilheimer et al. (5) to specific activities of 150–450 cpm/ng of HDL protein. Labeled rat lipoproteins contained somewhat higher amounts of ^{125}I in lipid (~10%) than did human lipoproteins (<2%). The percentage of radioactivity not precipitable by trichloroacetic acid (TCA) was always <2% for all labeled lipoproteins. BSA (Sigma Chemical Co.) was labeled with ^{125}I using lactoperoxidase to a specific activity of 300 cpm/ng.

Reconstitution of HDL with [^{3}H]Cholesteryl Linoleate: [1,2,6,7-^{3}H]Cholesteryl linoleate was reconstituted into hHDL apolipoproteins by the procedure of Hirz and Scanu (15). HDL was delipidated by the procedure of Scanu and Edelstein (28). Delipidated apolipoproteins were sonicated with a mixture of lipids such that the apolipoproteins constituted 57% by weight; egg phosphatidylcholine, 26% by weight; free cholesterol, 2% by weight; ester cholesterol ([^{3}H]cholesteryl linoleate) 11% by weight; and tripalmitin, 4% by weight. The mixture of apolipoproteins and lipid was subjected to sonic irradiation and the sonicated material was then adjusted to a density of 1.215 g/ml and centrifuged to isolate reconstituted HDL, which was dialyzed extensively against 0.15 M NaCl containing 0.01% EDTA, pH 7.4.

Reconstituted HDL had a weight ratio of protein/sterol of 2:1. The specific activity of the [^{3}H]cholesteryl linoleate was 5,077–9,787 dpm/nmol. Less than 1.6% of the ^{3}H was in free cholesterol. Radioactivity in these preparations migrated with native HDL on cellulose acetate electrophoresis, and the reconstituted preparation gave a line of identity with native HDL upon double immunodiffusion using a rabbit anti-human HDL antiserum.

Analytical Methods: Progesterone (11) and 20-α-hydroxypregn-4-en-3-one (24) was quantitated by specific radioimmunoassays. Protein was determined by the method of Lowry et al. (20) using BSA as a standard. In some experiments, ovaries were homogenized in 2 ml of 0.9% saline, and aliquots were added to an equal volume of cold 20% TCA. After standing at 4°C for 30 min, the mixture was centrifuged at 3,000 *g* for 10 min and the resultant supernatant was counted. Other aliquots of the homogenate were extracted with 5 vol of chloroform/methanol (2:1, vol/vol) and the radioactivity in the organic phase was quantified.

A single mean was derived for all animals in a given treatment group and the means were compared among groups with one-way analysis of variance and subsequent Newman-Keuls' test.

Perfusion of the Ovary and Collection of the Effluent: Labeled and/or unlabeled human or rat lipoproteins, ^{125}I-BSA, or ovine luteinizing hormone (LH) were delivered to the ovary using a modification of the perfusion method of Paavola (25). No attempt was made to separate the uterine artery from its companion vein since manipulation of these vessels brought about their constriction. Thus, the ligature securing the in-flow cannula was placed around both uterine vessels instead of around only the artery, as is the case for other animals. The ovary was then perfused, at a flow rate of 1–1.5 ml/min, in sequence for varying periods of time with (*a*) Krebs-Ringer-bicarbonate buffer (KRB; 37°C, 300 mOsmol, pH 7.4, 2 mg/ml glucose, without lipoproteins) to wash out blood; (*b*) KRB containing labeled and/or unlabeled lipoproteins, labeled albumin, or LH; and (*c*) lipoprotein-free KRB to wash out unbound label. The ovary was then either (*a*) excised, freed of adhering tissue, weighed, subjected to gamma spectrometry (counting efficiency: 70%), and then frozen for subsequent biochemical analyses or fixed by immersion in Bouin's fluid; or (*b*) perfused for an additional 45 min with cold 1% paraformaldehyde-1% glutaraldehyde-0.01% trinitrocresol (16) in 0.1 M sodium cacodylate buffer at pH 7.4, excised, counted using a gamma spectrometer, and processed for autoradiography as described below.

In some studies, the effluent from the ovary was collected as follows. After cannulating the uterine artery, a second cannula (No. 23 gauge needle attached to a short length of No. 60 polyethylene tubing containing heparin) was placed in the ovarian vein and secured with a ligature. The samples of ovarian effuent were then centrifuged as necessary to remove any blood, frozen, and subsequently subjected to steroid analysis. In these studies, 2% BSA was added to the KRB to facilitate secretion of steroids.

To study the conversion of [^{3}H]cholesteryl linoleate carried by reconstituted HDL into steroids, animals were pretreated with APP for 3 d prior to the experiment, which was performed on day 6 post-hCG injection. Ovaries were first perfused with KRB for 30 min and then for a 20-min period with KRB containing 15 μg of reconstituted HDL/ml (9.6×10^5 dpm/ml) with or without 333 μg of native HDL/ml.

The venous effluent was extracted with 6 vol of chloroform/methanol (12:1,

vol/vol). The organic phase was dried under nitrogen and the residue subjected to thin-layer chromatography on Whatman K5 plates (Whatman Laboratory Products Inc., Clifton, NJ) using hexane/ethyl acetate (7:3, vol/vol) as a solvent system. Areas where [^{3}H]progesterone and [^{3}H]20-α-hydroxypregn-4-en-3-one migrated, identified under UV light using authentic "cold" standards, were collected into vials for liquid scintillation counting using Biofluor (New England Nuclear, Boston, MA).

Autoradiography: Immersion-fixed ovaries were processed by routine methods for light microscopy and embedded in paraplast or glycolmethacrylate. Ovaries previously fixed by perfusion were washed overnight at 4°C in 0.1 M sodium cacodylate buffer; one-half was dehydrated in graded ethanols and embedded in low viscosity epoxy resin (32), and the other was embedded in glycolmethacrylate.

For light microscope autoradiography, sections of immersion- (5–6 μm paraffin, 2–3 μm glycolmethacrylate) or perfusion- (1–1.5 μm epoxy, 2–3 μm glycolmethacrylate) fixed tissue were placed on acid-cleaned, chrome alum-gelatin coated glass slides. Under a Wratten No. 2 red safelight, the slides were dipped in melted (43°C), undiluted Kodak NTB-3 nuclear emulsion and placed in light-tight boxes. After autoradiographic exposure in the dark at 4°C for varying periods of time, the sections were developed (undiluted Kodak D-19 developer, 2.5 min, 19°C), washed, and fixed by photographic means. Following washing and air-drying, they were stained with hematoxylin-eosin (paraffin) or methylene blue-azure II (glycolmethacrylate, epoxy) and viewed with a bright field microscope. To insure that no sites of uptake of ^{125}I-lipoprotein were overlooked, slides were processed at weekly intervals until the maximum number of silver grains was formed.

For electron microscope autoradiography, pale-gold thin sections of epoxy-embedded tissue were picked up on 0.5% celloidin-coated copper grids. The sections were coated with Ilford L-4 nuclear emulsion (Ilford Ltd., Ilford, Essex, England) (diluted 1:1 with distilled water) by the loop method of Caro and van Tubergen (8). After autoradiographic exposure, the sections were developed in freshly prepared D-19 for 2 min at 19°C, washed briefly with distilled water, fixed, and washed again. After air drying, the sections were treated with 1 N NaOH to remove gelatin from the emulsion, and with amyl acetate to remove celloidin; this greatly improved visibility of the tissue. They were then stained with uranyl acetate for 30 min and with lead citrate (26) for 5–15 min. For purposes of quantitation, cells to be photographed were selected in a predetermined manner to insure randomness, i.e., for each interval studied, the first 30–48 cells encountered that were overlain by one or more silver grains were photographed. The final prints were enlarged to 16,000 ×. Grains were considered to be associated with the plasma membrane if they were within 250 nm of this structure and to be intracellular if they were >500 nm from the plasmalemma.

Controls were carried out to rule out the presence of positive or negative chemography. The specificity of the autoradiographic localizations was evaluated by perfusing a 350-fold excess of unlabeled hHDL or hLDL to the ovary simultaneously with labeled lipoprotein.

RESULTS

Steroid Secretion by the Perfused Ovary

To determine whether the ovary remained functional during in situ perfusion, we examined its steroidogenic response to lipoproteins and LH. The pattern of steroid secretion was examined for ovaries of non-APP treated (control) and APP-treated rats perfused with lipoprotein-free KRB for 165 min, the maximum length of perfusion used in this work. APP, an adenine analogue, reduces lipoprotein secretion by the liver and produces a marked hypercholesterolemia (1) and reduces stores of ovarian cholesteryl esters (31). For these studies, effluent from the ovary was collected for successive 5-min periods and analyzed for progestin content. The total progestin secreted by ovaries of non-APP treated rats fluctuated from rat to rat but was consistently high in each animal for the first 15–20 min of perfusion, after which it declined to fairly constant levels (Fig. 1). This decline was anticipated since perfused ovaries are isolated from the circulatory system and therefore no longer exposed to trophic factors and lipoproteins normally present in plasma. A similar trend of progestin secretion was evident in rats treated with APP, although the level of steroid secretion was severalfold lower than that for non-APP treated animals (Fig. 1). This presumably reflects the depletion of endogenous sterol substrates as a result of APP treatment.

The response of the perfused ovary to exogenous lipoproteins was analyzed in APP-treated rats, since it was felt that an enhancement in sterol synthesis or secretion would be more readily detected in these animals. Ovaries were infused with hHDL at cholesterol concentrations of ~200 μg/ml, values which are roughly one-third to one-half of normal plasma cholesterol levels. Administration of hHDL increased the amount of progestin in the effluent by two- to threefold within 5 min after perfusate containing lipoprotein reached the organ, as compared with controls (Fig. 2). Steroid secretion remained elevated for 30–35 min, and then declined to pre-HDL infusion levels. In contrast to the situation for hHDL, where a statistically significant increment in progestin secretion was noted, infusion of hLDL to the ovaries of APP-treated rats did not significantly elevate the level of steroids in the effluent over the time periods we studied (Fig. 2). However, the mean values in the presence of LDL were greater than those for controls over the perfusion period.

The ability of the ovary in non-APP treated rats to respond to ovine LH administered after varying intervals of perfusion with KRB was also examined. When LH was infused at 50, 90, or 120 min after perfusion with KRB was begun, it brought about in each case an immediate and marked augmentation, from more than four- to tenfold, in steroid secretion (Fig. 3). As lipoproteins were not present in the perfusate, the steroids secreted were presumably from endogenous stores of cholesterol.

To determine whether cholesterol carried by the exogenous hHDL was being utilized as a substrate for steroidogenesis, some ovaries were infused with HDL reconstituted with [^{3}H]cholesteryl linoleate, and the effluent was analyzed for radioactive steroids. As shown in Table I, the effluent from ovaries perfused for 20 min with [^{3}H]cholesteryl linoleate-reconstituted HDL contained ^{3}H-labeled progestin. Moreover, the amount of [^{3}H]progestin in the outflow from the ovary decreased when the perfusate contained a 20-fold excess of unlabeled hHDL and ^{3}H-CE-HDL.

Uptake of ^{125}I-Human HDL by Perfused Ovaries

As shown in Fig. 4, the perfused ovaries accumulated ^{125}I-hHDL, with uptake reaching a maximum after 15 min of perfusion with label. The mean uptake of label by ovaries perfused for 15 min with ^{125}I-hHDL was 271,370 ± 42,243 cpm/ovary (mean ± SE, n = 7).

To determine whether uptake of ^{125}I-hHDL by the perfused ovary was specific, a 350-fold excess of either unlabeled human HDL or human LDL (0.87 mg of hHDL or hLDL protein/ml of perfusate) was administered to the ovary simultaneously with ^{125}I-hHDL. Specificity of accumulation was then evaluated after a 15-min perfusion with label, when lipoprotein uptake was maximal in our studies. When excess unlabeled hHDL was included in the perfusate, uptake of ^{125}I-hHDL was decreased by 70–95% (cpm/mg ovary, wet wt) (Figs. 4 and 5). In contrast, addition of an excess of unlabeled hLDL to the perfusate diminished uptake of ^{125}I-hHDL by only 32% (Fig. 5).

To establish the effect of temperature on the uptake of hHDL by the perfused ovary, we examined accumulation of ^{125}I-hHDL$_3$ at 37° and 4°C in APP-treated animals. Uptake at 37°C was carried out as described above. Uptake in the cold was accomplished by switching to perfusate maintained

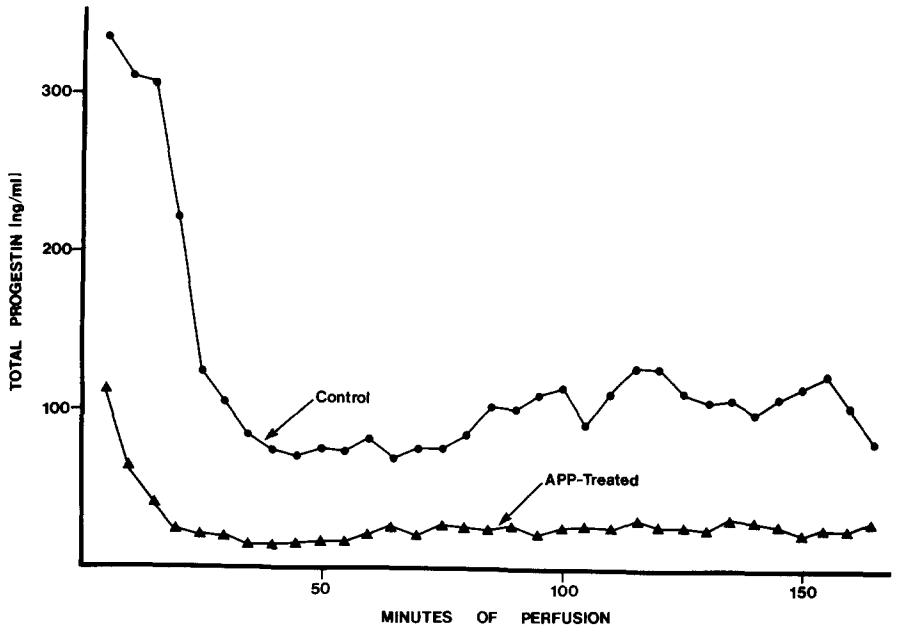

FIGURE 1 Steroid secretion by perfused ovaries of control (non-APP treated) and APP-treated rats. Ovaries were perfused for 165 min with KRB buffer and ovarian effluent was collected over 5-min intervals from control (n = 3) and APP-treated (n = 3) animals. Rats were treated with APP for the 3 d prior to the experiment, which was done on day 8 post-hCG injection. Progesterone and 20-α-hydroxypregn-4-en-3-one content of the effluent was determined and expressed as the sum of the two steroids.

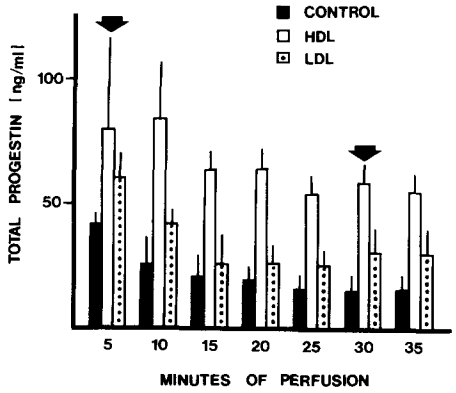

FIGURE 2 Effect of human HDL and human LDL on steroid secretion by perfused rat ovaries. Rats were treated with APP for three consecutive days before the experiment, which was done on day 8 post-hCG injection. Ovaries were perfused for 10 min with KRB buffer, for 30 min with buffer containing either hHDL (n = 3; 200 μg HDL cholesterol/ml of perfusate) or hLDL (n = 3; 200 μg of LDL cholesterol/ml of perfusate), and then with buffer. Ovaries of control rats (n = 3) were perfused with buffer alone. Ovarian effluent, collected over 5-min intervals, was assayed for progestin as described in the text. The arrow at the left indicates the initiation of lipoprotein infusion, that at the right the termination. Values presented are means ± SE. Analysis of variance reveals that there are differences among the three groups at 15–35 min of perfusion (F = 13.3, 7.0, 12.5, 19.3, and 22.1, respectively; df = 8). With the Newman-Keuls' test, statistical differences were located between the control and HDL-treated group at 15–35 min of perfusion ($P < 0.001$, except for 30 min, $P < 0.05$). Values for control and LDL-treated rats were not statistically different.

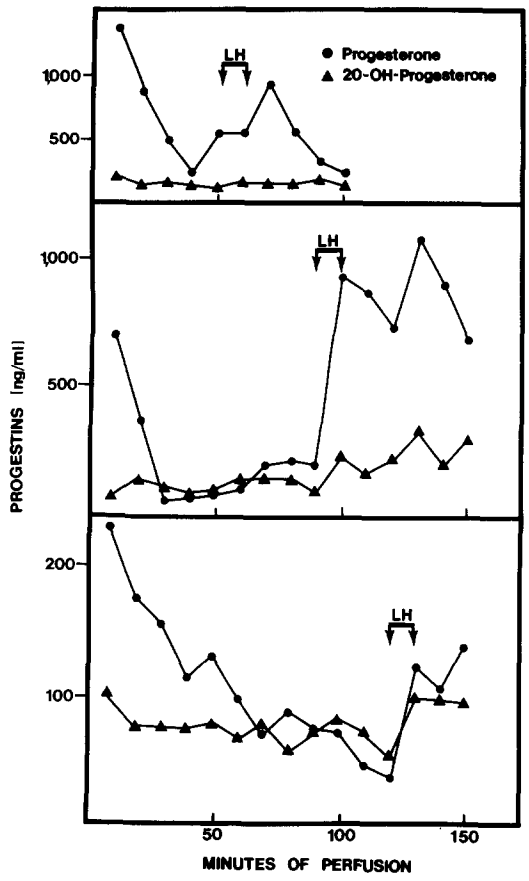

FIGURE 3 Effect of LH on steroid secretion by perfused rat ovaries. After 50 (*upper* panel), 90 (*middle* panel), or 120 (*lower* panel) min of perfusion with KRB buffer, ovine LH (100 ng/ml of perfusate) was infused into the ovaries for a period of 10 min (bar with arrows); this was followed by buffer alone. Ovarian effluent, collected over 10-min intervals, was analyzed for progestin content. Rats, treated for 3 d with APP prior to the experiment, were used on day 7 post-hCG injection. Each panel represents a separate animal.

at 4°C, and by surrounding the ovary with ice chips and flushing it with ice-cold saline. Perfusion of the ovary with ^{125}I-hHDL at 4°C reduced accumulation of the ligand by ~80% compared with ovaries perfused at 37°C. At 4°C, the ovary took up 425 ± 35 cpm/mg ovary (mean ± SE, n = 3), whereas at 37°C it accumulated 2274 ± 584 cpm/mg ovary (mean ± SE, n = 5).

Finally, the possibility that the ovary was selectively accumulating degraded materials, such as free or lipid-associated ^{125}I, was ruled out as follows. Ovaries from rats perfused at 37°C for 15 min with ^{125}I-hHDL$_3$ and washed for 3 min with KRB were homogenized and aliquots subjected to TCA precipitation and chloroform/methanol extraction. The amount

TABLE I

Metabolism of [1,2,6,7-^{3}H]Cholesteryl Linoleate Reconstituted into Human HDL by Perfused Rat Ovaries

Treatment	Perfusion No.	Ovarian wt	Radioactivity in venous effluent	Radioactivity in progesterone and 20-α-hydroxypregn-4-en-3-one	Conversion
		mg	*dpm × 10^{-6}*	*dpm × 10^{-4}*	%
Reconstituted ^{3}H-HDL	1	91.8	7.14	19.4	2.72
	2	75.5	3.86	17.49	4.53
	3	62.2	0.70	3.53	5.02
	4	104.1	0.55	2.69	4.62
	5	55.6	9.87	10.91	1.11
Reconstituted ^{3}H-HDL and "cold" HDL	6	101.3	7.77	8.51	1.09

Animals were pretreated with 4-APP for 3 d prior to the experiment, which was performed on day 6 post-hCG treatment. The ovaries were first perfused with KRB for 30 min and then for a 20-min period with KRB containing 18 μg/ml (9.6 × 10^5 dpm) reconstituted HDL (1, 2, 5, and 6), 21 μg/ml (0.95 × 10^5 dpm) reconstituted HDL (3 and 4), or reconstituted HDL and 333 μg/ml of unlabeled HDL (5 and 6). The ovarian effluent was collected for analysis of radioactive products as described in the text.

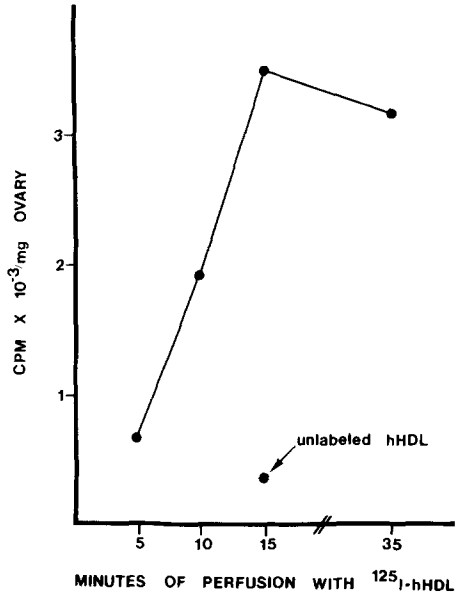

FIGURE 4 Time course of accumulation of ^{125}I-hHDL$_3$ by perfused rat ovaries. Rats (not treated with APP) were used on day 6 post-hCG injection. Ovaries were perfused briefly with KRB buffer to flush out the blood, then for 5–35 min with ^{125}I-hHDL$_3$ (10^6 cpm/ml of perfusate; specific activity, 400 cpm/ng of HDL protein), after which they were perfused for 3 min with buffer to remove unbound label. Ovaries were excised and counted. Each point represents a separate animal. This experiment was repeated twice with similar results.

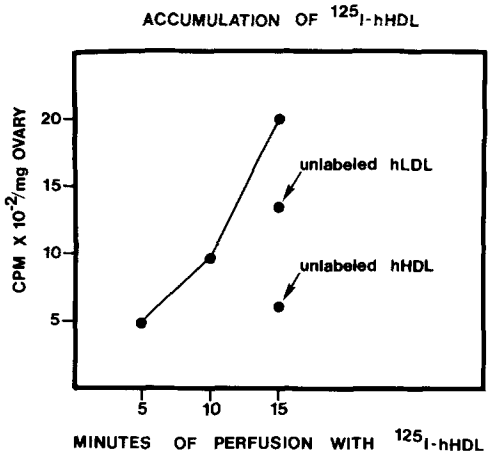

FIGURE 5 Specificity of lipoprotein uptake by perfused rat ovaries. Rats (not treated with APP) were used on day 6 post-hCG injection. After a brief rinse with KRB buffer, ovaries were perfused for 15 min with ^{125}I-hHDL$_3$ (10^6 cpm/ml of perfusate) with or without a 350-fold excess (0.87 mg/ml of perfusate) of either unlabeled hHDL or unlabeled hLDL. They were then perfused for 3 min with buffer alone to remove unbound label and handled as described in the legend to Fig. 4. Each point represents a separate animal. Similar results were obtained on three separate occasions for HDL and on two separate occasions for LDL.

of radioactivity in the TCA-soluble and organic phases was determined. TCA precipitated ~95% of the radioactivity in these ovaries and chloroform/methanol extracted <2% of the label (n = 5).

Effect of APP Treatment on Uptake of ^{125}I-hHDL by Perfused Ovaries

In our initial studies, uptake of ^{125}I-hHDL$_3$ was high in ovaries of rats that were primed with pregnant mares serum gonadotropin-hCG but that received no further treatment. However, subsequent studies indicated that the amount of uptake, although following the same pattern, was variable in these animals, with some ovaries accumulating <350 cpm/mg ovary. Our preliminary morphological studies suggested that ^{125}I-hHDL$_3$ uptake should be in excess of 500–1,000 cpm/mg tissue in order to carry out autoradiography successfully within a reasonable amount of time. We therefore examined uptake of ^{125}I-hHDL in rats that had been treated with APP for 3 d before use.

We found that perfusion of ^{125}I-hHDL$_3$ to ovaries of APP-treated rats resulted in at least a 2-fold increase in uptake of label compared to rats receiving gonadotropins alone. Uptake of label in APP-treated animals was rarely ≲1,000 cpm/mg ovary and reached as high as 6,000 cpm/mg ovary.

Pulse-Wash Studies with ^{125}I-hHDL

For these studies, ovaries of rats, either with or without APP treatment, were perfused first for 15 min with ^{125}I-hHDL, then for 3–150 min with KRB lacking both label and lipoproteins. While the pattern of uptake was similar in both non-APP treated and APP-treated rats, in the latter case the curve was shifted to the left (Figs. 6 and 7). The amount of label accumulated initially was high and more or less constant on a per milligram ovary basis for up to 15 min of wash for non-

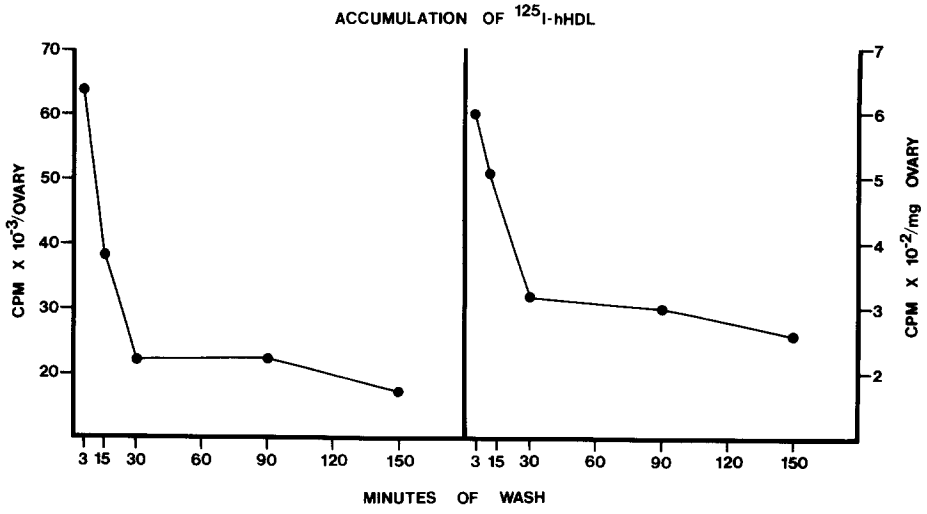

FIGURE 6 Pulse-wash studies in non-APP treated rats. Rats were used on day 7 post-hCG injection. Ovaries were perfused briefly with KRB buffer, "pulsed" for 15 min with ^{125}I-hHDL$_3$ (10^6 cpm/ml of perfusate) and then "washed" for 3–150 min with buffer alone. After removal, ovaries were counted. Each point represents a separate animal. This experiment was repeated twice with similar results. There was no APP treatment.

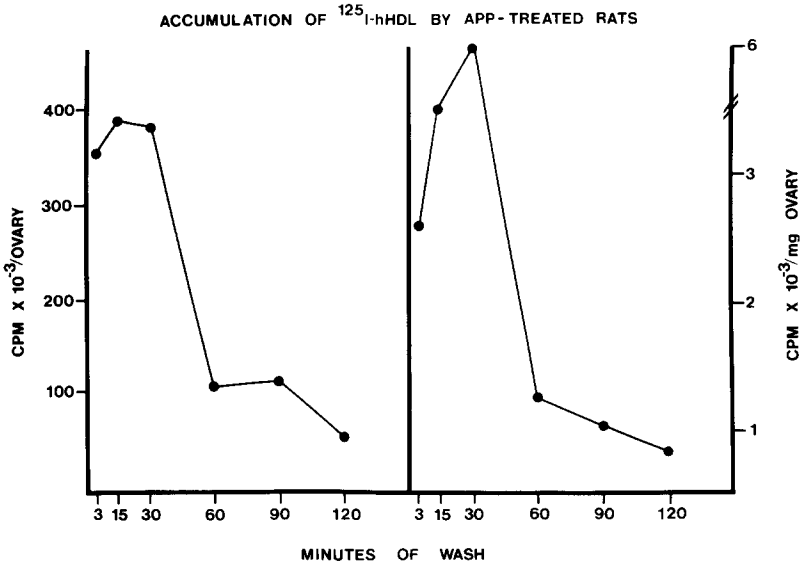

FIGURE 7 Pulse-wash studies in APP-treated rats. Rats were treated with APP for 3 d prior to the experiment, which was performed on day 7 post-hCG injection. The perfusion sequence and processing of ovaries was performed as described in the legend to Fig. 6, except that after labeling with ^{125}I-hHDL$_3$ ovaries were washed for 3–120 min with KRB buffer. Each point represents a separate animal.

APP treated rats and up to 30 min for APP-treated animals. By 30 (non-APP treated rats) or 60 (APP-treated rats) min of postlabel perfusion, the amount of radioactivity within the ovary declined by ~75%, and remained at the same level for the duration of the infusion period.

Autoradiographic Localization of ^{125}I-labeled Lipoproteins and Albumin

The compartments accumulating lipoprotein were localized in paraffin sections of immersion-fixed tissue by light microscope autoradiography after perfusion of the ovary for 15 min with ^{125}I-hHDL$_3$ and a brief buffer wash. In this material, silver grains were numerous over corpora lutea, but sparse over follicles, stroma, and ova (Fig. 8*a*). When excess unlabeled hHDL was perfused to the ovary along with ^{125}I-hHDL$_3$, the number of silver grains over corpora lutea was markedly reduced (Fig. 8*b*). In contrast, the level of labeling in this compartment was not appreciably altered when "cold" hLDL was infused to the ovary simultaneously with ^{125}I-hHDL$_3$ (Fig. 8*c*).

To identify the specific types of ovarian cells accumulating ^{125}I-hHDL$_3$, uptake of this lipoprotein was also studied in plastic sections of ovaries fixed in situ by vascular perfusion. The quality of morphological preservation in these preparations was superior to that of tissue fixed by immersion. Moreover, blood was washed out of the vascular bed and there was a slight shrinkage of the cells. As a result, the boundaries of adjacent cells were discernible, allowing the cells taking up ^{125}I-hHDL to be recognized easily.

After perfusing for 15 min with ^{125}I-hHDL$_3$ and washing for 3 min to remove unbound label, it was clear that in such preparations most silver grains were concentrated over luteal cells (Fig. 9). Silver grains were associated with the cell surfaces and also occurred over their cytoplasm. Silver grains were especially abundant along those surfaces of luteal cells that faced capillaries (Fig. 10*a*), although they were also observed at interfaces between adjacent luteal cells (Fig. 10*b*). In ~20–25% of the luteal cells in a given field, silver grains were near the nucleus (Fig. 10*c*). In all cases, a variable number of grains was clustered in or near blood vessels.

Silver grains occurred in locations similar to those just described after being washed with lipoprotein-free buffer for 15 (non-APP treated rats) and 30 (APP-treated rats) min (Fig. 11*a*). However, in ovaries "washed" for longer periods with buffer (i.e., 30–150 min for non-APP treated rats and 60–120 min for APP-treated rats) the number of grains over luteal cells was drastically reduced (Fig. 11*b*). The concentration of silver grains in or near blood vessels appeared unchanged as compared with that observed at shorter wash times.

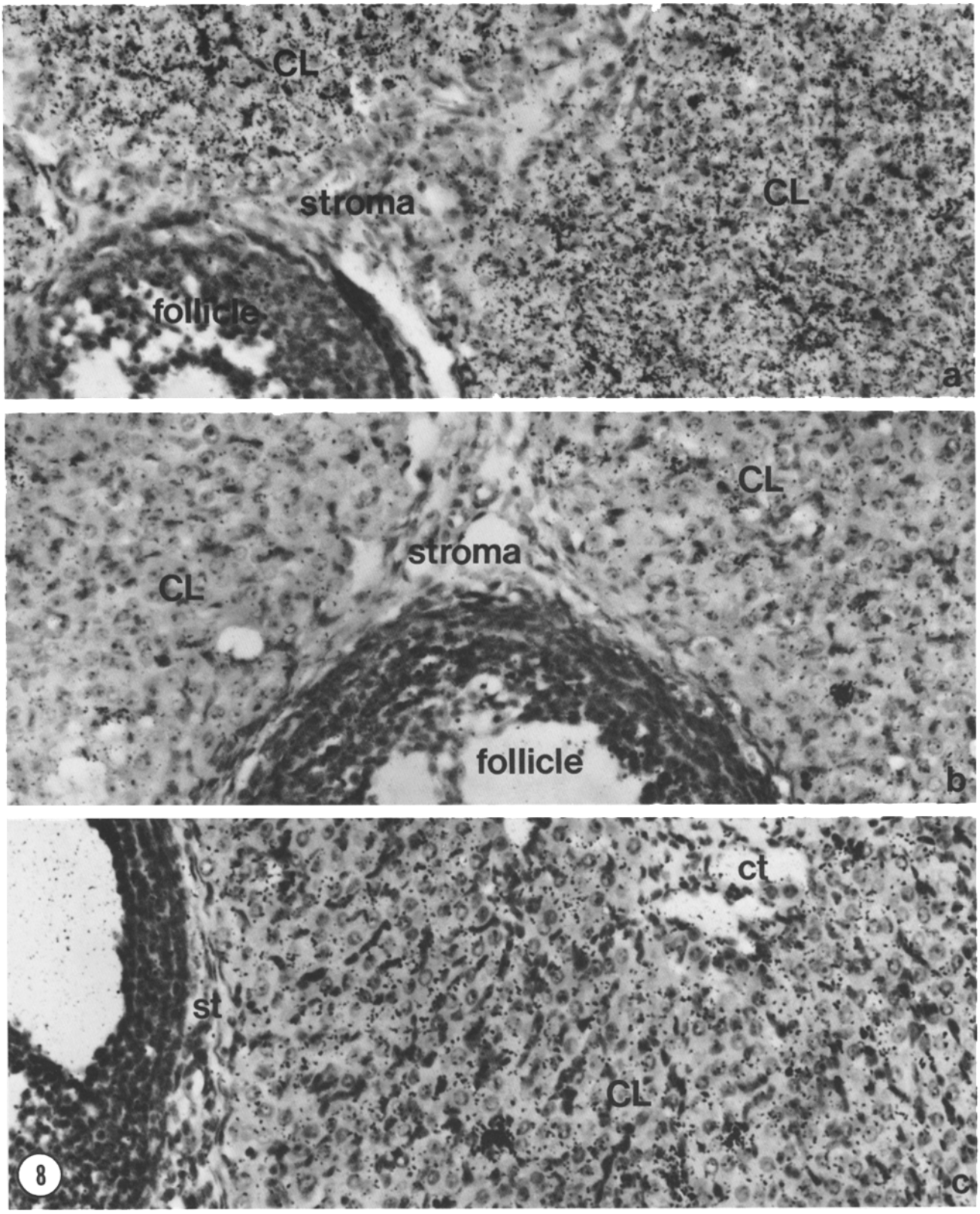

FIGURE 8 Light microscope autoradiographs of immersion-fixed, paraffin-embedded ovaries perfused with ^{125}I-human HDL_3. Ovaries were perfused briefly with lipoprotein-free KRB buffer, then for 15 min with ^{125}I-$hHDL_3$ (10^6 cpm/ml of perfusate; specific activity, 400 cpm/ng of HDL protein) with or without a 350-fold excess (0.87 mg/ml of perfusate) of unlabeled hHDL or hLDL, followed by a brief buffer wash to remove unbound label. (*a*) Silver grains (small black dots) were numerous over corpora lutea, but sparse over follicle and stromal cells in ovaries perfused with ^{125}I-$hHDL_3$. (*b*) In contrast, few silver grains occurred over corpora lutea (*CL*) of ovaries perfused simultaneously with ^{125}I-$hHDL_3$ and excess unlabeled hHDL. (c) Inclusion of excess unlabeled hLDL in the perfusate with ^{125}I-$hHDL_3$ did not markedly alter the number of silver grains over corpora lutea. *CL*, corpus luteum; *ct*, connective tissue at center of corpus luteum; *st*, stroma. All, no APP treatment; day 6 post-hCG injection; 14-d autoradiographic exposure; × 600.

As shown for immersion-fixed, paraffin-embedded tissue, the concentration of grains associated with luteal cells in plastic sections of perfusion-fixed material decreased when excess unlabeled hHDL was added to the perfusate along with ^{125}I-hHDL$_3$ (Fig. 10, *d* and *e*). There was a similar paucity of silver grains over corpora lutea when ^{125}I-hHDL$_3$ was perfused to ovaries prechilled to 4°C (Fig. 10*f*). These comparisons of relative grain concentrations were made among tissue sections exposed in the dark for similar amounts of time.

Some ovaries were perfused with ^{125}I-rat HDL to rule out the possibility that heterologous (human) lipoprotein might have a distribution among various ovarian compartments or cell types different from that for homologous (rat) lipoproteins. As shown in Fig. 11, *c* and *d*, silver grains were numer-

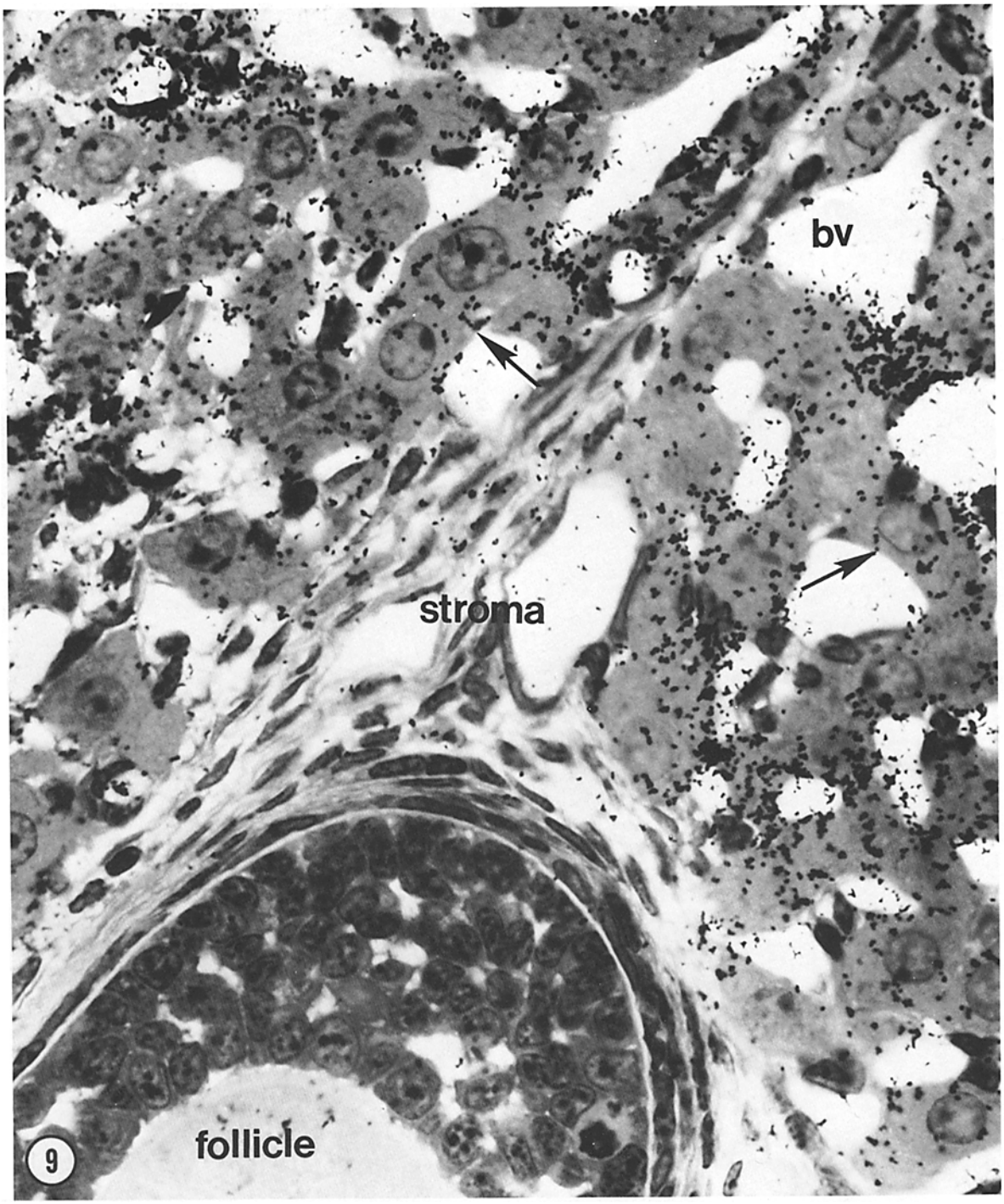

FIGURE 9 Localization of hHDL$_3$ in light microscope autoradiographs of perfusion-fixed, glycolmethacrylate-embedded ovary. After a brief perfusion with buffer, the ovary was infused with ^{125}I-hHDL$_3$ (10^6 cpm/ml of perfusate) for 15 min and washed for 3 min with buffer, after which it was perfused-fixed in situ with FGC-cacodylate. The quality of morphological preservation in these preparations was markedly improved compared with ovaries fixed by immersion. As a result, it was clear that silver grains were primarily associated with luteal cells (arrows); few grains were observed over cells of follicles or stroma. Luteal cells can be identified by their abundant cytoplasm and large, round nuclei which often contain prominent nucleoli. The small, dark irregularly shaped nuclei belong primarily to endothelial cells. Blood cells were washed out of the vascular bed by the perfusion (*bv*, blood vessel). APP-treated; day 7 post-hCG injection; 6-wk autoradiographic exposure; × 1,100.

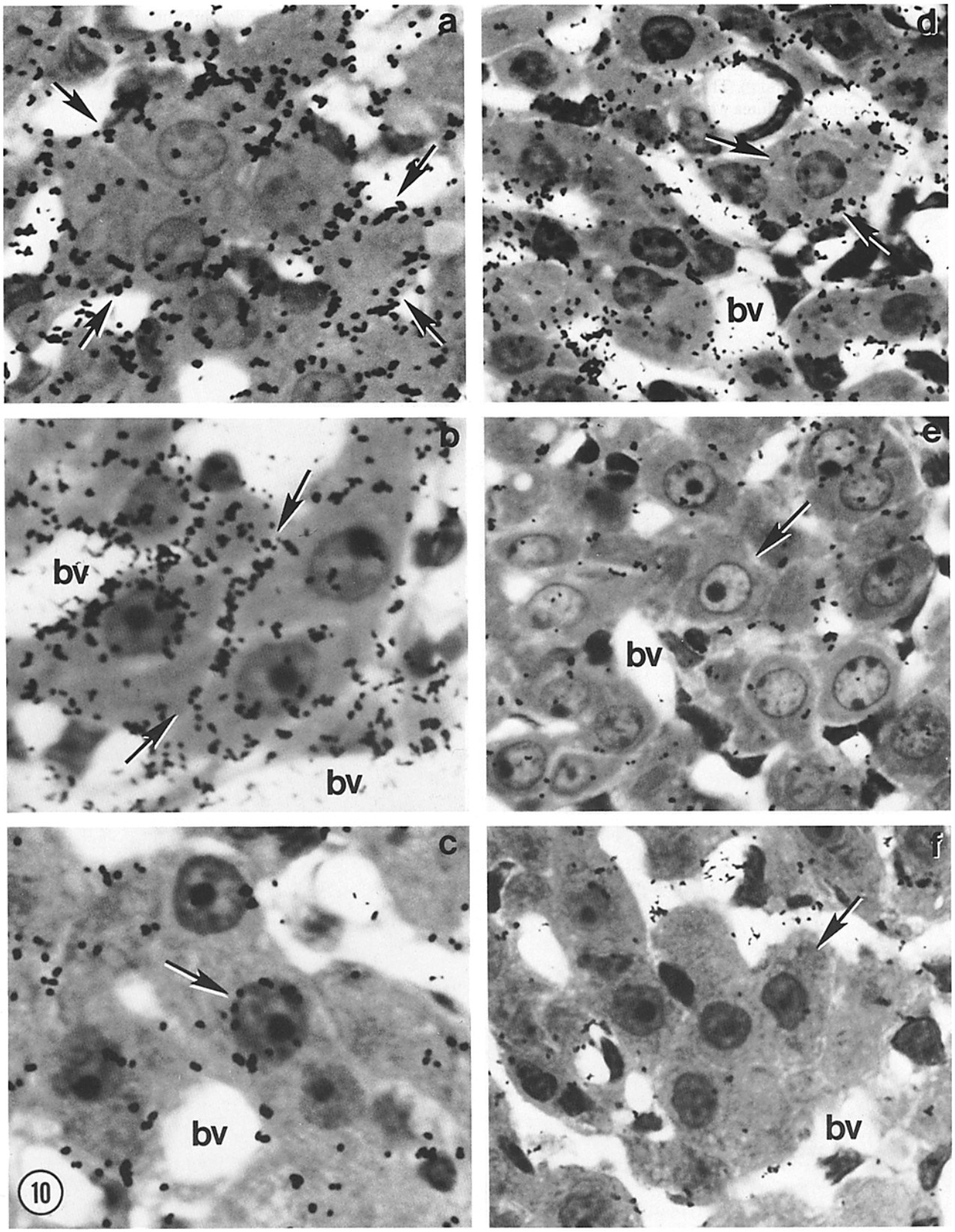

FIGURE 10 Light microscopic autoradiographs of ovaries perfused with ^{125}I-hHDL$_3$. (*a–c*) Detail of ^{125}I-hHDL$_3$ distribution in corpora lutea. The experiment was done as described in the legend to Fig. 9. Silver grains were especially numerous along those luteal cell surfaces facing the vascular bed (*a*, arrows), although some were also observed at interfaces between adjacent luteal cells (*b*, arrows). Silver grains were frequently near the nucleus, often encircling it (*c*, arrow). All, × 1,800. (*d–f*) Competition control and effect of temperature on ^{125}I-hHDL$_3$ uptake. At 37°C, ovaries perfused with ^{125}I-hHDL$_3$ (10^6 cpm/ml of perfusate) showed numerous silver grains which occurred over the cytoplasm and along the surfaces of luteal cells (*d*, arrows). In contrast, in autoradiographs of ovaries perfused with either ^{125}I-hHDL$_3$ and an excess of unlabeled hHDL at 37°C (*e*, arrow) or ^{125}I-hHDL at 4°C (*f*, arrow), few silver grains were over luteal cells. Blood vessels, *bv*. All, × 1,200. *a*, *b*, and *d–f*: APP-treated; day 7 post-hCG injection; perfusion-fixation; 6-wk autoradiographic exposure. *c*: No APP treatment; day 6 post-hCG injection; perfusion-fixation; 7-mo autoradiographic exposure.

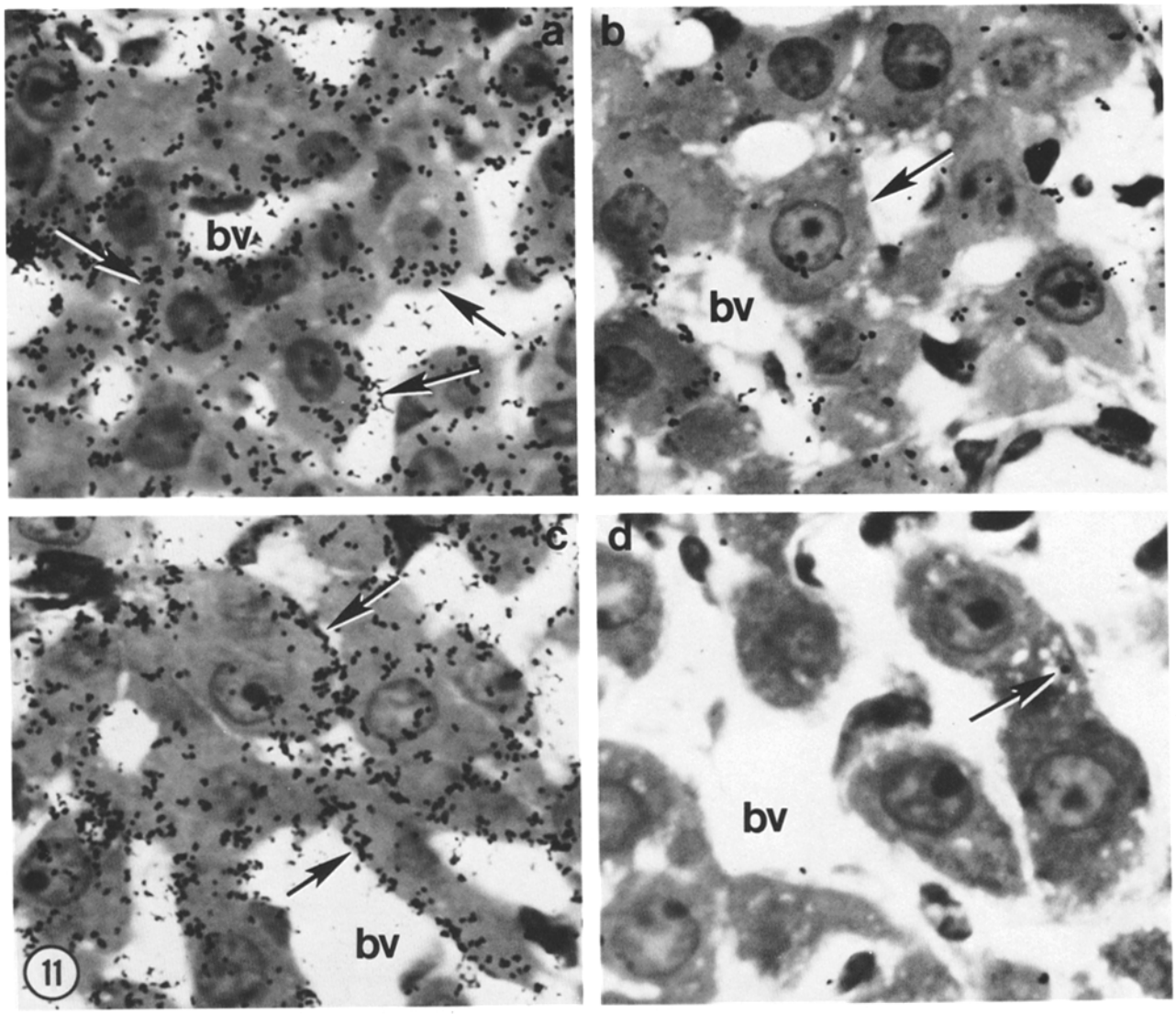

FIGURE 11 (*a* and *b*) Uptake of ^{125}I-hHDL$_3$, pulse-wash studies. Silver grain distribution in ovaries perfused for 15 min with ^{125}I-hHDL$_3$ (10^6 cpm/ml of perfusate) and washed for up to 15 (non-APP treated rats) or 30 (APP-treated rats) min was the same as that in ovaries washed for 3 min (compare with Fig. 9); i.e., grains were concentrated over luteal cells (*a*, arrows). However, after longer washes the number of grains associated with luteal cells was markedly reduced (*b*, arrow; 120 min of wash). Blood vessels, *bv*. Both, APP-treated; day 7 post-hCG injection; 21-d autoradiographic exposure; × 1,200. (*c* and *d*) Uptake of ^{125}I-rat HDL by perfused rat ovaries. In autoradiographs of ovaries perfused for 15 min with homologous lipoprotein (^{125}I-rat HDL; 10^6 cpm/ml of perfusate; specific activity, 400 cpm/ng of HDL protein) and washed for 3 min, silver grains were numerous along luteal cell surfaces and over their cytoplasm (*c*, arrows). In ovaries washed for longer periods after labeling, grains were sparse over luteal cells (*d*, arrow indicates a silver grain over a luteal cell). Blood vessels, *bv*. Both, APP-treated; day 7 post-hCG injection; 14-d autoradiographic exposure. c, × 1,200; d, × 1,800.

ous over luteal cells at 3 min of wash, whereas at 90 min of wash few grains occurred over luteal cells. At the later wash period, most of the grains were associated with blood vessels.

Finally, we examined the uptake of ^{125}I-BSA by the perfused ovary and compared its distribution with that of ^{125}I-HDL (rat and human), since albumin may be present as a contaminant in some lipoprotein preparations. In each of three animals, the results were identical. After perfusion for 15 min with ^{125}I-BSA followed by a 3-min buffer wash, silver grains were concentrated over blood vessels and over cells that resembled macrophages (Fig. 12) but were sparse over luteal cells. This pattern of grain distribution contrasted markedly with that of ^{125}I-human or rat HDL.

An examination of silver grain distribution in electron micrographs confirmed that luteal cells, identified by their characteristic fine structure, were the predominant type of cell that accumulated ^{125}I-hHDL$_3$. In electron microscope autoradiographs, as described above for the light microscope autoradiographic studies, silver grains were more abundant after shorter periods of wash (3–30 min) than after longer periods (60–120 min). The grain distribution over labeled luteal cells was quantified after various periods of wash as described above. After shorter periods of wash (e.g., 3–30 min), 65% of 236 grains in 48 cells were associated with the plasma membrane (i.e., on or within 250 nm of this structure), with 35% over the interior of the luteal cell. Of the plasma membrane grains, >50% were localized over processes, i.e., thin cytoplasm-filled extensions of the plasma membrane (Fig. 13*a*). It appeared that most of the plasma membrane grains were associated with regions of the plasmalemma that lacked a

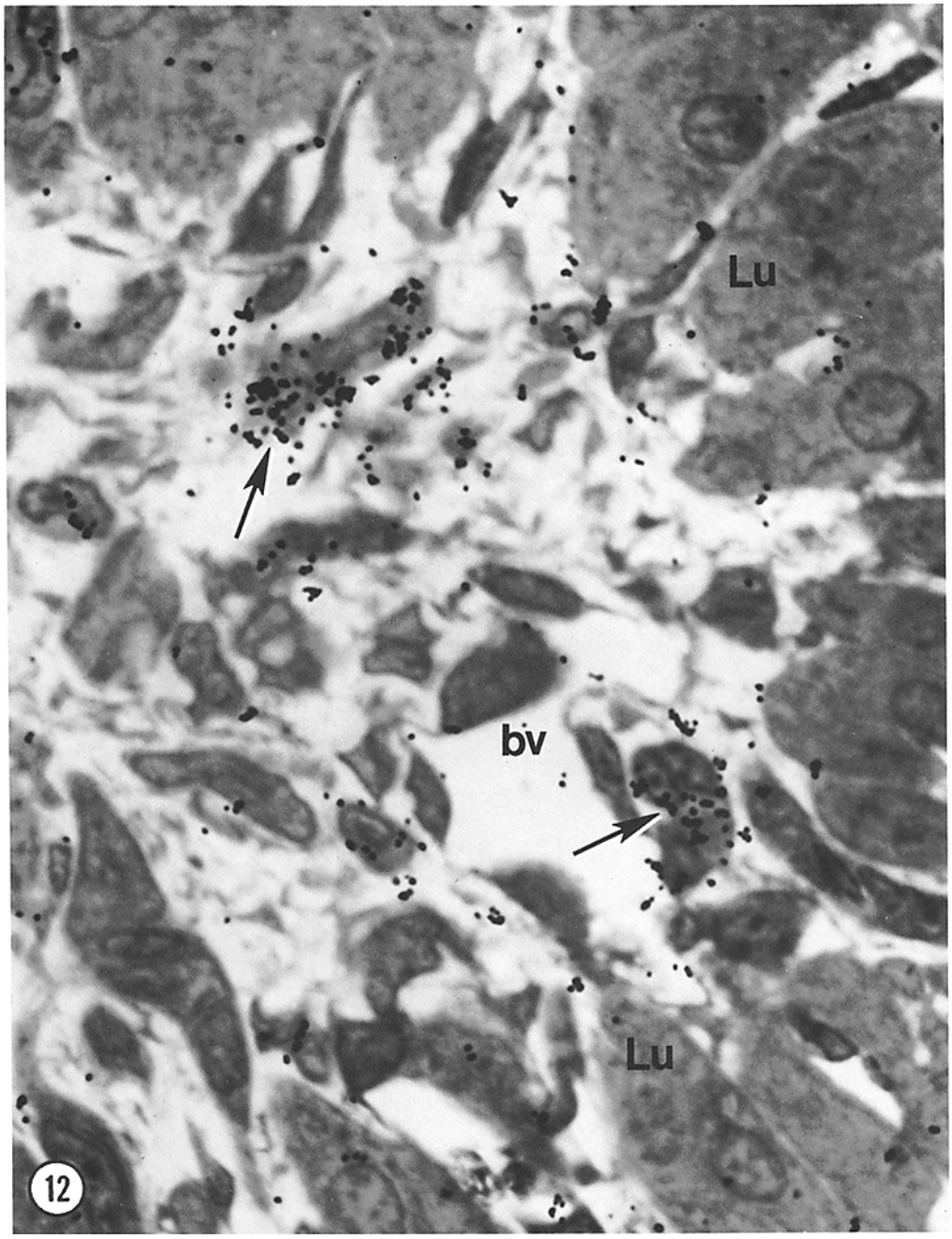

FIGURE 12 Light microscope autoradiograph showing localization of ^{125}I-bovine serum albumin. This field from a perfusion-fixed ovary contains several luteal cells and a portion of the connective tissue filling the center of the corpus luteum. After perfusion for 15 min with ^{125}I-BSA (10^6 cpm/ml of perfusate; specific activity, 300 cpm/ng protein) and a brief buffer wash, few silver grains were associated with luteal cells (*Lu*). Instead, they were heavily concentrated over cells that resembled macrophages (arrows). Blood vessel, *bv*. Compare with Figs. 9 and 10 *c*. APP-treated; day 6 post-hCG injection; 1-mo autoradiographic exposure; × 1,500.

morphologically identifiable bristle coat (Fig. 13*b*). After longer wash periods there appeared to be a shift in silver grain distribution. By 90 min of wash, the percentage of grains along the plasma membrane had declined slightly, to 51%, with a corresponding rise in that over the cytoplasm, to 49% (118 grains in 31 cells). A further decrease in the percentage of grains over the plasma membrane, to 34%, was observed at 120 min of wash; at this time 66% of 167 grains in 30 cells were associated with luteal cell cytoplasm (Fig. 13*c*).

DISCUSSION

Our results indicated that the in situ perfused ovary of the gonadotropin-primed immature rat is a suitable model for the study of lipoprotein metabolism. The ovary remains functional during the periods of perfusion used, since it remains able to secrete steroid hormones and to respond to trophic stimuli and lipoproteins. In APP-treated rats, the addition of $hHDL_3$ to the perfusate brought about a stimulation of progestin secretion by the ovary, raising it two- to threefold above that for ovaries not receiving lipoproteins. This response occurred even though the levels of $hHDL_3$ used supplied cholesterol at concentrations only one-third to one-half of the normal circulating values for this sterol. It is unlikely that the elevated levels of progestin were merely a consequence of heightened secretion. After perfusion of the ovary with HDL reconstituted with [^{3}H]cholesteryl linoleate, tritium-labeled progestin appeared in the effluent, indicating that sterol carried by HDL was converted to steroid. Infusion of LH also produced a vigorous secretory response in non-APP treated rats, even when this gonadotropin was delivered to the ovary 120 min after the perfusion was initiated.

Our studies further demonstrated that perfused ovaries take

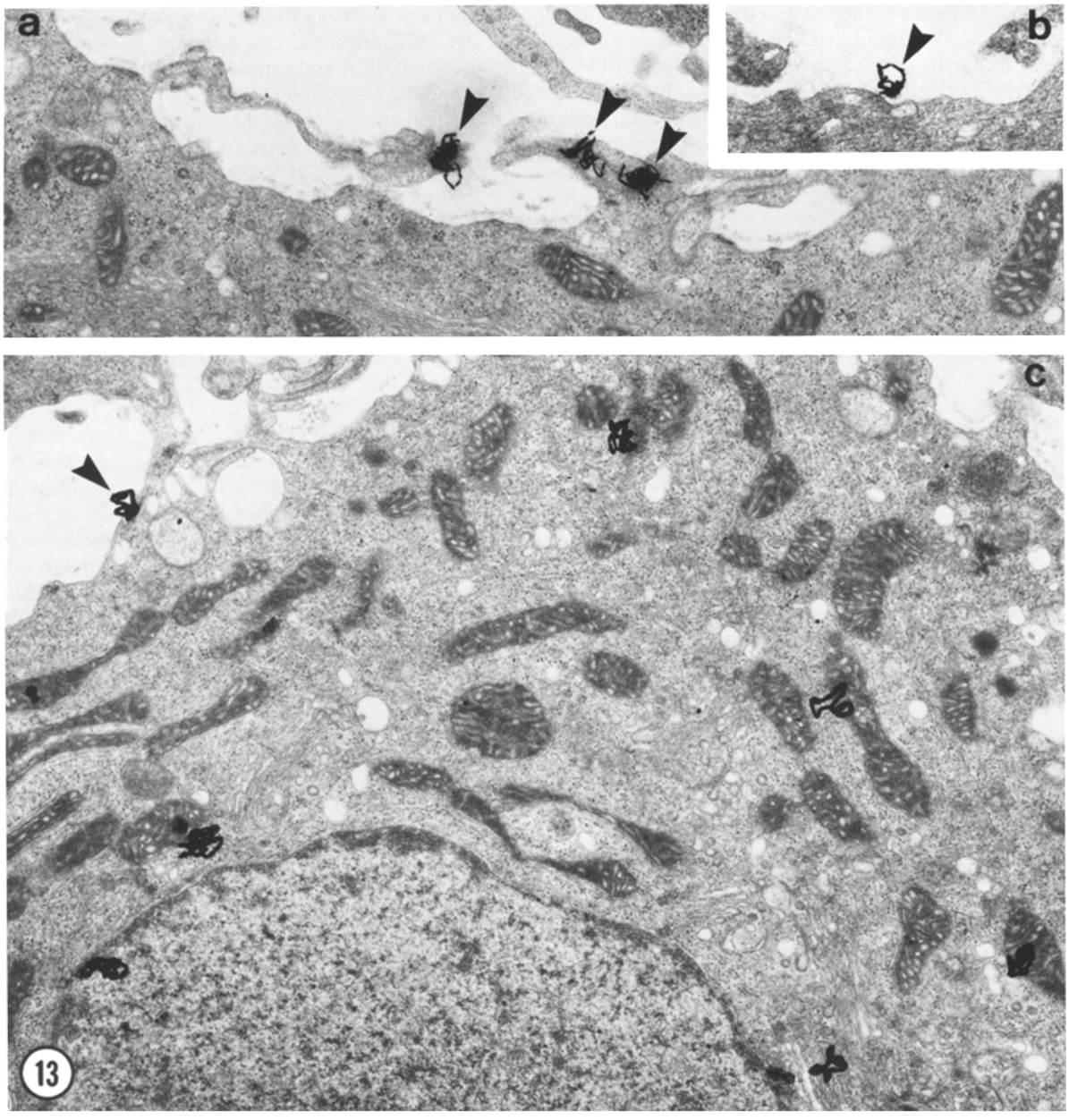

FIGURE 13 Electron microscope autoradiographs of rat ovaries perfused with ^{125}I-hHDL$_3$. After 15-min perfusion with ^{125}I-hHDL$_3$ (10^6 cpm/ml of perfusate) and a brief buffer wash, silver grains occurred primarily along the surfaces of luteal cells, often over villus-like processes of the plasma membrane (*a*, arrowheads). Most of these grains seemed to be associated with segments of the plasmalemma that lacked a bristle coat (*b*, arrowhead). After longer wash periods, an increased number of grains were observed over the cytoplasm of luteal cells (*c*) and fewer grains were observed at the cell surface (arrowhead). All, APP-treated; day 7 post-hCG injection; perfusion-fixation; 5-mo autoradiograpic exposure. *a*, × 19,100; *b*, × 18,900; *c*, × 18,800.

up ^{125}I-labeled human HDL$_3$ by a process that is temperature dependent and lipoprotein specific. At 37°C, accumulation of ^{125}I-hHDL$_3$ by the ovary reached a maximum after 15 min of perfusion with label. Thus, the time course for uptake of radiolabeled hHDL by the perfused ovary was remarkably similar to that for both dispersed ovarian cells and ovaries in vivo (30, 33). When binding was carried out in the cold, however, uptake of ^{125}I-hHDL$_3$ by the perfused ovary was reduced by 80%. The inclusion of excess unlabeled hHDL in the perfusate markedly suppressed the accumulation of ^{125}I-hHDL$_3$, suggesting that the uptake of labeled ligand by the perfused ovary involves a finite number of binding sites. We also found that unlabeled hLDL competed less effectively for ^{125}I-hHDL$_3$ uptake than did unlabeled hHDL, indicating a lipoprotein specificity to the uptake process. Taken together, these data suggest that the accumulation of ^{125}I-hHDL$_3$ occurred at the physiologic binding site, presumably the HDL receptor.

Treatment of gonadotropin-primed immature rats with APP reduced plasma cholesterol concentrations from 50 mg/dl to <15 mg/dl. In these animals, uptake of ^{125}I-hHDL$_3$ by perfused ovaries was severalfold greater than that for ovaries of rats not treated with APP. This finding is presumably due to the reduced occupancy of HDL receptors by endogenous lipoproteins and, perhaps, to an increase in HDL binding sites as a consequence of lowered plasma cholesterol levels.

We identified corpora lutea as the ovarian compartment which most actively accumulates ^{125}I-hHDL$_3$. These observations constitute the first morphological localization of HDL accumulation in intact steroid secreting tissue. By using ^{125}I-human HDL essentially free of apolipoprotein E as our probe, we insured that only the binding sites for HDL were visualized, and not those for LDL. Moreover, the lipoprotein-specific nature of our autoradiographic localization was verified by competition controls in which ovaries were perfused simultaneously with ^{125}I-hHDL$_3$ and an excess of unlabeled hHDL or hLDL. In addition, both ^{125}I-rat HDL and ^{125}I-human HDL labeled autoradiographically the same compartments in the perfused ovary. Thus, we can assume that the use of a heterologous lipoprotein as a probe did not give rise to an abnormal pattern of binding among ovarian structures. Finally, by perfusing some ovaries with ^{125}I-BSA, we ruled out the possibility that albumin, a contaminant of some lipoprotein preparations, was localized instead of HDL.

Besides localizing ^{125}I-hHDL$_3$ to corpora lutea, light and electron microscope autoradiography have pinpointed luteal cells as the main type of cell that accumulates this lipoprotein. Schuler et al. (30) suggested, in an earlier study of dispersed cells, that HDL was taken up by ovarian parenchymal cells. However, the precise identity of the cells was not established in that work. In discussing the possible mechanisms involved in the metabolism of HDL by adrenal gland in vivo, Kovanen et al. (19) pointed out that binding sites for HDL need not reside on adrenocortical parenchymal cells but could occur on other nearby cells, such as capillary endothelial cells. Our findings, however, appear to rule out this possibility as a major mechanism by which sterol is delivered to luteal cells. On the contrary, they imply that luteal cells are directly responsible for obtaining the substrate they use in the synthesis of steroids.

Our light microscopic autoradiographic observations suggested that ^{125}I-hHDL$_3$ accumulates at the surfaces of luteal cells. Uptake was especially heavy on those surfaces that faced the vascular spaces. However, grains were also observed along the interfaces between adjacent luteal cells, indicating that binding sites for the lipoprotein were not restricted to the perivascular surfaces of luteal cells. These impressions were confirmed by electron microscope autoradiography, which clearly demonstrated that binding sites for ^{125}I-hHDL$_3$ were associated with the plasma membranes of luteal cells. These findings support the suggestion, largely from biochemical studies, that the surfaces of steroid-secreting cells bear specific binding sites for HDL. For example, Christie et al. (10) have reported that uptake of ^{125}I-hHDL is greatest in that fraction of rat ovaries containing the highest 5′-nucleotidase activity, an enzyme considered to be a marker for plasma membrane. Of the silver grains we found associated with luteal cell plasma membranes, 50% occurred over processes, slender cytoplasm-filled projections of the plasmalemma. Receptors for ^{125}I-hCG are also particularly numerous along villous processes of rat luteal cells (13, 22). The majority of the binding sites for ^{125}I-hHDL$_3$, whether on processes or not, do not appear to be concentrated over coated pits or over linear bristle-coated segments of the plasma membrane. On the other hand, LDL receptors are associated with such specializations in both human fibroblasts (4) and rat granulosa cells in vitro (L. G. Paavola and J. F. Strauss, unpublished observations). Thus, HDL binding sites of rat luteal cells appear to differ from the classical LDL receptors in several ways, including their biochemical properties and location along the plasma membrane.

An intriguing observation in the current study was the presence of silver grains near the nucleus. On the basis of cell size and the resolution of autoradiography with ^{125}I, it seems likely that these perinuclear grains arose from a radioactive source within the cell, rather than from ^{125}I-hHDL$_3$ associated with the cell surface. It is therefore tempting to speculate that grains in this location reflect possible internalization of all or part of the HDL particle. Other data also suggest that at least some HDL apolipoprotein may be internalized by luteal cells. Schuler et al. (30), who noted that after brief exposure of dispersed ovarian cells to proteases only part of the cell-associated ^{125}I-HDL was released, have suggested that the fraction of radioactivity not susceptible to enzymic degradation may represent internalized HDL.

Even though rat ovaries clearly use HDL as a source of sterol, the mechanisms involved in liberating cholesterol from HDL particles remain vague. Several suggestions have been made, including the facilitation of cholesterol uptake from HDL by plasma membrane lipases (17). It is also possible that, after interaction with the plasma membrane, HDL is internalized and processed in a manner similar to that described for LDL (6). Although our observations and those of others suggest that internalization of HDL may occur, additional work is necessary to clarify the relationship, if any, between ingestion of HDL and release of sterol to the cell.

After a 15-min pulse with ^{125}I-hHDL$_3$ and a 30- (non-APP treated rats) or 60- (APP-treated rats) min wash with buffer lacking label, the amount of radioactivity associated with the ovary declined by ~75% when compared with that present in this organ at shorter periods of wash, and then remained more or less constant throughout the rest of the wash. Autoradiographs of this material revealed that there was a striking decrease in the number of silver grains associated with luteal cells as wash time was increased; the grains that remained were in a perivascular location. The basis for the decrease in the level of radioactivity in the ovary, specifically the luteal cells, after longer wash periods cannot be established from the current data. However, it may reflect, among other things, (*a*) dissociation of intact ^{125}I-hHDL$_3$ from the cell surface to the vascular bed, (*b*) internalization and degradation of ^{125}I-hHDL$_3$, or (*c*) both. The grains localized in perivascular areas may represent accumulation of ^{125}I-hHDL$_3$ or products of its degradation by macrophages.

Steroid-secreting tissues of several species possess LDL receptors and utilize LDL as a source of cholesterol for steroid hormone synthesis (7, 10, 29). In the case of rats, biochemical data indicate that ovarian granulosa cells, when in vitro, can use LDL as a sterol source (27). Morphological studies on the uptake of gold-labeled hLDL by rat granulosa cells in culture suggest that this lipoprotein interacts with the plasma membrane and is internalized by these cells (L. G. Paavola and J. F. Strauss, unpublished observations). However, unlike the situation for a number of species, including humans, where LDL is the main cholesterol-carrying lipoprotein, HDL is the principal sterol-transporting lipoprotein in rats (12). Indeed, rodents seem to be somewhat unusual in that their steroidogenic tissues can use both LDL and HDL (3, 12). Nevertheless, since HDL appears to be the preferred cholesterol carrier in vivo for rats, we have dealt specifically with the mechanisms of HDL uptake and have not addressed the issue of LDL receptors in the in situ perfused ovary.

In conclusion, the data presented here provide evidence

that ^{125}I-hHDL$_3$ is taken up by in situ perfused ovaries of rats, and that this accumulation involves a specific interaction between the lipoprotein and a binding site located on the plasma membrane of luteal cells. We have alluded to the possibility that HDL or some portion of it may be internalized by luteal cells. Studies in progress are aimed at providing further information on how the ovary processes HDL at the cellular level.

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