Biochem. J. (1992) 287, 841–848 (Printed in Great Britain)



# Characterization and isolation of a high-density-lipoprotein-binding protein from bovine corpus luteum plasma membrane

Kevin FERRERI and K. M. Jairam MENON*
Departments of Obstetrics and Gynecology and Biological Chemistry, University of Michigan, Ann Arbor, MI 48109-0278, U.S.A.

The ovary uses the cholesterol from high-density lipoproteins (HDL) as a substrate source for steroid hormone production. It is not clear, however, how ovarian cells acquire the lipoprotein cholesterol. This study describes the characterization and isolation of a high-affinity-binding protein for apolipoprotein E-free HDL from the plasma-membrane fraction of bovine corpora lutea. Plasma membranes were prepared by differential centrifugation with 5–6-fold enrichment of 5′-nucleotidase activity. The binding of $^{125}$I-HDL to the plasma membranes was time-dependent, and there appeared to be a single high-affinity site with a $K_d$ of 6.7 $\mu$g of HDL/ml of assay buffer. The binding was not affected by high concentrations of low-density lipoproteins or the $Ca^{2+}$ chelator EDTA, nor by changes in pH in the range 6.5–9.0. The binding was affected by the salt concentration in the buffer, with a dose-dependent increase that reached a maximum at 150–250 mM-NaCl. Binding was increased in the presence of high concentrations of KCl and KBr, and most significantly increased by high concentrations of bivalent metal ions. Ligand-blot analysis under reducing conditions revealed that the binding protein was a single polypeptide of about 108 kDa that was associated with the plasma-membrane fraction. This HDL-binding protein was purified to homogeneity by solubilization with Triton X-100, poly(ethylene glycol) precipitation, DEAE-Sephadex chromatography, and preparative SDS/PAGE. The purified binding protein is a single polypeptide of 108 kDa that retains high affinity and specificity for HDL as assayed by ligand blotting.

## INTRODUCTION

Steroid-producing tissues require large amounts of cholesterol for hormone production. Although they possess the means to synthesize cholesterol, the major steroid-producing organs, the adrenal and the ovary, are unable to maintain physiological levels of steroid output without importing exogenous substrate (Andersen & Dietschy, 1978; Schreiber *et al.*, 1982; Strauss *et al.*, 1982). This is accomplished by the uptake of plasma-borne cholesterol in the form of lipoprotein particles via specific high-affinity cell-surface lipoprotein receptors (Rajendran *et al.*, 1983).

The metabolism of low-density lipoproteins (LDL) and the structure and function of the LDL receptor have been well characterized (Brown & Goldstein, 1986). In most tissues, including steroidogenic organs, the number of LDL receptors increases when the cellular cholesterol level is low. These receptors bind the LDL and carry it through an endocytotic pathway to the lysosomes, where the LDL particles undergo degradation, thereby making cholesterol available for the metabolic needs of the cell (Schreiber *et al.*, 1982; Gotto *et al.*, 1986).

In contrast with this, relatively little is known about the metabolism of high-density lipoproteins (HDL). In most tissues, it appears that HDL serves to remove excess cholesterol (Oram, 1986). In steroidogenic tissues, however, HDL appears to function much like LDL by supplying cholesterol substrate (Strauss *et al.*, 1982; Andersen & Dietschy, 1981). Likewise, the number of HDL receptors in these tissues increases when the demand for cholesterol is high (Chen *et al.*, 1980; Ghosh & Menon, 1987). Unlike LDL, however, the mechanism in which cholesterol is exchanged between HDL particles and the target tissue is still unknown. In some cell types, the HDL remains at the cell surface, with cholesterol exchange occurring by passive diffusion (Karlin *et al.*, 1987; Oram *et al.*, 1987; Slotte *et al.*, 1987; Johnson *et al.*, 1988). Other cells, including ovarian luteal cells, appear to internalize HDL by a non-lysosomal endocytotic pathway (Paavolo & Strauss, 1983; Toth *et al.*, 1986, 1988; Rajan & Menon, 1987; Rahim *et al.*, 1991) that requires microtubules (Rajan & Menon, 1985). Recent studies in our laboratory have shown that HDL binding to rat luteal cells is up-regulated biphasically in both low- and high-cholesterol states, and therefore may function to efflux or influx cholesterol, depending on the status of the cell (Talavera & Menon, 1989; Ferreri *et al.*, 1991).

In our continuing study of lipoprotein support of ovarian steroidogenesis, we have now characterized and isolated an HDL-binding protein from the plasma membranes of the bovine corpus luteum.

## MATERIALS AND METHODS

### Materials

Electrophoresis chemicals, Triton X-100 and pre-stained molecular-mass standards were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Nitrocellulose was purchased from Gelman Scientific (Ann Arbor, MI, U.S.A.). Phenylmethanesulphonyl fluoride (PMSF), leupeptin and E64 were purchased from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Benzamidine and dithiothreitol (DTT) were purchased from Calbiochem (San Diego, CA, U.S.A.). Butylated hydroxytoluene (BHT) and SDS high-molecular-mass markers were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

### Lipoproteins

HDL (density 1.063–1.125 g/ml) and LDL (density 1.019–1.063 g/ml) were isolated from normal human plasma by ultracentrifugation after density adjustment with KBr as previously

Abbreviations used: LDL, low-density lipoproteins; HDL, high-density lipoproteins; INT, 2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium; PBS, phosphate-buffered saline [9 mM-$KH_2PO_4$ (pH 7.5)/150 mM-NaCl]; DTT, dithiothreitol; PEG, poly(ethylene glycol); PMSF, phenylmethanesulphonyl fluoride; BHT, butylated hydroxytoluene.

* To whom correspondence should be addressed.

described (Hwang & Menon, 1983). The HDL was chromatographed on a heparin–Affigel-10 column to remove the apolipoprotein-E-containing particles (Ferreri & Menon, 1990). Lipoproteins were radio-iodinated by a modified McFarlane (1958) procedure (Hwang & Menon, 1983) to a specific radioactivity of 800–1200 c.p.m./ng. Lipoprotein concentrations are expressed in terms of their protein content as determined with the Pierce BSA protein assay kit at 60 °C with BSA as standard.

### Membrane preparation

Plasma membranes from bovine corpora lutea were prepared by the differential-centrifugation method of Powell *et al.* (1976). Unless otherwise indicated, all steps were conducted at 4 °C. Fresh corpora lutea were obtained from Murco Meat Packer (Plainwell, MI, U.S.A.) and stored frozen at −70 °C until used. The frozen tissue was scraped from the capsule, weighed, minced, and homogenized with 10 vol. of homogenization buffer (0.3 M-sucrose/1 mM-$NaHCO_3$) containing 1 mM-PMSF. The homogenate was filtered through one layer and then four layers of sterile gauze, and then centrifuged at 6000 *g* for 10 min. The supernatant was collected and the pellet resuspended in 8 vol. of homogenization buffer and re-centrifuged. The combined 6000 *g* supernatants were centrifuged at 35000 *g* for 30 min. The supernatants were pooled. The top layers of the pellets were scraped off with a spatula and resuspended in 4 vol. of homogenization buffer and re-centrifuged at 35000 *g* for 30 min. After removal of the supernatant, the top layer of the pellet was collected by gentle swirling in 0.5 vol. of homogenization buffer. The combined 35000 *g* supernatants were centrifuged at 80000 *g* for 90 min and the pellets and supernatants collected separately. The subfractions are designated as follows: 6p is the 6000 *g* pellet, 35b is the bottom layer of the 35000 *g* pellet, 35t is the top layer of the 35000 *g* pellet, 80p is the 80000 *g* pellet, and 80s is the 80000 *g* supernatant. All pellets were resuspended in homogenization buffer containing 1 mM-PMSF, 1 mM-benzamidine, 2 μM-leupeptin and 2 μM-E64 (proteinase inhibitors). The activity of the plasma-membrane enzyme 5′-nucleotidase (EC 3.1.3.5) was measured in each subfraction (Morré, 1971) with $P_i$ release quantified as described by Ames & Dubin (1960). The amount of the mitochondrial enzyme succinate:2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium (INT) reductase (EC 1.3.99.1) was also quantified in each subfraction (Morré, 1971). Protein was measured with the Pierce BCA protein assay kit at 60 °C with BSA as standard.

### Binding assays

Binding assays were performed in five replicates. Each assay mixture contained 200 μg of protein, 1 μg of $^{125}$I-labelled apolipoprotein E-free HDL, and increasing concentrations of unlabelled lipoprotein in a final volume of 500 μl of assay buffer [20 mM-Tris/HCl (pH 7.4), 150 mM-NaCl, 1% (w/v) BSA and 0.01% (w/v) merthiolate]. The assay mixtures were incubated at 37 °C for 60 min and then aspirated separately through 0.45 μm-pore cellulose acetate filters (Millipore Corp., Bedford, MA, U.S.A.) with 2 ml of ice-cold assay buffer and washed with 4 ml of ice-cold assay buffer. Bound radioactivity was measured with a γ-radiation counter (Tracor Analytic, Elk Grove Village, IL, U.S.A.). Binding parameters were determined by the linear subtraction method (Van Zoelen, 1989) and by non-linear curve fitting using the LIGAND computer program (BioSoft, Milltown, NJ, U.S.A.).

### Isolation procedure

Subfractions 35t and 80p, obtained by differential centrifugation and which contained most of the 5′-nucleotidase activity, were pooled and used as the starting plasma-membrane fraction. This fraction was extracted by adjusting the protein concentration to 5 mg/ml in buffer A [125 mM-Tris/HCl (pH 7.4), 150 mM-KCl, 5 mM-EDTA, 250 mM-sucrose, 1% (v/v) Triton X-100, 2 mM-DTT, 1 mM-PMSF, 1 mM-benzamidine, 2 μM-leupeptin and 2 μM-E64] and stirring for 30 min. The detergent-solubilized protein fraction was separated from the insoluble portion of the membranes by centrifugation at 150000 *g* for 60 min. To remove excess detergent and concentrate the sample, poly(ethylene glycol) [PEG; 50% (w/v) in 9 mM-$KH_2PO_4$ (pH 7.5)/150 mM-NaCl (PBS)] was added to a final concentration of 12% (w/v) (Marshall *et al.*, 1985). After stirring the solution for 30 min at 4 °C, the precipitate was collected by centrifugation at 28000 *g* for 30 min. The pellet was washed by resuspension in 1 vol. of PBS and re-pelleted by centrifugation to remove traces of PEG. The pellet was then solubilized in buffer B [20 mM-Tris/HCl, pH 8.0, 150 mM-KCl, 1 mM-EDTA, 1 mM-DTT, 20% (v/v) glycerol, 0.1% Triton X-100, 1.7 mM-CHAPS, 100 μM-BHT and proteinase inhibitors as above] and adjusted to a protein concentration of 0.7–0.8 mg/ml. This sample was pumped through a DEAE-Sephadex A-50 column overnight at 5 °C with a peristaltic pump (3.5 mg of protein/ml of packed resin). The column was washed with 3 vol. of the same buffer, and eluted by adjusting the concentration of KCl to 300 mM. The eluted protein was concentrated 10-fold in an Amicon Centricon-30 microconcentrator unit and the concentrate was precipitated with an equal volume of methanol/chloroform (4:1, v/v) (Wessel & Flugge, 1984). The precipitate was dissolved in SDS/PAGE sample buffer [0.0625 M-Tris/HCl (pH 6.8), 0.1% glycerol, 2% (w/v) SDS, 0.00125% (w/v) pyronin Y and 5% (w/v) β-mercaptoethanol] and electrophoresed on a preparative 6%-polyacrylamide gel (Laemmli, 1970). The protein bands were made visible with Coomassie Blue (Hunkapiller *et al.*, 1983). The band that migrated with an apparent molecular mass of 108 kDa and which coincided with ligand-blot activity was excised and electroeluted in 10 mM-$NH_4HCO_3$/0.02% SDS/0.01% DTT at 4 W for 4 h in an ISCO concentrator (Lincoln, NE, U.S.A.).

### Electrophoresis and ligand blotting

Samples were precipitated with an equal volume of methanol/chloroform (4:1, v/v) (Wessel & Flugge, 1984), dried with air, and solubilized with 1 vol. of 5% SDS and 1 vol. of SDS/PAGE sample buffer by heating at 60 °C for 10 min. Appropriate portions of the samples and the molecular-mass standards were placed into the wells of a discontinuous polyacrylamide gel (6%T, 2.67%C) and electrophoresed at 30 mA for 5 h. Gels were silver-stained as described by Hochstrasser *et al.* (1988) to assess the purity of each sample.

For ligand-blot analysis, the separated proteins were electrophoretically transferred onto nitrocellulose sheets at 300 mA for 5–6 h. The nitrocellulose sheets were then incubated at 37 °C in 100 ml of blocking buffer [20 mM-Tris/HCl (pH 7.4), 0.01% merthiolate, 1% (w/v) non-fat dry milk, 1% BSA] for 2 h. The buffer was replaced with buffer containing 100 μg of LDL/ml and 1 μg of $^{125}$I-HDL/ml, and the blots were shaken at 37 °C for a further 2 h. Finally, the blots were rinsed twice with ice-cold blocking buffer, washed five times for 5 min each with the same buffer and rinsed briefly with cold PBS. The sheets were air-dried and autoradiographed. The apparent molecular mass of each protein was determined by comparison with pre-stained molecular-mass standards (Bio-Rad).

## RESULTS

Since lipoproteins circulate in the blood, the HDL receptor was expected to be enriched on the cell surface. Therefore bovine corpora lutea were homogenized and subfractionated by the

**Table 1. Subfractionation of bovine corpora lutea**

Freshly frozen bovine corpora lutea (8 g) were homogenized and subfractionated as described in the Materials and methods section. The protein concentrations of each subfraction were determined with the Pierce BCA protein assay kit. 5'-Nucleotidase (EC 3.1.3.5) activity was measured as described by Morré (1971). Samples (20 $\mu$g of protein) from each subfraction were incubated at 37 °C for 30 min in 0.25 ml of buffer containing AMP substrate. $P_i$ release was quantified as described by Ames & Dubin (1960). Succinate:INT reductase (EC 1.3.99.1) activity was determined as described by Morré (1971). A sample (100 $\mu$g of protein) from each subfraction was incubated at 37 °C for 60 min in 0.5 ml of buffer containing succinate and the substrate dye INT. The reduced dye was quantified after ethyl acetate extraction by measuring the $A_{420}$. Values represent the means of at least five replicates. Enzyme activities are expressed as nmol/min per mg.

| | Protein | | 5'-Nucleotidase | | | Succinate:INT reductase | | |
|---|---|---|---|---|---|---|---|---|
| Subfraction | Protein (mg) | % protein* | Specific activity | % total activity* | Fold enrichment† | Specific activity | % total activity* | Fold enrichment† |
| Homogenate | 515 | | 40.6 | | | 4.2 | | |
| 6p | 115 | 22 | 14.0 | 8 | 0.3 | 4.7 | 25 | 1.1 |
| 35b | 46 | 9 | 114.6 | 25 | 2.8 | 6.6 | 14 | 1.6 |
| 35t | 22 | 4 | 221.3 | 24 | 5.5 | 3.8 | 4 | 0.9 |
| 80p | 41 | 8 | 245.1 | 48 | 6.0 | 1.7 | 3 | 0.4 |
| 80s | 257 | 50 | 5.0 | 6 | 0.1 | 5.0 | 59 | 1.2 |
| Recovery | 481 | 93 | | 111 | | | 105 | |

* Percentages were calculated as 100 × (total activity of subfraction)/(total activity of homogenate).
† Fold enrichment was calculated as (specific activity of subfraction)/(specific activity of homogenate).

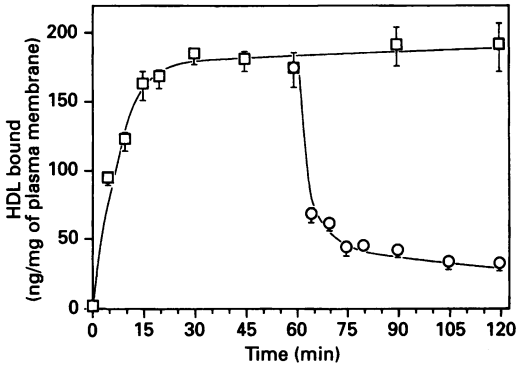


**Fig. 1. Time course of $^{125}$I-HDL binding to luteal plasma membranes**

Each assay tube contained 200 $\mu$g of plasma-membrane protein, 1 $\mu$g of $^{125}$I-HDL, and 0, 1, 3, 7, 15, 31, 63 or 127 $\mu$g of unlabelled HDL in 0.5 ml of buffer containing 20 mM-Tris/HCl, pH 7.4, 150 mM-NaCl, 0.01 % merthiolate and 1 % BSA. The samples were incubated at 37 °C for the times indicated, collected on cellulose acetate filters, and rinsed with ice-cold buffer. Bound radioactivity was quantified by a $\gamma$-radiation counter. Non-specific binding was determined by addition of a 200-fold excess of unlabelled HDL. Specific association (□) was calculated for each time point as total HDL bound minus non-specifically bound HDL. After a 60 min incubation, 194 $\mu$g of unlabelled HDL was added to certain tubes, and the dissociation (○) of the receptor–ligand complex was monitored. All assays were done in five replicates.

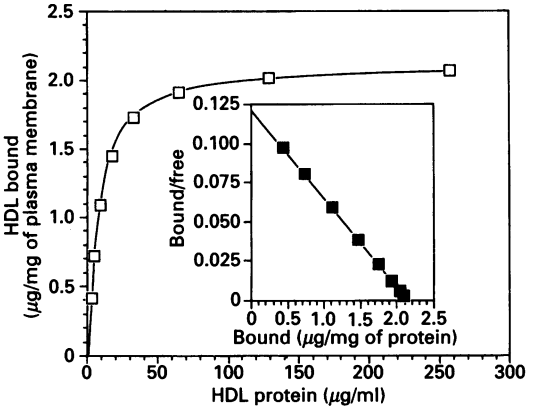


**Fig. 2. Concentration-dependent binding of $^{125}$I-HDL to luteal plasma membranes**

Each assay tube contained 200 $\mu$g of plasma-membrane protein, 1 $\mu$g of $^{125}$I-HDL, and 0, 1, 3, 7, 15, 31, 63 or 127 $\mu$g unlabelled HDL in 0.5 ml of buffer containing 20 mM-Tris/HCl, pH 7.4, 150 mM-NaCl, 0.01 % merthiolate and 1 % BSA. The samples were incubated at 37 °C for 60 min and then collected on cellulose acetate filters and rinsed with ice-cold buffer. Specific binding (□) was determined mathematically by using the linear subtraction method (Van Zoelen, 1989) and agreed well with experimental values obtained when non-specific binding was estimated with an excess of unlabelled ligand. All assays were done in five replicates. Inset: Scatchard transformation of the equilibrium binding data (■).

method of Powell *et al.* (1976). In order to identify the fraction(s) containing the plasma membrane, samples of each subtraction were assayed for the presence of specific marker enzymes. Two subfractions, 35t and 80p, were enriched 5–6-fold in the activity of the plasma-membrane marker enzyme 5'-nucleotidase and were depleted of the activity of the mitochondrial marker enzyme succinate: INT reductase (Table 1). Therefore 35t and 80p were pooled and designated as the plasma-membrane fraction.

Incubation of the plasma-membrane fraction with $^{125}$I-labelled apolipoprotein E-free HDL at 37 °C demonstrated increasing binding of HDL with time that reached a maximum at 30–45 min (Fig. 1). The bound radiolabelled HDL was displaced in a time-dependent fashion by addition of an excess of unlabelled HDL (Fig. 1). These results suggest that the labelled HDL is in equilibrium with the same binding sites as the unlabelled HDL.

Equilibrium binding studies of radiolabelled HDL to the plasma-membrane fraction showed saturability and high affinity (Fig. 2). Scatchard analysis of the binding was linear (Fig. 2, inset), indicating a single class of binding sites with a $K_d$ of 6.7 $\mu$g/ml and a $B_{max.}$ of 2.1 $\mu$g of HDL bound/mg of plasma-membrane protein.

The binding specificity of these sites was examined by competition between a constant amount of $^{125}$I-HDL and increasing concentrations of either LDL or HDL (Fig. 3). LDL competes poorly with HDL binding, displacing less than 20 % of the

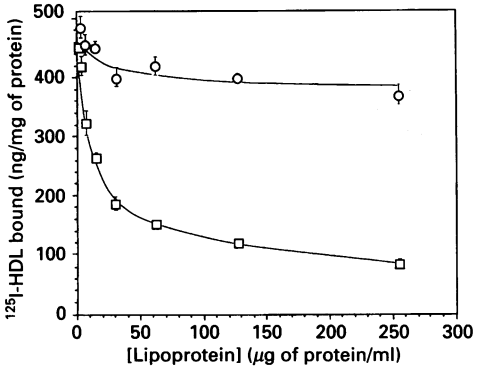


**Fig. 3. Effect of increasing concentrations of LDL and HDL on the binding of $^{125}$I-HDL to luteal plasma membranes**

Each assay tube contained 200 μg of plasma-membrane protein, 1 μg of $^{125}$I-HDL and the indicated concentrations of unlabelled LDL (○) or HDL (■) in 0.5 ml of buffer containing 20 mM-Tris/HCl, pH 7.4, 150 mM-NaCl, 0.01 % merthiolate and 1 % BSA. The samples were incubated at 37 °C for 60 min and then collected on cellulose acetate filters and rinsed with ice-cold buffer. Bound radioactivity was quantified by a γ-radiation counter. All assays were done in five replicates.

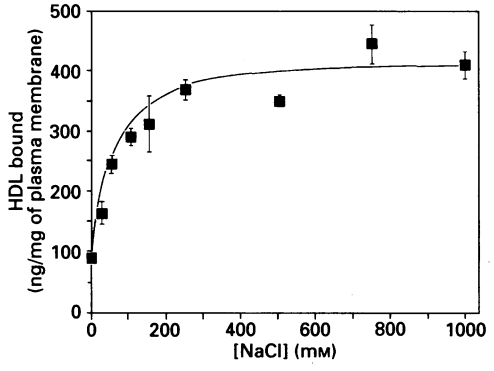


**Fig. 4. Effect of increasing concentrations of NaCl on the binding of $^{125}$I-HDL to luteal plasma membranes**

Each assay tube contained 200 μg of plasma-membrane protein, 1 μg of $^{125}$I-HDL and 0, 25, 50, 100, 150, 250, 500, 750 or 1000 mM-NaCl in 0.5 ml of buffer containing 20 mM-Tris/HCl, pH 7.4, 0.01 % merthiolate and 1 % BSA. The samples were incubated at 37 °C for 60 min and then collected on cellulose acetate filters and rinsed with ice-cold buffer. Specific binding (■) was determined by subtracting the binding in the presence of 194 μg of unlabelled HDL from the total binding. All assays were done in five replicates.

labelled HDL. On the other hand, unlabelled HDL displaces about 80 % of the labelled ligand. These results suggest that the binding sites are specific for apolipoprotein E-free HDL.

In the case of LDL and apolipoprotein E-containing HDL, the $Ca^{2+}$ chelator EDTA completely abolished binding to their receptors (Hui *et al.*, 1986; Yamamoto *et al.*, 1984), whereas low levels of $Ca^{2+}$ led to increased binding (approx. 2-fold increase in binding in the presence of 1–3 mM-$CaCl_2$). Therefore the effects of $Ca^{2+}$ and EDTA on the binding of apolipoprotein E-free HDL to the bovine ovarian receptor were examined. Increasing concentrations of either $Ca^{2+}$ or EDTA caused only minor changes in the interaction of HDL with the membranes when the assays were performed in the presence of 0.9 % NaCl, which coincides

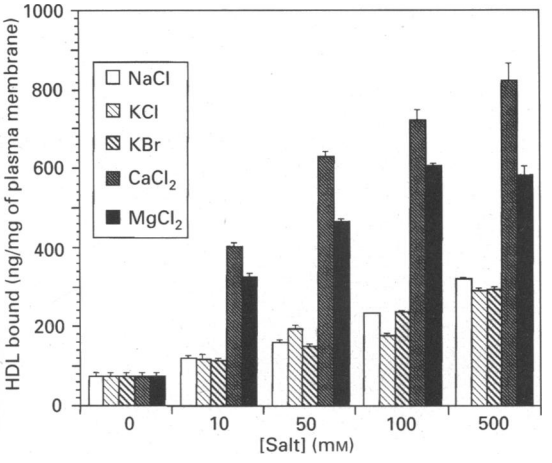


**Fig. 5. Effect of various salts on $^{125}$I-HDL binding**

Each assay tube contained 200 μg of plasma-membrane protein, 1 μg of $^{125}$I-HDL and the indicated concentrations of NaCl, KCl, KBr, $CaCl_2$ or $MgCl_2$ in 0.5 ml of buffer containing 5 mM-Tris/HCl, pH 7.4, 0.01 % merthiolate and 1 % BSA. The samples were incubated at 37 °C for 60 min and then collected on cellulose acetate filters and rinsed with ice-cold buffer. Specific binding was determined by subtracting the binding in the presence of 194 μg of unlabelled HDL from the total binding. All assays were done in five replicates.

with the description of HDL binding in other tissues and species (Chen *et al.*, 1980; Hwang & Menon, 1983; Hui *et al.*, 1986). Taken together, these findings imply that the interaction of HDL with its receptor is not dependent on ions chelated by EDTA.

In contrast with other species (Chen *et al.*, 1980; Hwang & Menon, 1983; Hui *et al.*, 1986), the interaction of the bovine plasma membranes with HDL exhibited a dependency on the NaCl concentration. In the absence of NaCl, the HDL binding was decreased to 25 % of its maximal value (Fig. 4). The binding increased in a dose-dependent manner to a maximum between 150 and 250 mM-NaCl. In order to determine if this effect was specific for NaCl, the effect of several salts as tested. Assays containing univalent salts (NaCl, KCl and KBr) all exhibited similar dose-dependent increases in HDL binding (Fig. 5), suggesting that the effect is not specific to NaCl. However, the binding observed with the bivalent salts $CaCl_2$ and $MgCl_2$ yielded binding values 2–3-fold higher than with the univalent salts (Fig. 5). It should be noted that the salt concentrations used in these studies are much greater than the normal plasma levels (142 mM-$Na^+$, 5 mM-$K^+$, 1.5 mM-$Ca^{2+}$, 1.5 mM-$Mg^{2+}$ and 103 mM-$Cl^-$; Guyton, 1977). Together with the results of binding in the presence of low levels of $Ca^{2+}$ and EDTA, these results suggest that the salt effects are due to the increased ionic strength of the buffer rather than a specific ion requirement. It is also possible that low salt concentrations might affect the physical state of the membranes, such as aggregation resulting in a decreased ligand binding.

It has been reported that the binding of HDL to dog liver membranes is markedly dependent on pH, with a distinct maximum at pH 7.5 (Hui *et al.*, 1986). Therefore the pH-dependence of HDL binding to luteal plasma membranes was examined. Changing the pH in the range 6.5–9.0 had only a mild effect of HDL binding (results not shown). However, a substantial increase in non-specific binding was observed below pH 6.5 that was due in part to the precipitation of $^{125}$I-HDL. The resultant uncertainty in the data prevented conclusions as to the effect of lower pH levels.

The ovarian subfractions were also subjected to ligand-blot

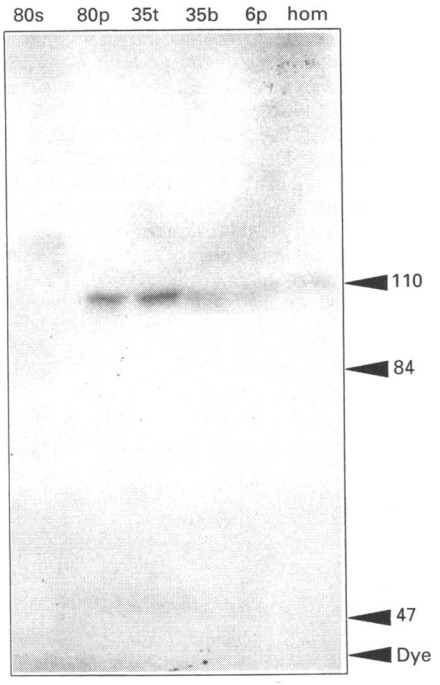


**Fig. 6. Ligand-blot analysis of bovine luteal subfractions**

Samples of the homogenate and each subfraction (200 $\mu$g each) were precipitated with 1 vol. of methanol/chloroform (4:1, v/v), electrophoresed on an SDS/6%-polyacrylamide gel under reducing conditions, and transferred to nitrocellulose. The nitrocellulose was gently shaken in a buffer containing 20 mM-Tris/HCl, pH 7.4, 150 mM-NaCl, 0.01% merthiolate and 1% BSA, plus 1% (w/v) non-fat dry milk at 37 °C for 2 h. The blot was then incubated for 2 h at 37 °C in the same buffer containing 1 $\mu$g of $^{125}$I-HDL/ml and 100 $\mu$g of LDL/ml. The blot was rinsed twice and washed five times for 5 min each with ice-cold blocking buffer, dried and autoradiographed. Key: hom, homogenate; 6p, 6000 *g* pellet; 35b, 35000 *g* pellet bottom; 35t, 35000 *g* pellet top; 80p, 80000 *g* pellet; 80s, 80000 *g* supernatant. The positions and sizes (kDa) of the prestained molecular-mass markers that were transferred to the nitrocellulose are indicated.

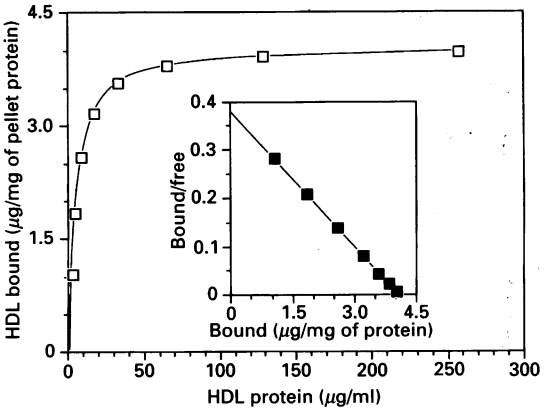


**Fig. 7. Concentration-dependent binding of $^{125}$I-HDL to the pellet from the PEG precipitation**

Each assay tube contain 200 $\mu$g of the PEG pellet protein, 1 $\mu$g of $^{125}$I-HDL and 0, 1, 3, 7, 15, 31, 63 or 127 $\mu$g of unlabelled HDL in 0.5 ml of buffer containing 20 mM-Tris/HCl, pH 7.4, 150 mM-NaCl, 0.01% merthiolate and 1% BSA. The samples were incubated at 37 °C for 60 min and then collected on cellulose acetate filters and rinsed with ice-cold buffer. Specific binding (□) was determined mathematically by the linear subtraction method (Van Zoelen, 1989). All assays were done in five replicates. Inset, Scatchard transformation of the equilibrium binding data (■).

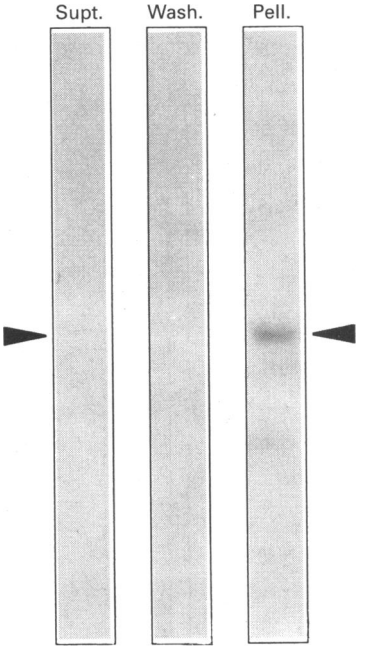


**Fig. 8. Ligand-blot analysis of the supernatant, wash and pellet from the PEG precipitation**

The plasma-membrane fraction was adjusted to 5 mg of protein/ml and solubilized with 1% (v/v) Triton X-100. PEG was added to a final concentration of 12% (w/v) and the precipitate collected by centrifugation for 30 min at 28000 *g*. The supernatant (Supt.) was removed and the pellet was resuspended in 1 vol. of buffer containing 10 mM-potassium phosphate, pH 7.5, and 150 mM-NaCl, and recentrifuged for 30 min at 28000 *g*. The supernatant (Wash) was removed and the pellet (Pell.) was resuspended in buffer containing 20 mM-Tris/HCl, pH 8.0, and 150 mM-KCl. Samples (100 $\mu$g each) of the Supt., Wash and Pell. were subjected to ligand-blotting analysis as described in the Materials and methods section, and the HDL-binding protein was detected by autoradiography. The position of the 108 kDa HDL-binding protein on the autoradiogram is indicated by the arrows.

analysis. Ligand blotting involves the separation of molecules according to size by electrophoresis, transfer to nitrocellulose, and incubation in the presence of radiolabelled ligand. The sizes of individual bands that bind ligand were revealed by autoradiography. In the bovine corpus luteum, only one species of HDL-binding protein, of about 108 kDa, was detected by ligand blotting (Fig. 6). Subfractions 35t and 80p, which contain most of the plasma membranes, are enriched in this protein (Fig. 6). Ligand blotting was performed after denaturation and reduction of the sample with SDS and $\beta$-mercaptoethanol, which suggests that the 108 kDa band consists of a single polypeptide chain. An excess of LDL was included in the binding mixtures to ensure the specificity of the binding protein.

To characterize better the role of HDL in the corpus luteum, an attempt was made to purify the 108 kDa HDL-binding protein. Plasma-membrane fractions were adjusted to a protein concentration of 5 mg/ml with buffer A, which contained 1% Triton X-100, and stirred for 30 min at 4 °C. This procedure solubilized about 80% of the protein in the plasma-membrane fraction. The soluble protein fraction was refractory to analysis by conventional binding assays, and therefore the purification of the binding protein was followed by ligand-blotting analysis of each step of the purification procedure.

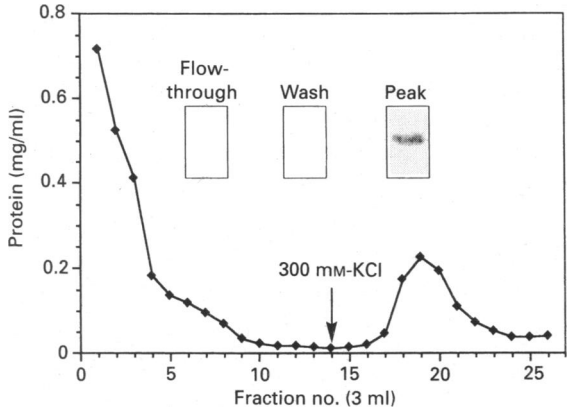


**Fig. 9. Elution profile of DEAE-Sephadex column**

The pellet from the PEG precipitation was resuspended in buffer B at a protein concentration of 0.75 mg/ml. The sample was recycled at 4 °C overnight through a DEAE-Sephadex column (6 cm × 1.5 cm) at a load of 3.5 mg of protein/ml of resin. The column was washed with 3 vol. of the same buffer and then eluted by adjusting the KCl concentration to 300 mM (arrow). Samples (100 $\mu$g each) of the pooled fractions from the flow-through and wash, and the peak eluted with 300 mM-KCl, were analysed by ligand blotting, and the autoradiograms are shown in the insets. Approx. 5% of the protein loaded on to the column was eluted in the 300 mM-KCl fraction.

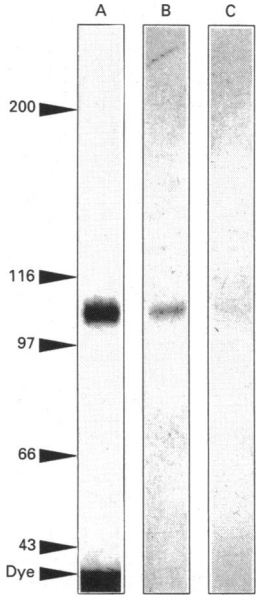


**Fig. 10. Silver staining and ligand-blot analysis of the purified HDL receptor**

The eluted peak from the DEAE-Sephadex column was concentrated by Amicon Centricon-30 ultrafiltration and precipitated with 1 vol. of methanol/chloroform (4:1, v/v). The precipitate was solubilized in SDS/PAGE sample buffer containing 5% (v/v) $\beta$-mercaptoethanol and electrophoresed on a 6%-polyacrylamide gel. The gel was stained with Coomassie Blue and the band at 108 kDa was excised and electroeluted. Samples (0.5 $\mu$g) of the eluted protein were electrophoresed on a 6% gel and subjected to silver staining (lane A), ligand-blot analysis (lane B), and ligand-blot analysis in the presence of a 100-fold excess of unlabelled HDL (lane C). The positions and sizes (kDa) of the molecular-mass markers from the silver-stained gel are indicated.

**Table 2. Purification of the 108 kDa HDL-binding protein**

The bovine ovarian HDL-binding protein was purified to apparent electrophoretic homogeneity from freshly frozen bovine corpora lutea as described in the Materials and methods section. Each step was assayed by ligand-blot analysis for the presence (+) or absence (−) of an HDL-binding protein. The HDL-binding activity of the homogenate, plasma-membrane fraction and the PEG pellet samples was also assayed by a conventional filtration-binding assay, and the calculated maximal binding values are given as $\mu$g of HDL bound/mg of sample protein. Abbreviation: n.a., not available (soluble forms of the receptor could not be assayed by conventional binding assays, because the receptor is not retained by the cellulose acetate filters used to separate the bound $^{125}$I-HDL from the unbound $^{125}$I-HDL).

| Sample | Protein (mg) | Ligand blot | HDL bound ($\mu$g/mg of sample) |
|---|---|---|---|
| Homogenate | 3853 | + | 0.53 |
| Plasma membrane | 471 | + | 2.13 |
| Solubilized protein | 388 | + | n.a. |
| PEG precipitation: | | | |
| Supernatant | 137.5 | − | n.a. |
| Wash | 163.1 | − | n.a. |
| Pellet | 78.6 | + | 4.04 |
| DEAE column: | | | |
| Flow-through | 71.6 | − | n.a. |
| Peak | 3.96 | + | n.a. |
| Electroeluted protein | 0.01 | + | n.a. |

PEG was added to the solubilized sample at a final concentration of 12% (w/v). At this concentration of PEG, only 20% of the protein was precipitated. PEG precipitation has been shown to remove most of the cellular proteins and detergent from Triton-solubilized samples while recovering most of the receptors for binding studies (Marshall *et al.*, 1985). Binding analysis of the PEG pellet showed saturable high-affinity binding to a single site with a $K_d$ of 3.3 $\mu$g/ml and a $B_{max.}$ of 4.0 $\mu$g of HDL bound/mg of PEG pellet (Fig. 7). Ligand-blotting analysis of the PEG precipitate shows the presence of the 108 kDa band (Fig. 8, Pell). No HDL binding activity was detected in either the PEG supernatant (Fig. 8, Supt) or the PBS used to wash the pellet (Fig. 8, Wash). The recovery of the receptor calculated from the binding-assay results was 31.7%, with a 1.9-fold enrichment over the plasma-membrane preparation. However, the 108 kDa band was not detected in any of the other fractions by ligand-blotting assay. This could be due either to masking of binding sites during precipitation or the possibility of a binding species that was neither precipitated by PEG nor detected by ligand blotting. However, the Scatchard plots and the ligand-blotting results of the plasma membrane and the PEG pellet suggest that a single binding species is present in this tissue. The recovery may therefore be higher than that measured by the binding-assay procedure.

The PEG pellet was resolubilized in buffer B and recycled through a column of DEAE-Sephadex at 4 °C overnight. After washing the column with 3 vol. of buffer B, the KCl concentration of the buffer was adjusted to 300 mM. A small peak containing about 5% of the total protein (Fig. 9) was eluted from the column. Subsequent ligand-blot analysis of the flow-through, wash and pooled peak fractions revealed that the 108 kDa HDL-binding protein appeared to be quantitatively bound to the column and eluted with 300 mM-KCl (Fig. 9, insets).

The pooled fractions of the eluted peak were concentrated 10-fold with an Amicon Centricon-30 micro-concentrator and then precipitated with 1 vol. of methanol/chloroform (4:1, v/v)

(Wessel & Flugge, 1984). The sample was dissolved in SDS/PAGE sample buffer containing 5% β-mercaptoethanol and electrophoresed on a preparative 6% polyacrylamide gel. The protein bands were detected by staining with Coomassie Blue. The band with an apparent molecular mass of 108 kDa was excised and electroeluted from the gel in 10 mM-$NH_4HCO_3$ containing 0.02% SDS and 0.01% DTT (Hunkapiller *et al.*, 1983). The eluted protein was electrophoretically pure, as indicated by silver staining of an SDS/polyacrylamide gel (Fig. 9, lane A). This material retained its HDL-binding capability, as shown by ligand-blotting analysis using an identical sample (Fig. 10, lane B). Ligand blotting in the presence of a 100-fold excess of unlabelled HDL abolished the binding of the $^{125}$I-HDL to the 108 kDa band. These results suggest that the binding of HDL to the 108 kDa protein was specific and saturable (Fig. 10, lane C).

The purification scheme of the bovine HDL binding protein is summarized in Table 2. A typical purification started with 42 g of tissue and yielded about 10 μg of purified product (equivalent to 93 pmol of receptor, based on an apparent molecular mass of 108 kDa). As mentioned above, standard binding assays could not be used to measure the binding activity of the soluble sample. Therefore the binding activity of the purified binding protein was confirmed by ligand blotting. However, the nature of the ligand-blot assay did not permit precise quantitative determination of the specific HDL-binding activity of the 108 kDa band.

## DISCUSSION

The present study describes the characterization and isolation of a putative HDL receptor from the bovine corpus luteum. Ligand-blotting analysis reveals HDL-binding protein that appeared to be a single polypeptide with an apparent molecular size of 108 kDa. The binding protein was associated with the plasma-membrane subfraction of the tissue homogenate. The putative receptor was isolated in homogeneous form by sequential solubilization of the plasma-membrane fraction with Triton X-100, PEG precipitation, DEAE-Sephadex column chromatography, preparative PAGE and electroelution. The purified receptor retained high affinity and specificity for HDL binding, as measured by a ligand-blotting assay.

At present, the specific molecular associations that comprise the binding of HDL to its receptor are unknown. Previous studies have shown that the receptor exhibits loose binding specificity for apolipoproteins AI, AII, and possibly other homologous apolipoproteins (Rifici & Eder, 1984; Hwang & Menon, 1985; Mendel *et al.*, 1986; Fong *et al.*, 1987), but not for apolipoprotein B (Rajendran *et al.*, 1983; Hwang & Menon, 1983) or apolipoprotein E (Rifici & Eder, 1984). Furthermore, the binding is not inhibited by changes in pH or by high concentrations of salts, conditions that would be expected to disrupt charge–charge interactions between the receptor and the ligand. In contrast with other lipoproteins, the binding of HDL is not altered by EDTA, and therefore does not appear to be dependent on ions that are chelated by EDTA, such as $Ca^{2+}$ and $Mg^{2+}$. Each of these treatments inhibits binding of apolipoproteins B and E to their respective receptors (Hui *et al.*, 1986; Yamamoto *et al.*, 1984), putatively by perturbing ionic interactions promoted by $Ca^{2+}$ (Yamamoto *et al.*, 1984; Lalazar *et al.*, 1988).

Of the various conditions examined in the present study, only elevated salt concentrations substantially affected HDL binding. The lack of specificity of the cations tested which stimulated binding argues against specific ion requirements. The indifference of HDL binding to high NaCl concentrations has been interpreted as suggesting that the ligand-recognition mechanism is primarily hydrophobic (Mendel *et al.*, 1986). This hypothesis is supported by our observation that HDL binding is lower at low salt concentrations, since increased ionic strength stabilizes hydrophobic interactions. This interpretation is consistent with the observed enhancement of binding promoted by the bivalent cations $Ca^{2+}$ and $Mg^{2+}$, which because of their higher charge density could have yet an even greater stabilizing effect.

Several HDL-binding proteins of varying sizes have been described. Our laboratory has previously described a 58 kDa HDL-binding protein in the rat ovary that exhibited characteristics of the putative HDL receptor (Ferreri & Menon, 1990). Graham & Oram (1987) reported ligand-blotting characterization of several binding proteins present in various tissues, including bovine arterial endothelial cells. The consensus size of the HDL-binding proteins in the tissues studied was 110 kDa, a value close to the apparent 108 kDa mass of our purified bovine binding-protein preparation. The 110 kDa protein was up-regulated by elevated cellular cholesterol levels (Graham & Oram, 1987) and by γ-interferon (Oppenheimer *et al.*, 1988). Tozuka & Fidge (1989) isolated two binding proteins of 120 kDa and 100 kDa, which were both present in rat and human liver. Barbaras *et al.* (1990) isolated a single 80 kDa species from a mouse adipocyte cell line. Most recently, Bond *et al.* (1991) purified HDL-binding proteins of 100 kDa and 60 kDa from Hep3B cells. The 100 kDa protein was associated with a DTT-sensitive 210 kDa complex and a larger 400–450 kDa complex.

The function of the HDL receptor remains unclear. The ability of HDL particles to remove excess cholesterol from cells in culture has been demonstrated and appears to be part of the reverse cholesterol-transport model of cholesterol homoeostasis (Gotto *et al.*, 1986; Oram, 1986). Although it is plausible that an HDL receptor could take part in cholesterol removal, there is contradictory evidence on the precise role that binding may have in cholesterol transfer. Although binding is not required for the exchange of cholesterol between HDL and the cell membrane (Karlin *et al.*, 1987; Johnson *et al.*, 1988), some cells do internalize HDL particles, presumably by a receptor-dependent process (Paavolo & Strauss, 1983; Toth *et al.*, 1986, 1988; Rajan & Menon, 1987; Rahim *et al.*, 1991). Slotte *et al.* (1987) suggested that the function of HDL binding is to promote intracellular translocation of cholesterol from internal stores to the cell surface, thus making cholesterol available for diffusion-controlled efflux to HDL particles. This hypothesis is supported by findings from our laboratory that demonstrate participation of microtubules in lipoprotein metabolism (Rajan & Menon, 1985). Mendez *et al.* (1991) have recently shown that the binding of HDL to its receptor activates a protein kinase C (PKC)-dependent pathway that promotes the translocation of cholesterol from intracellular pools to the plasma membrane. Other studies have demonstrated that HDL binding promotes PKC-dependent phosphorylation of specific cellular proteins (Darbon *et al.*, 1986; Wu & Handwerger, 1991). Taken together, these studies support the concept that the HDL receptor may be coupled to a G-protein that sends a signal through PKC to communicate to the cell that the receptor is occupied and ready for sterol transfer.

Steroidogenic tissues, such as the corpus luteum, require larger amounts of cholesterol than other tissues, since a major portion of their cholesterol is used for hormone production. The regulatory mechanisms that control intracellular cholesterol levels are critical to the normal physiology of the cell. The corpus luteum is absolutely dependent on the uptake of exogenous cholesterol from both HDL and LDL for maximal progesterone production (Andersen & Dietschy, 1978; Schreiber *et al.*, 1982; Strauss *et al.*, 1982). We have demonstrated *in vivo* (Talavera &

Menon, 1989) and *in vitro* (Ferreri *et al.*, 1992) that the HDL receptor in pseudopregnant-rat ovaries is up-regulated biphasically at low cholesterol levels as well as when the cholesterol stores of the cells are overloaded. These results imply that the HDL receptor serves to promote both cholesterol influx from HDL and cholesterol efflux to HDL, depending on the status of the cell. Therefore the HDL-binding protein appears to be central to the maintenance of cellular cholesterol homoeostasis.

Further delineation of the role of HDL in the regulation of ovarian cholesterol concentrations at the molecular level will require an in-depth knowledge of the structure and metabolism of the HDL receptor. The present study provides evidence for the existence of a specific receptor that recognizes HDL particles and describes its isolation from the bovine corpus luteum. These results provide a starting point for the further structural characterization of the bovine HDL 'receptor' that will hopefully lead to a better understanding of the role of HDL in cholesterol homoeostatis.

This work was supported by N.I.H. grant HD 06656.

## REFERENCES

Ames, B. N. & Dubin, D. T. (1960) J. Biol. Chem. **235**, 769–775
Andersen, J. M. & Dietschy, J. M. (1978) J. Biol. Chem. **253**, 9024–9032
Andersen, J. M. & Dietschy, J. M. (1981) J. Biol. Chem. **256**, 7362–7370
Barbaras, R., Puchois, P., Fruchart, J.-C., Pradines-Figueres, A. & Ailhaud, G. (1990) Biochem. J. **269**, 767–773
Bond, M. M., Morrone, G., Venuta, S. & Howell, K. E. (1991) Biochem. J. **279**, 633–641
Brown, M. S. & Goldstein, J. L. (1986) Science **232**, 34–46
Chen, Y.-D. I., Kraemer, F. B. & Reaven, G. M. (1980) J. Biol. Chem. **255**, 9162–9167
Darbon, J.-M., Tournier, J.-F., Tauber, J.-P. & Bayard, F. (1986) J. Biol. Chem. **261**, 8002–8008
Ferreri, K. & Menon, K. M. J. (1990) Endocrinology (Baltimore) **126**, 2137–2144
Ferreri, K., Talavera, F. & Menon, K. M. J. (1992) Endocrinology (Baltimore), in the press
Fong, B. S., Salter, A. M., Jimenez, J. & Angel, A. (1987) Biochim. Biophys. Acta **920**, 105–113
Ghosh, D. K. & Menon, K. M. J. (1987) Biochem. J. **244**, 471–479
Gotto, A. M., Jr., Pownall, H. J. & Havel, R. J. (1986) Methods Enzymol. **128**, 3–41
Graham, D. L. & Oram, J. F. (1987) J. Biol. Chem. **262**, 7439–7442
Guyton, A. C. (1977) Basic Human Physiology: Normal Function and Mechanisms of Disease, pp. 42–52, W. B. Saunders Co., Philadelphia
Hochstrasser, D. F., Harrington, M. G., Hochstrasser, A.-C., Miller, M. J. & Merril, C. R. (1988) Anal. Biochem. **173**, 424–435
Hui, D. Y., Brecht, W. J., Hall, E. A., Friedman, G., Innerarity, T. L. & Mahley, R. W. (1986) J. Biol. Chem. **261**, 4256–4267
Hunkapiller, M. W., Lujan, E., Ostrander, F. & Hood, L. E. (1983) Methods Enzymol. **91**, 227–236
Hwang, J. & Menon, K. M. J. (1983) J. Biol. Chem. **258**, 8020–8027
Hwang, J. & Menon, K. M. J. (1985) J. Biol. Chem. **260**, 5660–5668
Johnson, W. J., Mahlberg, F. H., Chacko, G. K., Phillips, M. C. & Rothblat, G. H. (1988) J. Biol. Chem. **263**, 14099–14106
Karlin, J. B., Johnson, W. J., Benedict, C. R., Chacko, G. H., Phillips, M. C. & Rothblat, G. H. (1987) J. Biol. Chem. **262**, 12557–12564
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lalazar, A., Weisgraber, K. H., Rall, S. C., Jr., Giladi, H., Innerarity, T. L., Levanon, A. Z., Boyles, J. K., Amit, B., Gorecki, M., Mahley, R. W. & Vogel, T. (1988) J. Biol. Chem. **263**, 3542–3545
Marshall, S., Heidenreich, K. A. & Horikoshi, H. (1985) J. Biol. Chem. **260**, 4128–4135
McFarlane, A. S. (1958) Nature **182**, 53
Mendel, C. M., Kunitake, S. T. & Kane, J. P. (1986) Biochim. Biophys. Acta **875**, 59–68
Mendez, A. J., Oram, J. F. & Bierman, E. L. (1991) J. Biol. Chem. **266**, 10104–10111
Morré, D. J. (1971) Methods Enzymol. **22**, 130–148
Oppenheimer, M. J., Oram, J. F. & Bierman, E. L. (1988) J. Biol. Chem. **263**, 19318–19323
Oram, J. F. (1986) Methods Enzymol. **129**, 645–659
Oram, J. F., Johnson, C. J. & Brown, T. A. (1987) J. Biol. Chem. **262**, 2405–2410
Paavolo, L. G. & Strauss, J. F., III (1983) J. Cell Biol. **97**, 593–606
Powell, W. S., Hammerstrom, S. & Samuelsson, B. (1976) Eur. J. Biochem. **61**, 605–611
Rahim, A. T. M. A., Miyazaki, A., Morino, Y. & Horiuchi, S. (1991) Biochim. Biophys. Acta **1082**, 195–203
Rajan, V. P. & Menon, K. M. J. (1985) Endocrinology (Baltimore) **117**, 2408–2416
Rajan, V. P. & Menon, K. M. J. (1987) Biochim. Biophys. Acta **921**, 25–37
Rajendran, K. G., Hwang, J. & Menon, K. M. J. (1983) Endocrinology (Baltimore) **112**, 1746–1753
Rifici, V. A. & Eder, H. A. (1984) J. Biol. Chem. **259**, 13814–13818
Schreiber, J. R., Nakamura, K. & Weinstein, D. B. (1982) Endocrinology (Baltimore) **110**, 55–63
Slotte, J. P., Oram, J. F. & Bierman, E. L. (1987) J. Biol. Chem. **262**, 12904–12907
Strauss, J. F., III, MacGregor, L. C. & Gwynne, J. T. (1982) J. Steroid Biochem. **16**, 525–531
Talavera, F. & Menon, K. M. J. (1989) Endocrinology (Baltimore) **125**, 2015–2021
Toth, I. E., Szabo, D., Bacsy, E., Szalay, K. S., Hesz, A. & Szollar, L. G. (1986) Mol. Cell. Endocrinol. **44**, 185–194
Toth, I. E., Szabo, D., Szalay, K. S., Gyevai, A., Szollar, L. G. & Glaz, E. (1988) Clin. Biochem. **21**, 101–105
Tozuka, M. & Fidge, N. (1989) Biochem. J. **261**, 239–244
Van Zoelen, E. J. J. (1989) Biochem. J. **262**, 549–556
Wessel, D. & Flugge, U. I. (1984) Anal. Biochem. **138**, 141–143
Wu, Y. Q. & Handwerger, S. (1991) Program Annu. Meet. Endocr. Soc. 73rd p. 58 (abstr.)
Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L. & Russell, D. W. (1984) Cell **39**, 27–38

Received 12 February 1992/16 April 1992; accepted 7 May 1992