Isolation of a Porcine Liver Plasma Membrane Fraction That Binds Low Density Lipoproteins†

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ABSTRACT: Large amounts of injected radiolabeled low density lipoproteins have been found by others to accumulate primarily in the liver and studies in various types of isolated cells, including hepatocytes, have indicated the presence of specific cell membrane recognition sites for lipoproteins. In the present studies, the high affinity binding of radiolabeled low density lipoproteins ([^{125}I]LDL, d 1.020–1.063 g/mL) was measured in the major subcellular fractions of porcine liver homogenates. The nuclear and mitochondrial fractions were 1.9- and 1.4-fold enriched in binding activity with respect to unfractionated homogenates and contained 15% and 12% of the total binding activity, respectively. The microsomes, which contained most of the plasma membranes and endoplasmic reticulum, were approximately 4-fold enriched in binding and contained 73% of the binding activity. Microsomal subfractions obtained by differential homogenization and centrifugation procedures were 5.6–7.0-fold enriched in LDL binding and contained 54–58% of the homogenate binding activity. They were separated by discontinuous sucrose density gradient centrifugation into fractions which contained "light" and "heavy" plasma membranes and endoplasmic reticulum. The heavy membrane fraction was 2–4-fold enriched in binding with respect to the parent microsomes (16–22-fold with respect to the homogenate). There was no enrichment of binding activity in the other two fractions. Two plasma membrane "marker" enzymes, nucleotide pyrophosphatase and 5′-nucleotidase, were also followed. Of the two, binding in the sucrose density gradient subfractions most closely followed nucleotide pyrophosphatase, which was also most highly enriched (3.2–3.3-fold) in the heavy membrane fraction, but did not follow it exactly. The enzyme was 2-fold richer in the light membranes than in the parent microsomes, though the light membrane binding activity was only 0.4–1.4 times that of the parent microsomes. High affinity binding was time and temperature dependent, saturable, and inhibited by unlabeled low density lipoproteins but not by unrelated proteins. Binding was stimulated 2–3-fold by Ca^{2+}, was not affected by treatment with Pronase or trypsin and was inhibited by low concentrations of phospholipids and high density lipoproteins (HDL). Heparin–Mn^{2+} treatment of HDL did not affect its ability to inhibit [^{125}I]LDL binding. The LDL recognition site was distinct from the liver membrane asialoglycoprotein receptor; LDL binding was not inhibited by desialidated fetuin. We conclude that porcine liver contains a high affinity binding site that recognizes features common to both pig low density and high density lipoproteins. Further studies may elucidate the significance of this binding site in lipoprotein metabolism.

Plasma low density lipoproteins (LDL,[1] density 1.006–1.063 g/mL) transport most of the circulating cholesterol in man and are thought to be products of the degradation of very low density lipoproteins (VLDL, d < 1.006 g/mL) (Bilheimer et al., 1972). LDL appear to have a role in the control of cholesterol biosynthesis in a number of extrahepatic tissues (Andersen & Dietschy, 1976; 1977; Balasubramaniam et al., 1977a,b) and in cultured cells (Brown et al., 1973; Brown & Goldstein, 1976; Kayden et al., 1976; Ho et al., 1976; Weinstein et al., 1976; Assmann et al., 1975) and are degraded in the process (Weinstein et al., 1976; Goldstein & Brown, 1974). However, the major site of LDL removal in vivo is unknown and it is unclear whether LDL degradation is secondary to its regulatory function, or primarily proceeds as a separate process.

Specific high affinity recognition sites for LDL have been found on the surface of normal human fibroblasts (Anderson et al., 1976; Goldstein & Brown, 1977). LDL bind to these receptors, are internalized, and degraded in the lysosomes. The cholesterol released is available to satisfy cellular requirements. Cholesterol synthesis by the cell is suppressed and incoming cholesterol in excess of the cell's needs is esterified for storage (Goldstein & Brown, 1977). The receptor itself appears to be protein or glycoprotein and its importance in cellular LDL and cholesterol metabolism is indicated by the observation that fibroblasts from familial hypercholesterolemic patients, in which a functional LDL recognition site is absent or its function is impaired (Brown & Goldstein, 1976; Goldstein & Brown, 1974, 1977; Goldstein et al., 1975) are unable to internalize or degrade the lipoproteins which results in altered cholesterol metabolism by those cells.

When [^{125}I]LDL is injected into normal rats or swine, it rapidly accumulates in the liver (Hotta & Chaikoff, 1955; Sniderman et al., 1975; Calvert et al., 1975), which appears to constitute the major extravascular pool of LDL at all times after injection (Sniderman et al., 1975). Very little labeled LDL is taken up by any other tissue. Nakai et al. (1976) have recently demonstrated high affinity binding and degradation of high density lipoproteins (HDL_3 d 1.12–1.21 g/mL) by rat liver parenchymal cells. These observations suggested that the liver might absorb lipoproteins by a mechanism similar to that in fibroblasts, that is, by initial binding to specific recognition sites on the liver cell membrane. We have previously suggested that a homologous porcine system of serum LDL and isolated liver cell membranes might be useful in studying cell membrane lipoprotein interactions (Bachorik et al., 1976). This report further describes the specific high affinity binding of porcine LDL to pig liver membranes and the isolation of a

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[1] Abbreviations used: Tos-PheCH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; EDTA, disodium ethylenediaminetetraacetate; LDL and HDL, low and high density lipoproteins, respectively.

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plasma membrane fraction rich in LDL binding activity from liver microsomes.

Materials and Methods

Materials.

Blood and liver were obtained from freshly sacrificed adult swine at a local slaughterhouse. Blood was allowed to coagulate at room temperature and the clot was removed by filtration through four layers of cheesecloth, followed by centrifugation at 2000*g* for 30 min at 4 °C. Total serum cholesterol and triglyceride concentrations were determined (*Lipid Research Clinics Manual of Laboratory Operations,* 1974) and found to be within the normal range (Mahley et al., 1975). The serum was also examined by agarose gel electrophoresis for the presence of HDL_c, a class of high density lipoproteins that is induced in pigs by feeding a diet high in cholesterol (Mahley, et al., 1975). No HDL_c was detected in the serum used for the experiments.

Liver (20–25 g) was immediately cut into 0.5–1-cm pieces and immersed in ice-cold Krebs–Ringer phosphate buffer, pH 7.4, or in 0.25 M sucrose buffered to pH 8.0 with 5×10^{-3} M Tris-Cl, for transport to the laboratory.

NADH, 5′-AMP, and yeast alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Fetuin was obtained from Sigma Chemical Co., St. Louis, Mo. Neuraminidase (*Clostridium perfringins,* EC 3.2.1.18), covalently attached to agarose, was obtained from the same source and was equilibrated with 0.11 M Tris–maleate buffer, pH 5.3, for use. Tos-PheCH_2Cl-treated trypsin was purchased from Worthington Biochemicals, Freehold, N.J. Pronase was obtained from Calbiochem, San Diego, Calif., and was self-purified before use according to the procedure of Awad et al. (1972). Bovine lecithin (99%) was purchased from Supelco, Bellefonte, Pa., and bovine brain sphingomyelin (98–99%) was obtained from Miles Laboratories, Inc., Kankakee, Ill. Glucose 6-phosphate and horse heart cytochrome *c* were purchased from Sigma Chemical Co., St. Louis, Mo. ^{125}I was obtained from New England Nuclear, Boston, Mass. Rabbit antisera to porcine serum and porcine LDL were prepared as described by Fidge (1973).

Methods.

Isolation of Porcine Lipoproteins. VLDL ($d < 1.006$ g/mL), LDL (d 1.020–1.063 g/mL), and HDL (d 1.12–1.16 or d 1.12–1.21 g/mL) were isolated and purified by ultracentrifugation according to established procedures (Havel et al., 1955; Mahley & Weisgraber, 1974) using KBr for density adjustment. They were dialyzed against 8 L (two changes, 4 L each) of 0.05 M Tris-Cl, pH 7.4, containing 5×10^{-4} M EDTA and their purity was tested by agarose gel electrophoresis and immunoelectrophoresis. Agarose gel electrophoresis was performed by a modification of the method of Noble (1968). Electropherograms were stained with Sudan Black B or Coomassie Brilliant Blue R-250, to detect contamination with other serum lipoproteins or proteins, respectively. Immunoelectrophoresis was performed using antisera to pig serum and pig LDL.

VLDL and LDL were free of detectable contaminants. Most, but not all preparations of HDL isolated between d 1.12 and 1.21 g/mL contained small amounts of an unidentified component which had a somewhat slower electrophoretic mobility than HDL on agarose gel electrophoresis. This component stained for protein but not for lipid. It was confined to the density range 1.16–1.21 g/mL, and could not be separated from HDL by gel filtration on Sepharose 6B. The protein was not characterized further. The HDL used in the experiments reported here was therefore isolated between densities 1.12 and 1.16 g/mL. It was free of contamination by the unidentified protein as well as by the other serum proteins and lipoproteins.

Radioiodination of Porcine LDL. LDL was radiolabeled with ^{125}I according to the procedure of McFarlane (1958), as modified by Bilheimer et al. (1972), and freed of unreacted label by gel filtration on Sephadex G-25 followed by dialysis against 12 L (three changes, 4 L each) of 0.15 M NaCl containing 0.05% EDTA, pH 7.4. The [^{125}I]LDL was >99% precipitable with trichloroacetic acid and 95% precipitable with antiserum to porcine LDL. The various preparations used had specific activities between 200 and 300 cpm/ng LDL protein, and 2–3% of the total label was associated with LDL lipids.

Preparation of Liver Membranes. Binding experiments were performed with three kinds of preparations: (1) a crude plasma membrane enriched fraction obtained by low-speed centrifugation of liver homogenates; (2) microsomes and other major cell fractions obtained from liver homogenates; and (3) submicrosomal fractions obtained by differential centrifugation and sucrose density centrifugation.

Crude Plasma Membrane Enriched Fraction. Liver (4 g) was added to 40 mL Krebs–Ringer phosphate buffer, pH 7.4, homogenized with a Polytron homogenizer (Brinkman Instruments, Westbury, N.Y.), and centrifuged at 600*g* for 10 min at 4 °C. The pellet was discarded and the supernatant fraction was centrifuged at 4000*g* for 10 min. The resulting pellet was washed and resedimented four times using the same buffer, twice at 4000*g* for 10 min and twice at 10 000*g* for 5 min. The final preparation was resuspended in Krebs–Ringer phosphate buffer for use.

Isolation of Major Cell Fractions. The liver fractionation procedure was modified from the method of Touster et al. (1970). All manipulations were performed at 2–6 °C. Liver (10–20 g) was finely minced on an ice-cold glass plate with the aid of a razor blade. The mince was added to 3 volumes of 0.25 M sucrose buffered with 5×10^{-3} M Tris, pH 8.0, mixed with a spatula, and expressed either through four layers of cheesecloth or the coarse sieve of a tissue press to remove fibrous material. The suspension was then homogenized in a 55-mL glass homogenizer with one stroke of a Teflon pestle rotating at approximately 1000 rpm. The homogenate was centrifuged at 1000*g* for 10 min at 2 °C and two fractions were obtained, the postnuclear supernatant fraction and the nuclear pellet (Figure 1). The nuclear pellet was washed by resuspension in the homogenizing medium, and rehomogenized with one stroke of the slow rotating pestle. The suspension was then centrifuged at 1000*g* for 10 min. The pellet was similarly washed three additional times and is designated "washed nuclear pellet" (Figure 1). The washings were combined but kept separate from the original postnuclear supernatant fraction (Figure 1). The postnuclear supernatant fraction and the combined nuclear washes were then centrifuged at 10 000*g* for 25 min to sediment the mitochondrial pellets (Figure 1). The two mitochondrial pellets were kept separate. Each was washed twice by resuspension in homogenizing medium and rehomogenization as described above. The mitochondrial pellet washes were added to their respective post-mitochondrial supernatant fractions, and the fractions were centrifuged at 78 000*g* for 90 min to sediment microsomes (Figure 1). The microsomal and mitochondrial fractions isolated from the postnuclear supernatant fraction are designated microsomes I and washed mitochondria I, respectively. The corresponding fractions isolated from the combined nuclear pellet washes are termed micro-

somes II and washed mitochondria II (Figure 1). The four fractions were analyzed separately. The two 78 000*g* supernatant fractions were combined and are designated "Combined Soluble Fraction" (Figure 1). The washed nuclear pellet and the different mitochondrial and microsomal fractions were resuspended in cold Tris buffer (5×10^{-3} M, pH 8).

Subfractionation of Microsomes. A rapidly sedimenting subfraction of microsomes I was obtained as follows. The mitochondrial pellet was removed from the postnuclear fraction of the liver homogenate as described above, and the resulting post-mitochondrial supernatant fraction was centrifuged at 78 000*g* for 15 min. The microsomal pellet obtained is designated 15′ microsomes I.

Microsomes I and 15′ microsomes I were further subfractionated by discontinuous sucrose density centrifugation. All sucrose solutions were buffered with Tris-Cl (5×10^{-3} M, pH 8.0). Sucrose (57% w/w) was slowly added with constant mixing to a suspension of microsomes in 5×10^{-3} M Tris, pH 8.0. The final mixture contained 52–53% sucrose and 1–2 mg/mL microsomal protein. Ten milliliters of this mixture was overlayed with 10 mL of 34%, 10 mL of 30%, and approximately 9 mL of 0.25 M sucrose and centrifuged at 78 000*g* for 16 h at 2 °C in a swinging bucket rotor (Beckman type SW-27). The microsomes separated into three subfractions which accumulated at the tops of the 30, 34, and 53% layers. Except as indicated in the text, they were collected by pump from the top of the tube. The subfractions of microsomes I were diluted with 5×10^{-3} M Tris, pH 8.0, and harvested by centrifugation at 78 000*g* for 90 min at 2 °C. Subfractions of 15′ microsomes I were similarly diluted and harvested except the centrifugation time was reduced to 30 min. Each of the membrane fractions was then suspended in a convenient volume of 5×10^{-3} M Tris-Cl, pH 8.0, for use.

Enzyme Assays. The plasma membrane "marker" enzymes, nucleotide pyrophosphatase and 5′-nucleotidase, were used to estimate the enrichment of plasma membranes in the various cell fractions. Glucose-6-phosphatase and NADH:cytochrome *c* reductase served as markers for rough and smooth endoplasmic reticulum, respectively, and cytochrome oxidase was used as a mitochondrial marker.

Nucleotide pyrophosphatase was assayed at 37 °C, pH 9.5, as suggested by Solyom & Trams (1972). Reduced nicotinamide mononucleotide produced from NADH was determined spectrophotometrically after oxidation of the remaining substrate using alcohol dehydrogenase (Jacobson & Kaplan, 1957). Glucose-6-phosphatase and 5′-nucleotidase were determined at pH 6.5 and 9.0, respectively, as described by Solyom & Trams (1972). The inorganic phosphate released from glucose 6-phosphate and 5′-AMP was determined by the method of Bartlett (1959). Cytochrome oxidase was measured at 24 °C as described by Applemans et al. (1955), using reduced horse heart cytochrome *c* prepared as described by Cooperstein & Lazarow (1951). NADH:cytochrome *c* reductase was determined by a modification of the method of Mackler & Green (1956). All enzymatic activities are expressed as μmol of product generated per h per mg/mL enzyme protein except cytochrome oxidase and NADH:cytochrome *c* reductase, which are expressed as ΔA_{550nm} per minute per mg/mL enzyme protein.

[^{125}I]LDL Binding Assays. Binding assays were performed as described previously (Bachorik et al., 1976). Membranes were incubated with [^{125}I]LDL (5 μg/mL) in 150 or 300 μL of Krebs-Ringer phosphate buffer, pH 7.4, containing bovine serum albumin (40 mg/mL) to minimize nonspecific interactions. Incubations were performed at 23 °C with and without unlabeled LDL (500 μg/mL). High affinity binding was estimated as the difference between the amount of [^{125}I]LDL associated with the membranes in the presence and absence of the unlabeled lipoprotein. Membranes were excluded from control incubations. After the incubation the membranes were reisolated and washed by centrifugation through a layer of 0.25 M sucrose in Krebs-Ringer phosphate buffer, pH 7.4, containing bovine serum albumin (40 mg/mL). When the incubations were performed with the crude plasma membrane enriched fraction (600–4000*g*) isolated from Polytron homogenates, they were harvested from the assay medium by centrifugation at 10 000*g* for 5 min at room temperature. When binding was assayed in the subcellular and submicrosomal fractions, they were separated from the assay system by centrifugation at 105 000*g* for 90 min at 2 °C. 15′ microsomes I and its subfractions were separated in a similar manner except the centrifugation time was reduced to 30 min.

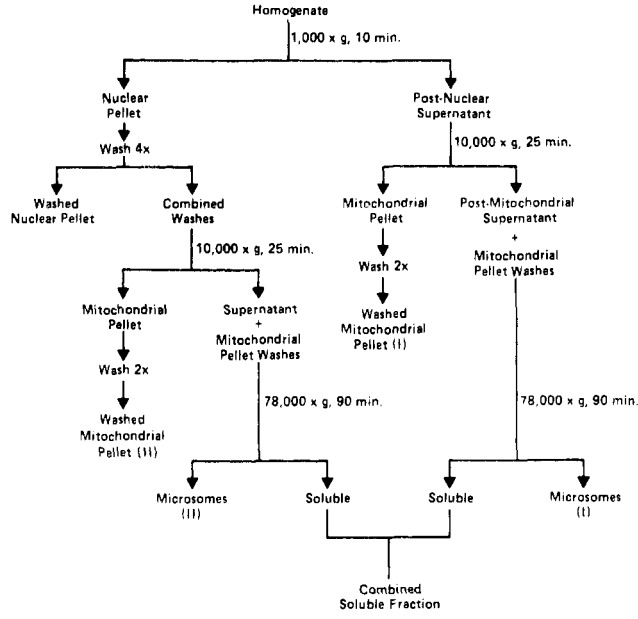

FIGURE 1: Schematic representation of the fractionation of porcine liver homogenate.

In several experiments with the heavy membrane fraction isolated from 15′ microsomes I (see below), the membranes were reisolated by filtration through 0.22-μm filters (Millipore Corp., Bedford, Mass.) followed by washing seven times with cold Krebs-Ringer phosphate buffer containing bovine serum albumin. The filters were prepared for use by soaking overnight with pooled human plasma containing 1–2 mg/mL LDL protein to minimize adherence of labeled LDL. 15′ microsomes were completely retained by the filters.

Preparation of Desialofetuin. Fetuin (6 mg) was incubated with insolubilized neuraminidase (0.8 U) in 0.11 M Tris-maleate buffer, pH 5.3, for 2.5 h at 37 °C, eluted with 4 volumes of 0.1 M Tris-Cl, pH 7.2, and dialyzed against 5×10^{-3} M Tris-Cl, pH 7.2. The treatment removed 75% of the sialic acid from the molecule.

Preparation of Lipids. Phosphatidylcholine (1 mg) or sphingomyelin (0.6 mg), dissolved in 60 μL of methanol, were rapidly added to 1–1.5 mL of Krebs-Ringer phosphate buffer, pH 7.4, containing bovine serum albumin, 40 mg/mL. The resulting mixtures were clear or slightly turbid and were centrifuged at 10 000*g* for 30 min. Lipid concentrations were established by determination of the phosphate content (Bartlett, 1959) of extracts (Folch et al., 1957) of the clear supernatant solutions.

Treatment of HDL with Heparin and $MnCl_2$. HDL (*d*

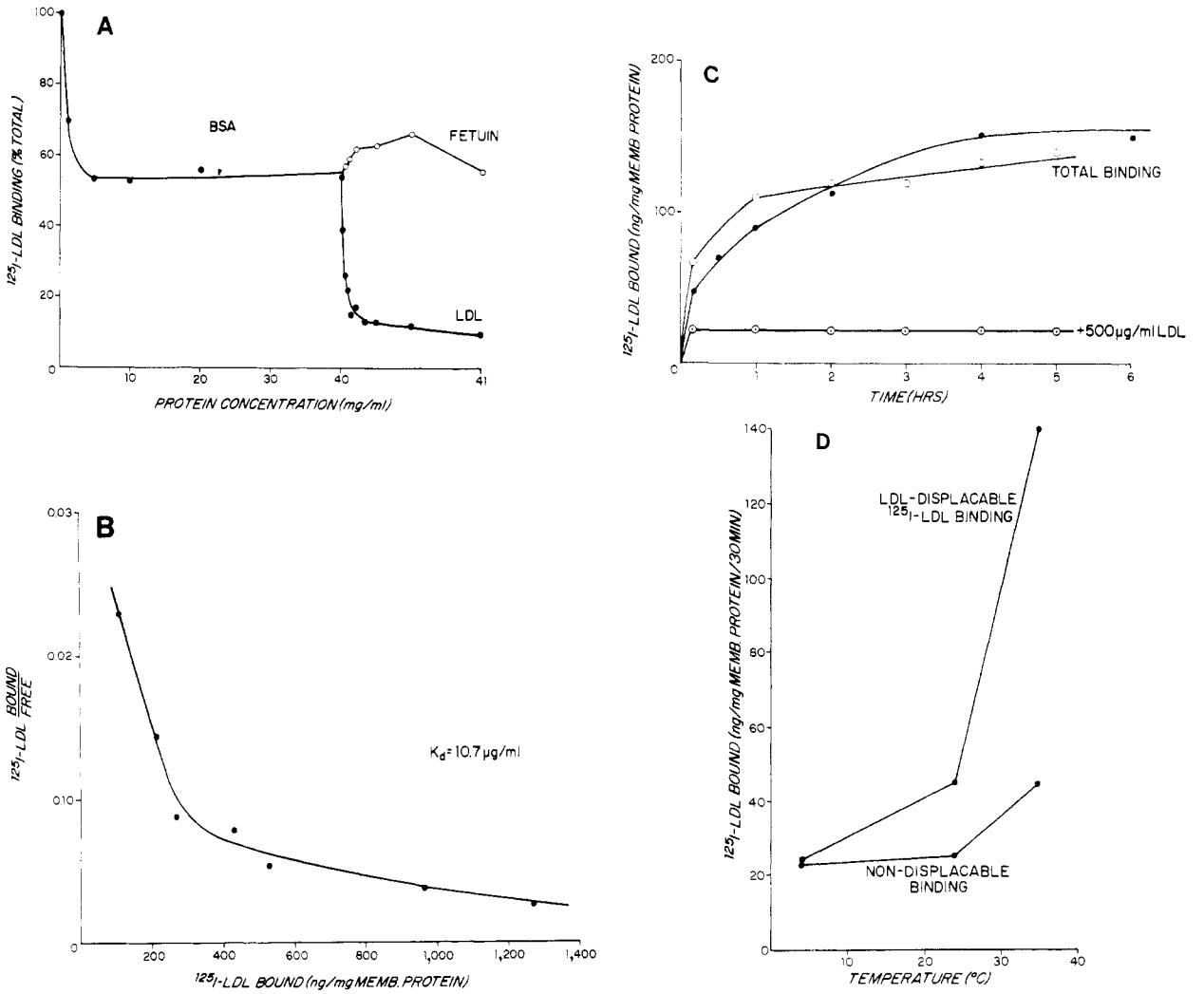

FIGURE 2: LDL binding characteristics of crude plasma membrane enriched fraction. (A) LDL specific and nonspecific binding. Composite of two experiments. Membranes (0.66 mg/mL membrane protein) were incubated with [^{125}I]LDL (5 µg/mL) at 23 °C for 4 h. In the first experiment the incubation medium contained increasing concentrations (0–40 mg/mL) of bovine serum albumin. In the second experiment the incubation medium contained 40 mg/mL bovine serum albumin and increasing concentrations (0–1 mg/mL) of either unlabeled LDL or fetuin. Results are expressed as % of total binding. One hundred percent is the amount of binding in the absence of either bovine serum albumin or unlabeled LDL. The protein concentrations indicated on the abscissa refer to bovine serum albumin in the range 0–40 mg/mL and bovine serum albumin + unlabeled LDL or fetuin in the range 40–41 mg/mL. (B) Scatchard plot of [^{125}I]LDL binding. Membranes (0.66 mg of membrane protein/mL) were incubated with [^{125}I]LDL (5–500 µg/mL) in a medium containing bovine serum albumin (40 mg/mL) for 4 h at 23 °C. (C) Time dependence of LDL-displaceable [^{125}I]LDL binding. Membranes were incubated in a medium containing bovine serum albumin (40 mg/mL) at 24 °C. The figure illustrates the results of two experiments: membrane protein concentration 1.0 mg/mL, [^{125}I]LDL, 6 µg/mL (●—●); membrane protein concentration 0.66 mg/mL, [^{125}I]LDL, 5 µg/mL in the presence (⊙—⊙), and absence (○—○) of unlabeled LDL, 500 µg/mL. (D) Temperature dependence of LDL-displaceable [^{125}I]LDL binding. Membranes (1.0 membrane protein/mL) were incubated with [^{125}I]LDL (5 µg/mL) in a medium containing bovine serum albumin (40 mg/mL) for 30 min. Nondisplaceable binding is that which could not be inhibited by unlabeled LDL (500 µg/mL). LDL-displaceable [^{125}I]LDL binding was calculated as the difference between total [^{125}I]LDL bound and nondisplaceable binding.

1.12–1.16 g/mL) was dialyzed against 0.15 M NaCl containing 2.6×10^{-3} M EDTA, pH 7.4. The lipoprotein (3.1 mg/mL) was incubated at 0 °C in the presence of heparin sulfate, 0.76 mg/mL and 0.032 M $MnCl_2$, for 30 min. The lipoprotein precipitate was collected by centrifugation at 1500*g* for 30 min, washed twice with a solution of heparin sulfate (0.76 mg/mL) and $MnCl_2$ (0.032 M), and redissolved in 1 mL of 10% $NaHCO_3$. Both the redissolved precipitate and the unprecipitated HDL fractions were freed of heparin sulfate and $MnCl_2$ as described by Mahley & Innerarity (1977). Seventy-three percent of the starting material was recovered. The conditions of treatment were sufficient to precipitate 76% of the HDL, based on recovered HDL protein.

Treatment of Membranes with Proteolytic Enzymes. Heavy membranes from 15′ microsomes I (800 µg of membrane protein) were incubated for 3.25 h at 37 °C with or without trypsin or Pronase (4.2%, w/w) in 500 µL of 0.05 M Tris-Cl, pH 8.0. The Pronase system also contained 5×10^{-3} M $CaCl_2$. The reaction was complete as judged by the increase in A_{280nm} of Cl_3CCOOH-soluble material. The reaction mixtures were quenched in an ice bath, and the membranes were harvested by centrifugation through a layer of 0.25 M sucrose containing 20 mg/mL bovine serum albumin, pH 7.4, for 30 min at 88 000*g*, and resuspended in bovine serum albumin, 20 mg/mL, pH 7.4. Binding assays were performed as described and membranes were reisolated from the assay systems by the filtration procedure. Calculations were based on the initial protein content of the membranes.

In another experiment, 575 µg of membrane protein was incubated for 4 h at 37 °C with or without trypsin (7.3%, w/w), in 500 µL of Tris-Cl, pH 7.0. The reaction was complete as judged by the increase in A_{280nm} of Cl_3CCOOH-soluble ma-

TABLE I: Distribution of [^{125}I]LDL Binding Activity in Fractions Isolated from Porcine Liver Homogenate.

fraction	protein		LDL-displaceable [^{125}I]LDL binding[a] (ng/mg of membrane protein)	purification	%	nucleotide pyrophosphatase act.[c]	purification	%	5′-nucleotidase act.	purification	%	glucose-6-phosphatase act.	purification	%	cytochrome oxidase act.	purification	%
	mg	%[b]																
homogenate	1791		47.2	1		1.20	1		0.62	1		2.19	1		2.67	1	
nuclear pellet	166	10.3	89.2	1.9	15.0	1.58	1.3	13.0	1.98	3.2	32.4	0.99	0.5	4.9	3.62	1.4	12.6
mitochondria[d]	189	11.8	66.2	1.4	11.9	0.61	0.5	5.7	0.03	0.1	0.4	0.32	0.2	1.7	17.42	6.5	69.6
microsomes	367	22.8	192.8	4.1	72.7	3.68	3.1	67.1	1.52	2.5	54.8	7.86	3.6	85.5	2.29	0.9	17.7
soluble	885	55.1				0.32	0.3	14.2	0.14	0.2	12.2	0.30	0.1	7.8	0	0	0
total act. recovered in all fractions (%)		90			115			94			91			86			99

[a] Calculated as the difference between [^{125}I]LDL bound in the presence and absence of unlabeled LDL (500 μg/mL). [b] Expressed as % of recoverable activity. [c] Enzyme activities are expressed as μmol per h per mg/mL cell fraction protein, except cytochrome oxidase, which is expressed as ΔA_{550nm} per min per mg/mL cell fraction protein. [d] Data for mitochondria and microsomes were calculated from assays performed individually on mitochrondria I and II and Microsomes I and II.

terial. The membranes were harvested as above, and the binding assays were performed with the ultracentrifugal procedure.

Other Procedures. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Apoproteins of porcine lipoproteins were prepared by extraction with ethanol:ether (3:1, v/v) (Brown et al., 1969) and examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Weber & Osborn, 1969).

Results

Studies with a Crude Plasma Membrane Enriched Fraction. The membrane fraction isolated from Polytron homogenates of liver between 600 and 4000*g* has been previously estimated to be four–fivefold enriched in plasma membranes (Bachorik et al., 1976) based on the activities of the "marker" enzymes nucleotide pyrophosphatase and 5′-nucleotidase. These preparations bound [^{125}I]LDL, but about half of the total binding was nonspecific when a background protein was not included in the incubation system (Figure 2A). Approximately 40% of the total binding was inhibited by including bovine serum albumin, 5 mg/mL, in the incubation system. Increasing the albumin concentration to 40 mg/mL had no further effect on the amount of [^{125}I]LDL bound. The inclusion of low concentrations of unlabeled LDL in incubation systems containing 40 mg/mL albumin caused a marked inhibition of [^{125}I]LDL binding. In contrast, similar concentrations of an unrelated protein, fetuin, had no effect on LDL binding (Figure 2A). The Scatchard plot (Figure 2B) of the concentration dependence of [^{125}I]LDL binding to the crude plasma membrane preparations revealed the presence of at least two binding components, a high affinity component with an apparent K_d of approximately 11 μg/mL, and a low affinity component. Total [^{125}I]LDL binding was time dependent (Figure 2C) and reached a maximum by 4 h. The nonspecific component of total binding, estimated as the amount of [^{125}I]LDL bound in the presence of excess unlabeled LDL, was maximal at the shortest time measured. The temperature dependence of high affinity [^{125}I]LDL binding is illustrated in Figure 2D. High affinity binding was markedly influenced by temperature, whereas the effect on nonspecific interactions was minimal. LDL-displaceable [^{125}I]LDL binding was linear with membrane concentration up to about 1 mg/mL membrane protein.

Distribution of [^{125}I]LDL Binding Activity among Liver Fractions. Liver was fractionated as described in Methods (Figure 1) into nuclear, mitochondrial I and II and microsomal I and II fractions. Each was assayed individually. The results of the analyses of fractions I and II were combined to give total mitochondrial and total microsomal activities. The results of one such experiment are shown in Table I. Over 70% of the LDL displaceable [^{125}I]LDL binding activity was found in the microsomal fraction and the enrichment of binding activity was the greatest in this fraction (Table I). This fraction also contained most of the plasma membranes and endoplasmic reticulum, as indicated by the activities of nucleotide pyrophosphatase, 5′-nucleotidase, and glucose-6-phosphatase.

The distribution of [^{125}I]LDL binding activity between microsomes I and II is shown in Table II. Over 70% of the total microsomal [^{125}I]LDL binding activity, 60% of the nucleotide pyrophosphatase activity, and about half the 5′-nucleotidase activity was found in microsomes I, whereas 60–73% of the glucose-6-phosphatase activity was found in microsomes II (Table II). The data indicate that most of the binding and nucleotide pyrophosphatase activities were released during the

TABLE II: Distribution of Porcine Liver Plasma Membranes among Microsomes I and II.

microsomes from	protein mg	protein %	LDL-displaceable [^{125}I]LDL binding[a] total act.[b] ng	%	nucleotide pyrophosphatase total act.[c] μmol/h	%	5′-nucleotidase total act. μmol/h	%	glucose-6-phosphatase total act. μmol/h	%
prep no. 1[d]										
total microsomes[e]	367	100	70 751	100	1350	100	557	100	2885	100
microsomes (I)	156	43	51 402	73	814	60	268	48	1167	40
microsomes (II)	211	57	19 349	27	536	40	289	52	1718	60
prep no. 2										
total microsomes	382	100	196 959	100	1121	100	395	100	3997	100
microsomes (I)	126	33	140 741	72	676	60	200	51	1076	27
microsomes (II)	256	67	19 349	28	445	40	195	49	2921	73

[a] Calculated as the difference between [^{125}I]LDL bound in the presence and absence of unlabeled LDL (500 μg/mL). [b] Calculated as the product of specific activity (ng/mg membrane protein) and amount of microsomal protein (mg). [c] Enzyme activities were calculated as the product of the specific activity (μmol per h per mg/mL membrane protein) and amount of microsomal protein (mg). [d] Same preparation as in Table I. [e] Microsomes I and II were assayed separately and the data were used to calculate the values for total microsomes.

TABLE III: Distribution of [^{125}I]LDL Binding Activity among Subfractions of Microsomes I and 15′ Microsomes I.

	prep no.	fraction	LDL-displaceable [^{125}I]LDL binding ng/mg of membrane protein[a]	purification[b]	nucleotide pyrophosphatase act.[a]	purification[b]	5′-nucleotidase act.	purification	glucose-6-phosphatase act.	purification
(A)										
	1	microsomes I	329.5	7.0	5.22	4.4	1.72	2.8	7.48	3.4
		light membranes	121.2	2.6	11.69	9.7	6.86	11.1	1.82	0.8
		heavy membranes	741.0	15.7	16.65	13.9	3.25	5.2	6.03	2.8
		endoplasmic reticulum	304.6	6.5	5.16	4.3	0.32	0.5	11.78	5.4
(B)										
	2	15′ microsomes I	294.4	5.6	6.50	7.7	1.25	3.4	4.94	2.2
		light membranes	307.5	5.8	14.00	16.7	3.86	10.4	4.90	2.2
		heavy membranes	1165.8	22.0	21.50	25.6	3.88	10.5	0.98	0.4
		endoplasmic reticulum	364.6	6.9	4.98	5.9	0.66	1.8	9.59	4.3
	3	15′ microsomes I	176.1	6.4	5.14	6.7	1.12	4.3	ND[c]	ND
		light membranes	241.9	8.8	11.93	15.6	3.90	15.0	ND	ND
		heavy membranes	472.1	17.1	16.66	21.6	1.95	7.5	ND	ND
		endoplasmic reticulum	229.2	8.3	5.57	7.2	0.32	1.2	ND	ND

[a] Binding and enzyme activities are expressed as described in the legend to Table I. [b] Purification is expressed with respect to the starting homogenate which was assigned a value of 1.0. [c] ND, not determined.

original gentle homogenization, whereas a larger part of the internal membranes initially sedimented with the nuclear pellet and was released during subsequent rehomogenization and washing of the nuclear pellet.

It was also observed that about 70% of the protein in microsomes I sedimented within 15 min at 78 000*g*. This fraction is designated 15′ microsomes I and contained an approximately equal proportion of the LDL displaceable [^{125}I]LDL binding and marker enzyme activities.

This finding, and those in Table II led to the use of 15′ microsomes I for many of the subsequent studies. The 15′ microsomes I were usually 5.5–7.5-fold enriched in plasma membranes and [^{125}I]LDL binding activity. They account for approximately 40% of the homogenate binding activity. The major advantage of using this fraction is that it can be more readily separated from the incubation system used for the binding assays; when 15′ microsomes I or its subfractions were used, the centrifugation time was reduced from 90 to 30 min.

[^{125}I]LDL Binding Activity of Microsomal Subfractions. Microsomes I and 15′ microsomes I were subjected to ultracentrifugation in discontinuous gradients of 53%–34%–30%–0.25 M sucrose. They each separated into several subfractions. Defined protein bands accumulated near the tops of the 30% and 34% sucrose layers and at the top of the 53% layer and are designated "light membranes", "heavy membranes", and endoplasmic reticulum, respectively. The 53% layer contained the greatest accumulation of membrane protein. There was also some membrane material distributed in the region between the 30% and 34% bands and below the 34% band as evidenced by slight turbidity in these areas. The enrichment of [^{125}I]LDL binding activity and plasma membrane marker enzyme activities in the three defined bands are shown in Table IIIA for microsomes I and in Table IIIB for 15′ microsomes I. The activity patterns were similar in both microsomal fractions. The "light membranes" and "heavy membranes" were enriched in plasma membranes as indicated by the purification of nucleotide pyrophosphatase and 5′-AMPase in these fractions (Table III). 5′-AMPase was more highly enriched in the light membrane fraction. The enrichment in this fraction was usually about twice that of the heavy membrane fraction, as seen in preparations 1 and 3 (Table III) and in several other preparations (not shown) in which we did not measure [^{125}I]LDL binding or nucleotide pyrophosphatase.

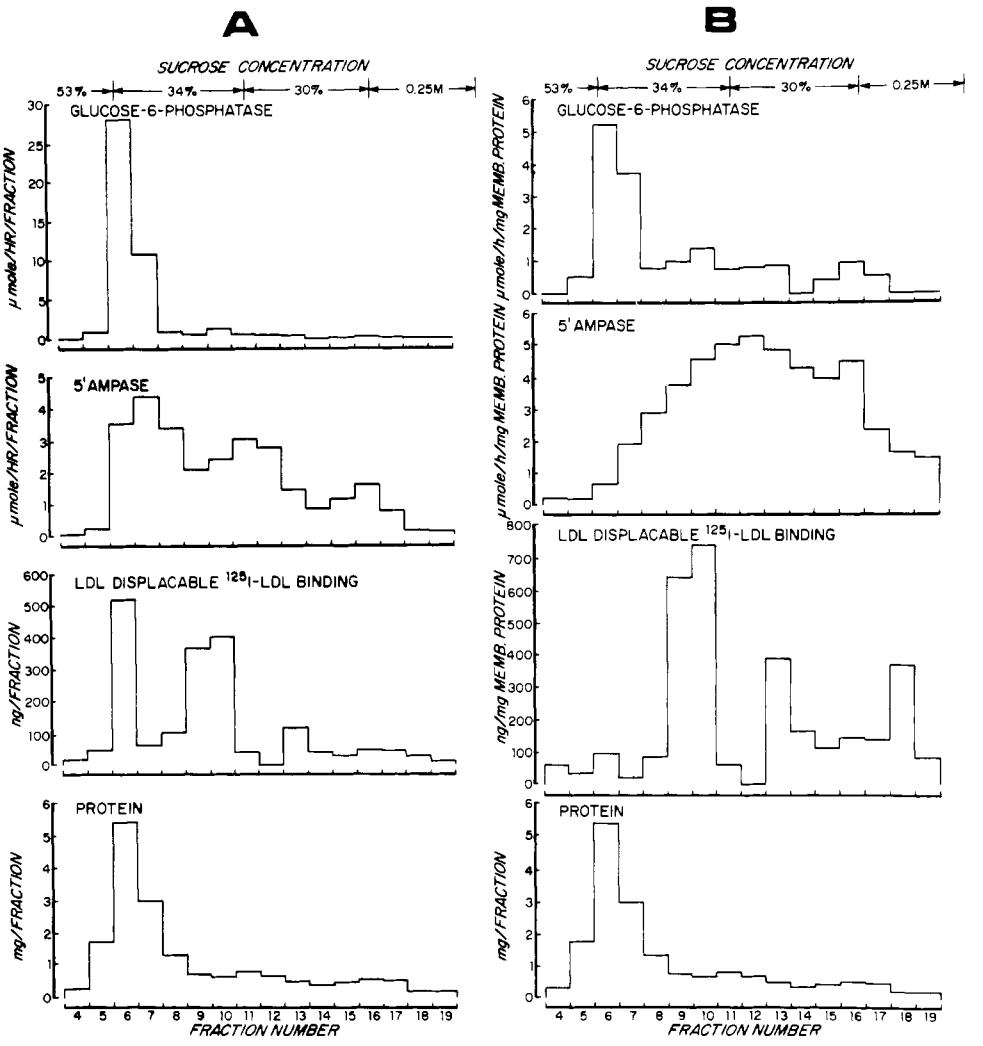

FIGURE 3: Distribution of (A) total LDL-displaceable [^{125}I]LDL binding, 5'-nucleotidase, and glucose-6-phosphatase activities and (B) specific activities of binding and the two enzymes among submicrosomal fractions. The 15' Microsomes I were separated on a discontinuous sucrose gradient as described in Methods and collected in equal fractions of approximately 2 mL, from the bottom of the tube. Enzyme and binding assays were performed as described in methods. LDL-displaceable [^{125}I]LDL binding is the difference between the amount of radioactivity associated with the membranes in the presence and absence of unlabeled LDL (500 μg/mL). The initial sucrose concentrations are indicated at the top of the figure.

The heavy membrane fraction was the most highly enriched in nucleotide pyrophosphatase activity and was the only fraction in which [^{125}I]LDL binding activity was enriched over that of the parent microsomes. The heavy membranes isolated from 15' microsomes I contained an approximate 20-fold enrichment of binding activity with respect to the original liver homogenates. The 53% sucrose band primarily contained internal membranes as indicated by the concentration of glucose-6-phosphatase in this fraction.

The quantitative distribution of [^{125}I]LDL binding activity among the submicrosomal fractions was estimated by assaying the entire sucrose gradient. The 15' microsomes I were subjected to discontinuous sucrose density gradient ultracentrifugation as described above. The entire gradient was collected in fractions from the bottom of the tube and the membranes from each fraction were harvested and assayed individually. As seen in Figures 3A and 4A, most of the protein and internal membranes accumulated at the top of the 53% sucrose layer as indicated by the presence of most of the glucose-6-phosphatase and NADH:cytochrome *c* reductase activities in this region. This layer also contained an appreciable quantity of plasma membranes as evidenced by its association with about half of the total 5'-AMPase and about 60% of the total nucleotide pyrophosphatase activities. The remainder of the two plasma membrane marker activities were found in two peaks corresponding to the light and heavy membrane regions of the gradient. The LDL displaceable [^{125}I]LDL binding activity, 62–65%, was found in the plasma membrane fractions and, of this, 75–80% was localized in the heavy membrane-containing part of the gradient.

The specific activity profiles in Figures 3B and 4B indicate that LDL-displaceable [^{125}I]LDL binding is most highly concentrated in the heavy membrane region, which also contains the greatest enrichment of nucleotide pyrophosphatase. Both nucleotide pyrophosphatase and 5'-AMPase activities were found throughout the 30% and 34% sucrose layers, indicating that the membrane material observed as turbidity between the defined light and heavy membrane bands and below the heavy membrane band also contained some plasma membranes. Rough endoplasmic reticulum was primarily confined to the 53% sucrose band as reflected by the concentration of glucose-6-phosphatase in this area (Figure 3B). NADH:cytochrome *c* reductase was found in the 53% sucrose band, in the region below the 34% band and in an area roughly corresponding to the 30% band, suggesting the presence of smooth endoplasmic reticulum membranes (Figure 4B). The specific activity of this enzyme was slightly greater in the light membrane region than in the other two but the enzyme did not

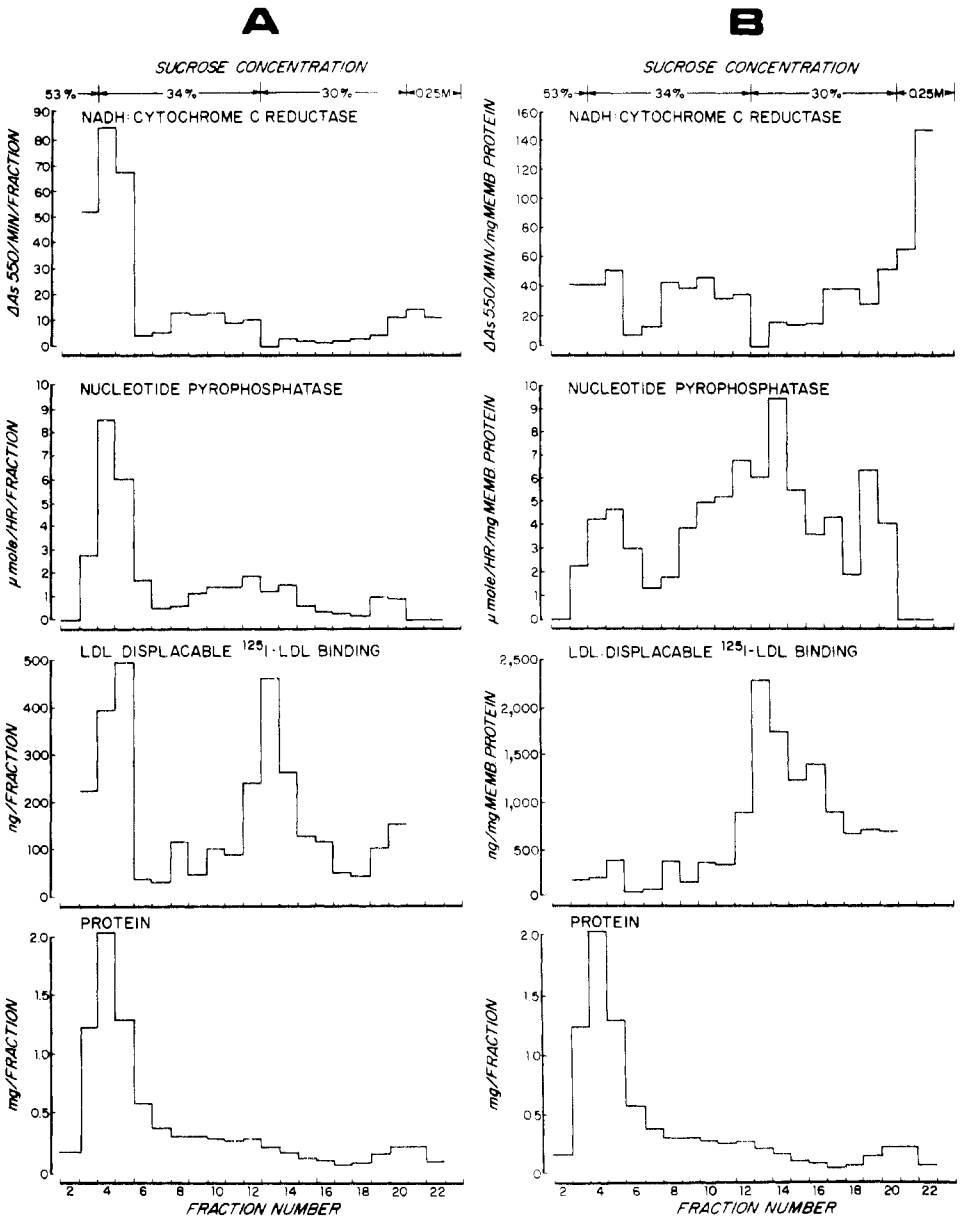

FIGURE 4: Distribution of (A) total LDL-displaceable [^{125}I]LDL binding, nucleotide pyrophosphatase, and NADH:cytochrome *c* reductase activities and (B) specific activities of binding and the two enzymes, among submicrosomal fractions. The 15′ Microsomes I were fractionated and assayed as indicated in the legend to Figure 3.

appear to significantly concentrate in any region.

Further Characterization of Heavy Membrane [^{125}I]LDL Binding. Heavy membrane preparations were examined to determine the extent to which [^{125}I]LDL binding in this fraction was similar to that observed in the crude membrane fractions prepared from Polytron homogenates of liver. The lipoprotein concentration dependence of [^{125}I]LDL binding to heavy membranes is illustrated in Figure 5A. Binding was saturable and was half-saturated at a lipoprotein concentration of about 10 μg/mL. Binding was markedly inhibited by low concentrations of unlabeled LDL, and HDL was as effective an inhibitor of [^{125}I]LDL binding as unlabeled LDL (Figure 5B). LDL displaceable [^{125}I]LDL binding was time dependent, whereas nondisplaceable binding did not show an appreciable time dependence (Figure 5C). Furthermore, LDL displaceable [^{125}I]LDL binding to heavy membranes was markedly influenced by temperature (Figure 5D), in contrast to nondisplaceable binding which was only minimally affected.

In view of the large nonspecific component of total binding observed in the crude plasma membrane fraction isolated from Polytron homogenates (Figure 2A), it was considered that the bovine serum albumin included as a background protein in the binding assay systems might bind to the membranes in such a way as to influence the subsequent findings with respect to *high affinity* binding. The binding of [^{125}I]LDL to heavy membranes was therefore measured in assay systems that contained either bovine serum albumin, human γ-globulins, or no background protein at all (Figure 6A). The total binding was about the same in all three systems, and low concentrations of unlabeled LDL caused a marked inhibition of [^{125}I]LDL binding whether or not a background protein was present. Furthermore, the residual binding in the presence of excess unlabeled LDL was approximately the same in all three systems. The results indicate (1) that the nonspecific component of heavy membrane binding is considerably less than that of the crude Polytron fraction, and (2) that high affinity binding is not a consequence of interactions between the background protein and the membranes.

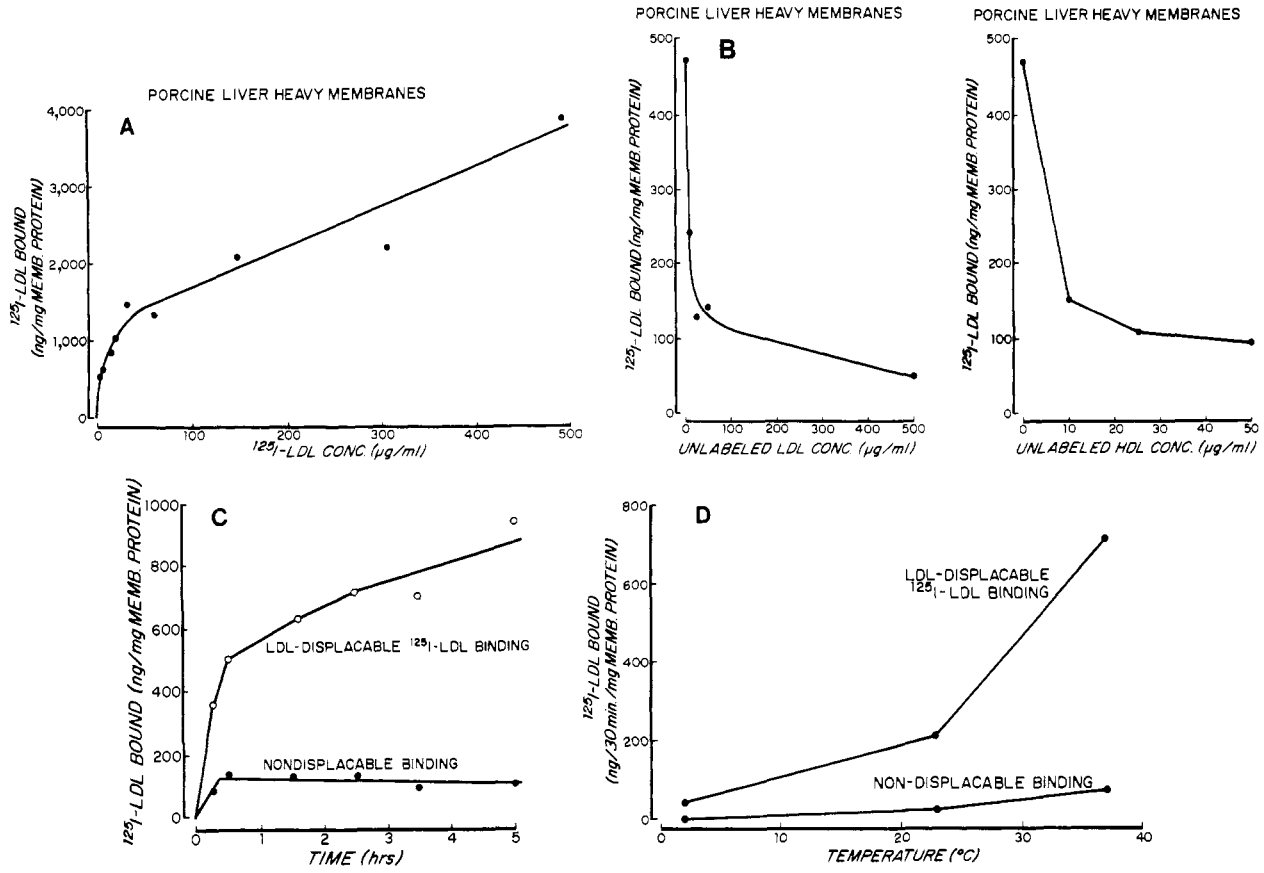

FIGURE 5: LDL-binding characteristics of porcine liver heavy membranes. (A) [^{125}I]LDL saturation curve. Membranes (0.27 mg/mL) were incubated for 4 h at 23 °C with [^{125}I]LDL (3-492 μg/mL). The incubation system also contained bovine serum albumin (40 mg/mL). (B) Inhibition of [^{125}I]LDL binding by unlabeled porcine LDL and HDL. Membranes (0.28 mg/mL) were incubated with [^{125}I]LDL (4 μg/mL) as described in A above. (Left panel) Inhibition by unlabeled LDL (10-500 μg/mL); (right panel) inhibition by unlabeled HDL (10-50 μg/mL). The incubation system also contained bovine serum albumin (40 mg/mL). (C) Time dependence of binding. Membranes (344 μg/mL) were incubated with [^{125}I]LDL (4.7 μg/mL) at 23 °C in a medium containing bovine serum albumin (40 mg/mL). LDL-displaceable [^{125}I]LDL binding is calculated as the difference between the amount of radioactivity associated with the membranes in the presence and absence of unlabeled LDL (500 μg/mL). Nondisplaceable binding is that in the presence of unlabeled LDL. (D) Temperature dependence of binding. Membranes (334 μg/mL) were incubated with [^{125}I]LDL (5 μg/mL) for 30 min in a medium containing bovine serum albumin (40 mg/mL). LDL-displaceable [^{125}I]LDL binding and nondisplaceable binding are as defined in C above.

Binding was not inhibited by up to 265 μg/mL of either intact fetuin, or that from which 75% of its covalently bound sialic acid had been removed, indicating that the LDL binding site is distinct from the hepatic membrane asialoglycoprotein receptor. Divalent cations stimulated, but were not absolutely required for binding. Ca^{2+} was more effective than Mg^{2+}, and stimulated binding two- to threefold at a concentration of 1×10^{-3} M (Table IV). Treatment of the membranes with either trypsin or Pronase had no effect on high affinity binding.

HDL Inhibition of [^{125}I]LDL Binding to Heavy Membranes. The inhibition of [^{125}I]LDL binding to heavy membranes by HDL was examined further to consider the possibility that its effect was due to the presence of small amounts of apoE in the HDL preparations. ApoE is a potent inhibitor of LDL binding in peripheral cell systems (see Discussion). Polyacrylamide gel electrophoretic analysis of HDL (*d* 1.12-1.16 g/mL) in the presence of sodium dodecyl sulfate failed to reveal detectable amounts of apoE (Figure 7). HDL was treated with heparin sulfate and $MnCl_2$ to remove trace amounts of apoE, if present. Similar electrophoretic analysis of both the precipitated and soluble subfractions failed to reveal traces of apoE in either fraction (Figure 7). Both subfractions effectively inhibited [^{125}I]LDL binding (Table V).

Phospholipid Inhibition of [^{125}I]LDL Binding. Phosphatidylcholine was a potent inhibitor of LDL binding at low concentrations and produced inhibition curves similar to those of LDL and HDL (Figure 6B). Sphingomyelin also inhibited binding, but was somewhat less effective. In other experiments (not shown), neither choline nor acetylcholine was an inhibitor.

Discussion

The present study describes the isolation of a plasma membrane enriched fraction from porcine liver microsomes, that is enriched about 20-fold in [^{125}I]LDL binding activity with respect to the starting homogenate. The isolation scheme that was finally adopted was developed by following LDL-displaceable [^{125}I]LDL binding and various marker enzymes in the major cell fractions obtained from liver homogenates by differential centrifugation. [^{125}I]LDL binding activity was primarily located in the microsomes, which also contained most of the plasma membranes and endoplasmic reticulum. The binding observed in the nuclear and mitochondrial fractions can be attributed, at least in part, to contamination with microsomal membranes, but the data do not exclude binding to organelles other than microsomes. It is interesting that Pricer & Ashwell (1976) have recently demonstrated the presence of the rat liver plasma membrane asialoglycoprotein receptor in several other cell organelles including the Golgi complex, the lysosomes, and the smooth microsomes. We had observed in early experiments that the [^{125}I]LDL binding activity of microsomes was somewhat greater when the nuclear pellet

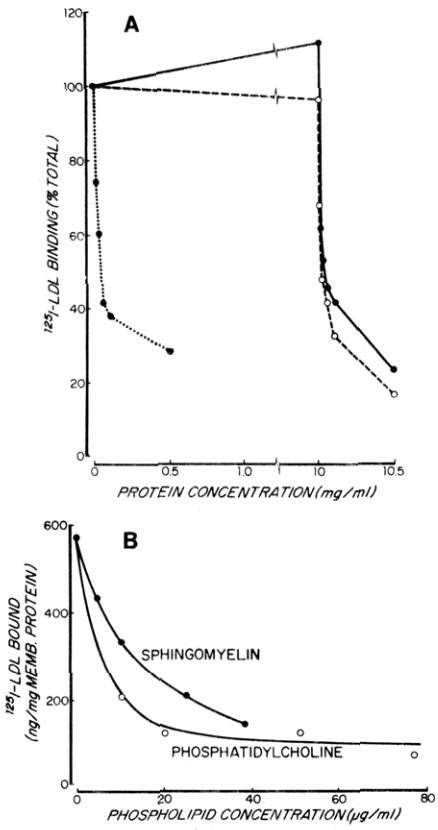

FIGURE 6: (A) Effect of background protein on [^{125}I]LDL binding to heavy membranes. Membranes (0.26 mg/mL membrane protein) were incubated at 23 °C for 4 h with [^{125}I]LDL (5.3 μg/mL) and increasing concentrations (0–0.5 mg/mL) of unlabeled LDL in the absence of background protein (●···●), or in the presence of 10 mg/mL bovine serum albumin (●—●), or 10 mg/mL human γ-globulins (○···○). One hundred percent is the amount of binding in the absence of either background protein or unlabeled LDL. The protein concentrations indicated on the abscissa refer to unlabeled LDL in the system without background protein, or to background protein + unlabeled LDL in the other two systems. Membranes were isolated from the binding assay system using the filtration procedure described in Methods. (B) Inhibition of [^{125}I]LDL binding by phospholipids. Heavy membranes were incubated with [^{125}I]LDL (3.7 μg/mL) for 3 h at 23 °C in the presence of increasing concentrations of phosphatidylcholine (○—○) or sphingomyelin (●—●).

TABLE IV: Effect of Divalent Cations on [^{125}I]LDL Binding to Heavy Membranes.[a]

addition	LDL-displaceable [^{125}I]LDL binding (ng/mg of membrane protein)	rel act.
A. none	419	1.0
Ca^{2+}, 1×10^{-3} M	848	2.0
Mg^{2+}, 1×10^{-3} M	572	1.4
B. none	286	1.0
Ca^{2+}, 1×10^{-3} M	793	2.8
C. none	289	1.0
EDTA, 1×10^{-3} M	257	0.9

[a] Membranes were incubated with [^{125}I]LDL (4.8–6.3 μg/mL) for 3 h at 23 °C in 5×10^{-3} M Tris-Cl, pH 7.4, containing 10 mg/mL bovine serum albumin and the additions indicated. Membranes were isolated from the assay system using the ultracentrifugal (A and B) or filtration (C) procedures described in Methods.

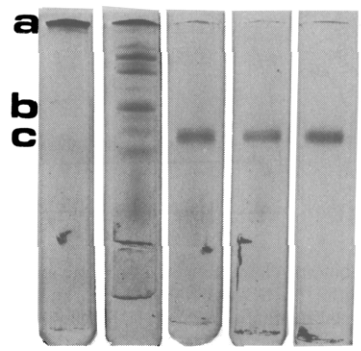

FIGURE 7: Polyacrylamide gel electrophoresis of porcine apolipoproteins. Porcine apolipoproteins were subjected to polyacrylamide gel electrophoresis in 10% gels in the presence of 0.1% sodium dodecyl sulfate. (Left to right) Apoproteins from: LDL (d 1.020–1.063 g/mL), VLDL ($d <$ 1.006 g/mL), heparin-Mn^{2+}-precipitable subfraction of HDL (d 1.12–1.16 g/mL), heparin-Mn^{+2} soluble subfraction of HDL, parent HDL (d 1.12–1.16 g/mL). LDL and VLDL gels contained 40 μg of protein; HDL gels contained 20 μg of protein. Protein bands are identified by comparison with electropherograms presented by Mahley et al. (1975): (a) apoB; (b) apoE; (c) apoA-I.

washes were not added to the post-nuclear supernatant fraction prior to isolation of microsomes. This finding suggested that the lipoprotein-binding membranes were released relatively readily during the initial gentle homogenization and that membranes subsequently released by rehomogenizing and washing the nuclear fraction had less binding activity. This was confirmed by isolating microsomes I and II from the post-nuclear supernatant fraction and the combined nuclear washes respectively. Most of the binding activity was released into the post-nuclear supernatant by the initial homogenization, accompanied by the majority of the microsomal nucleotide pyrophosphatase activity and about half of the 5′-nucleotidase. Microsomes II, released by repeated washing of the nuclear pellet, contained more glucose-6-phosphatase activity. The overall pattern of enzymatic and binding activities suggests that plasma membranes are more easily released than internal membranes and that the binding activity behaves similarly.

When Microsomes I were subfractionated by discontinuous sucrose density centrifugation, two plasma membrane fractions were obtained. The light membrane fraction was usually about twice as enriched in 5′-nucleotidase as the heavy membranes, whereas the latter were 1.4–1.5-fold richer in nucleotide pyrophosphatase. Only the heavy membranes were enriched in LDL-displaceable [^{125}I]LDL binding activity with respect to the unfractionated microsomes. Lutz & Frimmer (1970) obtained a light and a heavy plasma membrane fraction from porcine liver homogenates by a different procedure, and found a similar separation of nucleotide pyrophosphatase and 5′-AMPase activities. Light and heavy plasma membrane fractions have also been isolated from rat and mouse liver (Evans, 1969, 1970; Gurd et al., 1972; Evans & Gurd, 1971), and Evans (1970) has suggested that the light membranes originate primarily from the bile canalicular surface of the cell. Heavy membranes from rat and mouse liver have a higher insulin-binding activity than light membranes, and Evans et al. (1973) therefore concluded that the heavy fraction is enriched in membrane fragments derived from blood sinusoidal surface of the cell. If the same is true of heavy membranes from porcine liver, the [^{125}I]LDL binding activity is most concentrated in the fraction enriched in fragments derived from that portion of the cell membrane normally exposed to the circulating lipoproteins.

It was noted that, while the enrichment of binding activity followed the enrichment of nucleotide pyrophosphatase during

TABLE V: Effect of Heparin-$MnCl_2$ Treatment of HDL on Inhibition of [^{125}I]LDL Binding to Heavy Membranes.[a]

addition	[^{125}I]LDL bound (ng/mg of membrane protein)	%
A. none	747	100
HDL, 10 μg/mL	444	59
heparin-Mn^{2+} soluble, 10 μg/mL	329	44
heparin-Mn^{2+} precipitable, 10 μg/mL	497	67
B. none	760	100
HDL, 11 μg/mL	347	46
heparin-Mn^{2+} soluble, 11 μg/mL	321	42
heparin-Mn^{2+} precipitable, 12 μg/mL	392	52

[a] HDL preparations are those indicated in the legend to Figure 7. (A) Heavy membranes (229 μg/mL membrane protein) were incubated with [^{125}I]LDL (5.0 μg/mL) for 3 h at 23 °C in the presence of the indicated additions, and were reisolated using the filtration procedure described in Methods. (B) Heavy membranes (239 μg/mL) were incubated with [^{125}I]LDL (4.9 μg/mL) as above and were reisolated using the ultracentrifugal procedure described in Methods.

the gross fractionation of the liver, and most closely followed this enzyme when the microsomes were subfractionated, the two activities were not quite parallel in the submicrosomal fractions. The light membranes were always enriched in nucleotide pyrophosphatase with respect to the starting microsomes, but not in binding activity. One explanation is that binding activity could be associated with an internal membrane fraction that accumulates with heavy plasma membranes. The present study does not rule out this possibility, but the fact that binding activity is rather easily released by gentle homogenization makes it less likely. Another consideration is that marker enzymes are at best imperfect indicators of plasma membrane identity and this enzyme may simply not be an ideal marker for that portion of the cell membrane that contains lipoprotein binding activity. The use of other presumably more specific markers, such as insulin binding (Chang et al., 1975), may be helpful in further resolving the problem.

Experiments which characterized the binding of [^{125}I]LDL to a crude plasma membrane fraction showed that in the absence of a background protein, about half of the total binding was nonspecific, and could be prevented by the addition of bovine serum albumin. There was much less nonspecific binding in heavy membranes. The marked specificity of the membranes for LDL is demonstrated by the dramatic inhibition of [^{125}I]LDL binding by low concentrations of unlabeled LDL in the presence of 40 mg/mL bovine serum albumin. The Scatchard plot indicates that LDL is bound to at least two membrane sites, a high affinity saturable site and a much lower affinity, nonsaturable site. The apparent K_d of the high affinity site is approximately 11 μg/mL. This value agrees well with that determined in fibroblasts (Brown & Goldstein, 1976; Goldstein & Brown, 1974). High affinity binding in both the crude membrane preparations and heavy plasma membranes was substrate saturable, time and temperature dependent, and markedly inhibited by low concentrations of unlabeled LDL, but not by unrelated proteins, and therefore satisfied all the generally accepted criteria for binding to a specific recognition site that can be tested in subcellular fractions. Binding was stimulated two- to threefold by Ca^{2+}. This is similar to the magnitude of the Ca^{2+} stimulation observed in the post-nuclear membrane fraction isolated from cultured human fibroblasts (Basu et al., 1978). In contrast to the fibroblast receptor, however, liver membrane binding was not affected by treatment with either Pronase or trypsin. This could indicate that the recognition site is nonprotein in nature. Cell surface gangliosides, for example, have been shown to serve as receptors for cholera toxin (Cuatrecasas, 1973) and thyrotropin (Mullin et al., 1976). Alternatively, the recognition site may not be accessible to the enzymes. For instance, if the LDL molecule binds to the carbohydrate region of a membrane glycoprotein, the carbohydrate side chains might prevent enzyme interaction with the polypeptide portion of the molecule. Studies using membranes treated with various agents such as lipid solvents and lipid and carbohydrate-splitting enzymes are in progress and should be useful in deciding among the alternatives.

The LDL recognition site is distinct from the liver asialoglycoprotein receptor. LDL binding was not affected by high concentrations of desialidated fetuin, which has been shown to inhibit the binding of ^{125}I-labeled asialoorosomucoid to the purified asialoglycoprotein receptor by 50% at a concentration of 2 μg/mL (Hudgin et al., 1974).

HDL is a potent inhibitor of LDL binding to liver membranes. This contrasts sharply with the findings in human fibroblasts and human aortic smooth muscle cells (Brown & Goldstein, 1974; Brown et al., 1977; Miller et al., 1977). The fibroblast receptor is specific for apoB-containing lipoproteins and those containing apoE, the "arginine-rich" apoprotein, such as porcine HDL_c, a lipoprotein class induced by feeding the animals a high cholesterol diet (Bersot et al., 1976; Mahley & Innerarity, 1977; Brown & Goldstein, 1974) and the slight inhibition of [^{125}I]LDL binding to fibroblasts by high concentrations of HDL has been related to small amounts of "arginine-rich" apoprotein present in HDL preparations (Mahley & Innerarity, 1977). The presence of apoE does not account for the present findings for several reasons. First, lipoproteins were obtained from normolipidemic serum which did not contain detectable levels of HDL_c. Second, little if any HDL_c would be present in the density range at which we isolated HDL. Third, polyacrylamide gel electrophoresis under conditions employed by Mahley et al. (1975), but at twice the protein load employed by those authors, revealed no detectable apoE in the HDL preparations. Finally, treatment of HDL with heparin and $MnCl_2$, which precipitates the apoE containing fraction of HDL and completely abolishes its ability to inhibit LDL binding to human fibroblasts (Mahley & Innerarity, 1977), did not alter its inhibitory activity in liver membranes. The heparin-Mn^{2+} treatment was carried out under conditions that precipitated three quarters of the HDL in order to maximize the removal of trace amounts of apoE. The findings suggest that liver membranes recognize structural features other than, or in addition to, apoB and the "arginine-rich" apoprotein. This impression is strengthened by the marked inhibition of LDL binding by low concentrations of the major circulating phospholipids, particularly phosphatidylcholine, which is a component of both LDL and HDL. The specificity of the binding site may therefore be based, at least in part, on its recognition of the phospholipid component of the lipoprotein molecule, and the "LDL binding site" might be better thought of as a "lipoprotein binding site". This conclusion remains tentative, however, since the present work does not distinguish whether the lipids interfere with the LDL-recognition site on the membrane, or the membrane-recognition site on LDL.

It is, of course, hazardous to attribute physiological significance to the lipoprotein recognition site based on criteria that do not include an actual determination of at least one physio-

logical consequence of binding (Cuatrecasas, 1974; Cuatrecasas & Hollenberg, 1975). While nonspecific interactions usually have low affinities and are not saturable, Dana et al. (1977) have reported that [^{125}I]LDL binds to glass beads in a manner that mimics some of the characteristics of a receptor interaction, such as high affinity and substrate saturability. This particular interaction, however, was not time dependent; it was maximal within less than 1 min, the shortest time measured, which is a characteristic of nonspecific interactions (Cuatrecasas, 1974). It is not possible to determine physiological consequences of binding in subcellular fractions, nor is it presently clear what kind of physiological effect would be appropriate to monitor in the liver, but there is, nonetheless, evidence in vitro and in vivo that the high affinity association of lipoproteins with liver cells does occur. Rat liver parenchymal cells in suspension are capable of high affinity saturable binding of [^{125}I]HDL to sites that apparently also recognize LDL (Nakai et al., 1976). Unlabeled LDL inhibited the binding of [^{125}I]HDL to these cells by about 40% as the LDL protein concentration was increased to about 50 μg/mL. This compared with about 50% inhibition by the same concentration of unlabeled HDL. When the concentrations of LDL or unlabeled HDL were increased further, the latter was much more effective in further inhibiting [^{125}I]HDL binding. One interpretation of these data is that [^{125}I]HDL was bound at two (or more) high affinity sites and that LDL inhibited HDL binding to only one of them. Similar high affinity saturable HDL uptake by rat liver parenchymal cells, but not by nonparenchymal cells, has been observed with [^{3}H]cholesteryl ester labeled HDL (Drevon et al., 1977). We have not measured [^{125}I]HDL binding in the isolated membranes.

When [^{125}I]LDL is injected into pigs in vivo, the lipoprotein rapidly accumulates in the liver (Sniderman et al., 1975; Calvert et al., 1975), which constitutes the major extravascular pool of the lipoprotein. Liver associated LDL seems to be in rapid equilibrium with that of the plasma (Sniderman et al., 1975). It is probably not involved in the control of hepatic cholesterogenesis (Breslow et al., 1977), and liver uptake of the lipoprotein may not be entirely related to its catabolism (Sniderman et al., 1974). Sniderman et al. (1978) have recently presented evidence in humans suggesting the removal of cholesteryl esters from LDL during its association with the liver. The cholesteryl esters seem to be replaced by triglycerides and the modified lipoprotein is released back to the circulation. LDL may therefore function, in part, to transport cholesterol to the liver for removal or reutilization. The present studies suggest that it may do so by first binding the lipoprotein to specific recognition sites on the liver cell membrane.

The liver membrane recognition site differs from that of peripheral tissues in at least two ways. The first is the differential susceptibility of the two sites to proteolytic inactivation. The second is the observation that peripheral cells normally take up very little lipoprotein through the high affinity mechanism unless they have been cholesterol starved for some period of time, during which they adapt by increasing their synthesis of cholesterol and their ability to absorb LDL from the medium (Brown & Goldstein, 1976; Kayden et al., 1976; Ho et al., 1976). This process appears to function in vivo as well. Very little injected [^{125}I]LDL accumulates in peripheral tissues under normal conditions (Sniderman et al., 1975; Calvert et al., 1975), but when circulating levels of cholesterol are drastically reduced in the rat by the administration of 4-aminopyrazolo[3,4-*d*]pyrimidine, there is a marked increase in cholesterol synthesis in a number of extrahepatic tissues; this effect is suppressed by the infusion of LDL (Andersen & Dietschy, 1977). In contrast, the liver rapidly accumulates a large amount of injected [^{125}I]LDL in vivo under conditions of normal circulating levels of lipoproteins (Sniderman et al., 1975). Furthermore freshly isolated rat liver cells used in suspension, without previous cholesterol starvation, actively bind and degrade HDL (Nakai et al., 1976), and the binding is markedly inhibited by low concentrations of LDL, suggesting that LDL is also bound by freshly isolated liver cells. These studies and the present findings indicate that the ability of the liver to interact with LDL is not under the direct feedback control of LDL, as it is in peripheral cells.

The liver recognition site is half-saturated at a LDL protein concentration of about 11 μg/mL. It would be almost completely saturated at about ten times this level, or 110 μg/mL. Since the circulating LDL protein concentration in swine is about 400 μg/mL (Carew et al., 1976), the liver recognition site would normally be completely saturated. It would be expected, therefore, that the amount of LDL taken up by the liver would depend on the number of available recognition sites. It was indeed observed that the binding activity of the liver varied considerably in individual animals, judged by measurements in unfractionated liver homogenates. While the mean binding capacity of seven homogenates, measured at a substrate concentration of 5 μg/mL, was 51 ng/mg protein, the individual values varied fivefold, from 25 to 131 ng/mg. The factors responsible for the variation are presently unknown.

The amount of LDL bound to high affinity membrane sites in the total liver can be estimated from measurements in unfractionated homogenates. The size of the extravascular LDL pool, estimated from plasma decay curves, is about 20% of the vascular pool, and of this at least half is in the liver (Sniderman et al., 1975). The animals used in the present studies weighed approximately 90 kg at slaughter. Assuming a circulating plasma volume of 4320 mL, and an LDL protein concentration of 0.4 mg/mL, the size of the vascular pool would be 1782 mg of LDL protein. The extravascular space would therefore contain about 346 mg of LDL protein and the liver at least half this much, or 173 mg. The liver had a mean weight (seven animals) of 1600 g, contained about 103 mg of protein/g of liver, and bound an average of 51 ng of [^{125}I]LDL/mg of protein at a substrate concentration of about 5 μg/mL. Since this is in the linear portion of the substrate concentration curve, 112 ng/mg would be bound at half-saturation, or 224 ng/mg at complete saturation. Assuming that the liver recognition site is saturated under physiological conditions, it can be calculated that the liver would contain about 37 mg of LDL protein bound to membranes. The value would vary from 18 to 95 mg in individual animals. Based on these estimates, 10–55% of the liver associated LDL would be membrane bound.

The porcine liver membranes should be a useful system in which to investigate the mechanism of the lipoprotein binding reaction through enzymatic and chemical modification of both the substrate LDL and the membrane recognition site. Furthermore the availability of isolated membranes rich in LDL binding activity makes it feasible to try to identify and isolate a membrane protein or glycoprotein component that may be involved in lipoprotein recognition. A better understanding of the molecular nature of the interaction would add considerably to the understanding of these processes as they may normally occur in vivo, how they may be perturbed in the pathological situation, and might further suggest ways in which the functional membrane defects might be repaired or bypassed to restore normal lipoprotein metabolism.

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