Two Independent Lipoprotein Receptors on Hepatic Membranes of Dog, Swine, and Man

APO-B,E AND APO-E RECEPTORS

ROBERT W. MAHLEY, DAVID Y. HUI, THOMAS L. INNERARITY, and KARL H. WEISGRABER, *Gladstone Foundation Laboratories for Cardiovascular Disease and Departments of Pathology and Medicine, Cardiovascular Research Institute, University of California, San Francisco, California 94140*

ABSTRACT We have reported previously that canine livers possess two distinct lipoprotein receptors, an apoprotein (apo)-B,E receptor capable of binding the apo-B-containing low density lipoproteins (LDL) and the apo-E-containing cholesterol-induced high density lipoproteins (HDL_c), and an apo-E receptor capable of binding apo-E HDL_c but not LDL. Both the apo-B,E and apo-E receptors were found on the liver membranes obtained from immature growing dogs, but only the apo-E receptors were detected on the hepatic membranes of adult dogs.

In this study, the expression of the apo-B,E receptors, as determined by canine LDL binding to the hepatic membranes, was found to be highly dependent on the age of the dog and decreased linearly with increasing age. Approximately 30 ng of LDL protein per milligram of membrane protein were bound via the apo-B,E receptors to the hepatic membranes of 7- to 8-wk-old immature dogs as compared with no detectable LDL binding in the hepatic membranes of adult dogs (>1–1.5 yr of age). Results obtained by in vivo turnover studies of canine ^{125}I-LDL correlated with the in vitro findings. In addition to a decrease in the expression of the hepatic apo-B,E receptors with age, these receptors were regulated, i.e., cholesterol feeding suppressed these receptors in immature dogs and prolonged fasting induced their expression in adult dogs. Previously, it was shown that the apo-B,E receptors were induced in adult livers following treatment with the hypocholesterolemic drug cholestyramine. In striking contrast, the apo-E receptors, as determined by apo-E HDL_c binding, remained relatively constant for all ages of dogs studied (10–12 ng/mg). Moreover, the expression of the apo-E receptors was not strictly regulated by the metabolic perturbations that regulated the apo-B,E receptors.

Similar results concerning the presence of apo-B,E and apo-E receptors were obtained in swine and in man. The hepatic membranes of adult swine bound only apo-E HDL_c (apo-E receptors), whereas the membranes from fetal swine livers bound both LDL and apo-E HDL_c (apo-B,E and apo-E receptors). Furthermore, the membranes from adult human liver revealed the presence of the apo-E receptors as evidenced by the binding of 12–14 ng of HDL_c protein per milligram of membrane protein and <1 ng of LDL protein per milligram. The membranes from the human liver also bound human chylomicron remnants and a subfraction of human HDL containing apo-E. These data suggest the importance of the E apoprotein and the apo-E receptors in mediating lipoprotein clearance, including chylomicron remnants, by the liver of adult dogs, swine, and man.

INTRODUCTION

Recently, canine livers have been shown to have two distinct types of high affinity receptors that interact with plasma lipoproteins (1). One is the low density lipoproteins (LDL)[1] (apoprotein [apo-B,E]) receptor, which interacts with both apo-B-containing LDL and apo-E-containing high density lipoproteins, e.g., the

Address reprint requests to Dr. Mahley, Gladstone Foundation Laboratories for Cardiovascular Disease, P.O. Box 40608, San Francisco, Calif. 94140.

Received for publication 13 April 1981 and in revised form 23 June 1981.

[1] *Abbreviations used in this paper*: apo, apoprotein; apo-E HDL_c, cholesterol-induced high density lipoproteins containing only the E apoprotein; FCR, fractional catabolic rate; HDL, high density lipoproteins; LDL, low density lipoproteins; K_d, equilibrium dissociation constant.

cholesterol-induced apo-E HDL_c. This apo-B,E receptor is expressed on hepatic membranes of immature dogs (puppies) but is not detected on the hepatic membranes of adult dogs. The second receptor (the apo-E receptor) is present on the hepatic membranes of adult dogs, as well as on those of immature dogs. This is a unique receptor in that it binds the apo-E-containing lipoproteins (apo-E HDL_c) but does not interact with LDL. The importance of the E apoprotein in mediating the hepatic uptake of lipoproteins has previously been postulated (2–5). Furthermore, the high affinity processes responsible for the uptake of apo-E HDL_c and chylomicron remnants appear to be identical (5).

The apo-B,E receptor present on the hepatic membranes of immature dogs closely resembles or is identical to the LDL receptor present in fibroblasts (1, 6, 7). Although not detectable normally in the livers of adult dogs, the apo-B,E receptor can be induced on the hepatic membranes of adult dogs by treating the animals with the hypocholesterolemic drug cholestyramine (1). The apo-B,E and apo-E receptors appear to be distinct and separate receptors that are under independent control. The purpose of this study is to investigate further the properties of these two lipoprotein receptors in dogs and to investigate their roles in lipoprotein metabolism. Also, the observation that the adult canine liver lacks significant LDL binding activity is extended to adult swine and human hepatic membrane fractions.

METHODS

Animals. Beagle dogs were obtained from Marshall Research Animals (North Rose, N. Y.) and foxhounds were obtained from Brink Farm (Paola, Kans.). The animals had continuous access to water and were fed a normal dog chow diet (Purina dog meal, Ralston Purina Co., St. Louis, Mo.). Three adult foxhounds were fasted 3.5 d before the liver membranes were isolated. In addition, two 8-wk-old beagle puppies and two adult beagles were fed a semisynthetic diet containing 16% hydrogenated coconut oil and 5% cholesterol for 30 d to produce hypercholesterolemia (8).

Lipoprotein isolation, characterization, and iodination. Human LDL (d = 1.02–1.05) from fasted donors were isolated by centrifugation for 18 h at 59,000 rpm in a 60 Ti rotor (Beckman Instruments, Palo Alto, Calif.). The human LDL were washed by recentrifugation at d = 1.05 g/ml for 16 h at 59,000 rpm. Human HDL were prepared by ultracentrifugation (d = 1.063–1.21) for 48 h at 59,000 rpm, followed by recentrifugation for 24 h. The human HDL-with apo-E were isolated by heparin-Sepharose affinity chromatography, as described (9). Dogs were fasted for 16 h before the plasma lipoproteins were isolated. Canine LDL and HDL_1 were isolated from the plasma of normolipidemic foxhounds by ultracentrifugation (d = 1.02–1.063) in a 60 Ti rotor at 59,000 rpm for 18 h and were purified by Geon-Pevikon block electrophoresis (10). Eight different canine LDL preparations have been used in the various studies. Apo-E HDL_c from hypercholesterolemic dogs fed a coconut oil-cholesterol diet (8) were isolated by Geon-Pevikon electrophoresis from the ultracentrifugal fraction d = 1.006–1.02, as described (8). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the isolated apo-E HDL_c contained apo-E as the only protein constituent. Normal swine LDL were isolated from plasma obtained from a 1-yr-old mixed-breed Hampshire sow by ultracentrifugation (d = 1.02–1.063; 18 h at 59,000 rpm) and purified by Geon-Pevikon block electrophoresis. The isolated LDL from the dog, swine, and man contained the B apoprotein as the only detectable protein and migrated as a single band with β-mobility on paper electrophoresis. The isolated lipoproteins were dialyzed exhaustively against 0.15 M NaCl, 0.01% EDTA, pH 7, before use. Canine ^{125}I-apo-E HDL_c and human chylomicron remnants were iodinated by the Bolton and Hunter method, as described previously (11). Human, swine, and canine ^{125}I-LDL were prepared according to the procedure of Bilheimer et al. (12).

Liver samples and the preparation of liver membranes. Dogs, fasted overnight, were anesthetized with intravenous pentobarbital, and the livers were perfused in situ with saline to remove all blood. The livers were removed and placed in ice-cold phosphate-buffered saline, and membrane preparation was begun within 30 min.

Livers from fetal pigs were obtained from sows (Sinclair Research Farms, Columbia, Mo.) in the last 2 wk of gestation by cesarian section. Adult swine livers were obtained from a 1-yr-old domestic, mix-breed sow and a 10-yr-old female miniature swine (Sinclair Farms). Livers were removed from the animals, rinsed with ice-cold saline, and frozen on dry ice until the membranes were prepared. Membrane preparations were begun within 24 h.

Human livers were obtained from patients undergoing cholecystectomy, as described (13). A liver biopsy (2–7 g) was obtained from the right lobe of the liver and was placed immediately in ice-cold saline. The liver samples were immediately frozen in liquid nitrogen and stored on dry ice. Membranes were prepared within 72 h of surgery. Informed consent was obtained from all patients.

Liver membranes were prepared according to the procedure of Kovanen et al. (14), as described (1). Liver membranes sedimenting between 8,000 and 100,000 g were used for the binding assays. All animals were fasted overnight before the liver samples were obtained.

Binding of ^{125}I-lipoprotein to liver membranes. Liver membranes were resuspended in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1.0 mM $CaCl_2$ by forcing the suspension through a 22-gauge needle 10 times and by brief sonication of the suspension for 20 s in a Sonifier cell disrupter (Branson Sonic Power Co., Danbury, Conn.). The protein content of each membrane sample was determined by Lowry's method (15). The binding of lipoproteins to liver membranes was assessed by determining the amount of ^{125}I-labeled lipoproteins associated with the membranes, using the method of Basu et al. (16). Unless specifically noted, all assays were carried out at 0°C on ice in 100 μl of incubation buffer containing 50 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1.0 mM $CaCl_2$, and 20 mg/ml of bovine serum albumin. A typical assay tube contained 160 μg of membrane protein, 18.9 μg/ml of ^{125}I-LDL (335 cpm/ng) or 0.9 μg/ml of ^{125}I-apo-E HDL_c (687 cpm/ng) in the presence or absence of 60 mM EDTA. After incubation for 60 min, 75-μl aliquots were removed and layered onto 100 μl of fetal bovine serum in cellulose nitrate Airfuge tubes (Beckman Instruments). The tubes were centrifuged at 4°C in a Beckman Airfuge at 100,000 g for 30 min. The pellets were washed once with fetal bovine serum and were then separated from the tubes by slicing with a razor blade. The radioactivity in the pellets was determined in a gamma counter (Gamma 4000, Beckman Instruments). Specific calcium-dependent binding of ^{125}I-lipoproteins to the liver membranes was determined by subtracting the amount of ^{125}I-lipoprotein bound in the presence of EDTA from the

amount bound in the absence of EDTA. The maximum amount of lipoprotein bound to the membranes was determined from the Scatchard analysis (17), using various concentrations of the iodinated lipoproteins in the incubation medium (see figure legends for details of individual experiments). The equilibrium dissociation constants (K_d) were determined by plotting the ratio of receptor-bound to free lipoprotein against receptor-bound lipoprotein, as described by Scatchard (17). The slope of the resulting straight line was equal to $-1/K_d$, and the x-intercept in the Scatchard plot represented the maximum amount of lipoprotein bound per milligram of membrane protein. The values used for the molecular weight of LDL and apo-E HDL_c were 3×10^6 (20% protein) and 3.6×10^6 (15% protein), respectively (18). The molecular weight used for canine HDL_1 was 2.0×10^6 with a protein composition of 22.6% (10).

In vivo turnover studies. ^{131}I- or ^{125}I-labeled control dog LDL (0.5 mg of lipoprotein; 150 cpm/ng of protein) were injected into an exposed cephalic vein of foxhounds. Blood samples were obtained at various time intervals, and aliquots of the plasma were assayed for radioactivity. Plasma activities of each time interval were plotted as a percentage of the 5-min time point which was assumed to represent 100%. The curves were analyzed by the standard curve peeling technique, and the fractional catabolic rates were calculated by using the slopes and intercepts of the two exponentials, as described by Matthews (19). LDL protein levels in the plasma of adult and immature dogs were determined by a combination of ultracentrifugation and Geon-Pevikon block electrophoresis (10). Plasma (11 ml) from fasted animals was raised to $d = 1.063$ with KBr and centrifuged in a 50 Ti rotor for 18 h at 40,000 rpm. The LDL were isolated from the $d < 1.063$ fraction by Geon-Pevikon block electrophoresis and the LDL protein determined by the method of Lowry et al. (15).

Chylomicron remnant production. Human chylomicrons were obtained from the plasma of a type V familial hyperlipidemia patient with marked chylomicronemia. The plasma was refrigerated overnight at 4°C, and the top creamy layer was removed. This chylomicron-enriched fraction, raised to a $d = 1.02$, was washed twice by underlayering this fraction beneath a saline-EDTA solution ($d = 1.006$) and centrifuging for 20 min at 25,000 rpm with a SW28 rotor.

Chylomicron remnants were prepared by a slight modification of the procedure described by Florén et al. (20). Post-heparin plasma was obtained from a normal subject 10 min after injection of 100 U/kg of body wt of heparin. The post-heparin plasma was frozen at −20°C until used. A 20-ml chylomicron aliquot (2.77 mg triglyceride/ml) was added to 20 ml of 0.1 M Tris-HCl (pH 8.5) containing 10% fatty acid-free albumin, and the solution was dialyzed overnight against 0.1 M Tris-HCl (pH 8.5). 7 ml of post-heparin plasma were then added to 35 ml of the sample, and the solution was incubated for 26 h at 37°C. At the end of incubation, the solution was adjusted to a final density of 1.063 by KBr and refloated in a solution of 10 mM Hepes, pH 7.4, 1.1% NaCl, and 0.01% EDTA in the SW28 rotor by centrifugation for 3 h at 27,000 rpm. The top layer containing the chylomicron remnants was removed and dialyzed against saline. This procedure resulted in the hydrolysis of 59% of the triglycerides associated with chylomicrons.

RESULTS

The observation that the liver membranes from adult dogs lacked detectable high affinity receptors capable of binding LDL, whereas the liver membranes from immature dogs possessed LDL receptors, prompted us to determine in detail the effect of age on the expression of hepatic receptors (1). The age-dependence of the binding of LDL to the apo-B,E receptor on liver membranes was demonstrated in studies using beagles 7 wk–21 mo of age (Fig. 1A). Using a constant ^{125}I-LDL concentration of 18.7 μg/ml of protein, we determined that the LDL binding to hepatic membranes decreased with age from ~30 ng of LDL protein in 7- to 8-wk-old dogs to <1 ng in the 19- to 21-mo-old dogs. The two older dogs are considered to be mature animals. It was also shown that adult foxhounds (>24 mo of age) lacked detectable hepatic membrane receptors for LDL (1). The lack of significant LDL binding in adult animals has been confirmed in 10 adult dogs (beagles and foxhounds), using eight different canine LDL preparations (Table I).

The existence of high affinity binding of apo-E HDL_c to the liver membranes of mature dogs, as well as to membranes of the immature animals, suggested the presence of a unique apo-E receptor distinct from the apo-B,E receptor (1). As shown in Fig. 1B, the binding of apo-E HDL_c initially decreased with increasing age, and then began to level off in the 15- to 21-mo-old dogs at 10–12 ng of HDL_c protein bound per milligram of membrane protein. The initial portion of the curve represents HDL_c binding to both the apo-E and apo-B,E receptors, whereas, in the older dogs, the apo-E HDL_c binding reflects the predominance of the apo-E receptors. It was possible to estimate apo-E HDL_c binding specifically to the apo-E receptors (Fig. 1B, dashed line). These derived values suggested that the number of apo-E receptors expressed on the liver membranes of dogs of all ages remained relatively constant or decreased only slightly with age.

The value for apo-E HDL_c binding to the apo-B,E receptor was derived by knowing how much LDL was bound to the apo-B,E receptors (Fig. 1A) and from this value calculating the amount of HDL_c that would bind in comparison with LDL. It was possible to calculate the value for apo-E HDL_c, assuming that LDL and apo-E HDL_c bind at a ratio of 3.5:1. We have shown that the apo-B,E receptors of fibroblasts bind three to four times more LDL than apo-E HDL_c at receptor saturation (21). This derived value for apo-E HDL_c binding to the apo-B,E receptors was subtracted from the total apo-E HDL_c binding (Fig. 1B) to give the amount of apo-E HDL_c binding specifically to the apo-E receptors (Fig. 1B, dashed line). Although these were derived data involving several assumptions, it was apparent that the level of the apo-E receptor binding activity remained relatively constant for all ages.

An alternative approach in the estimation of apo-E HDL_c binding to the apo-B,E receptor in puppy liver membranes vs. the amount of apo-E HDL_c bound to the apo-E receptor was to take advantage of the sensitivity of the apo-B,E receptor to inhibition at high salt con-

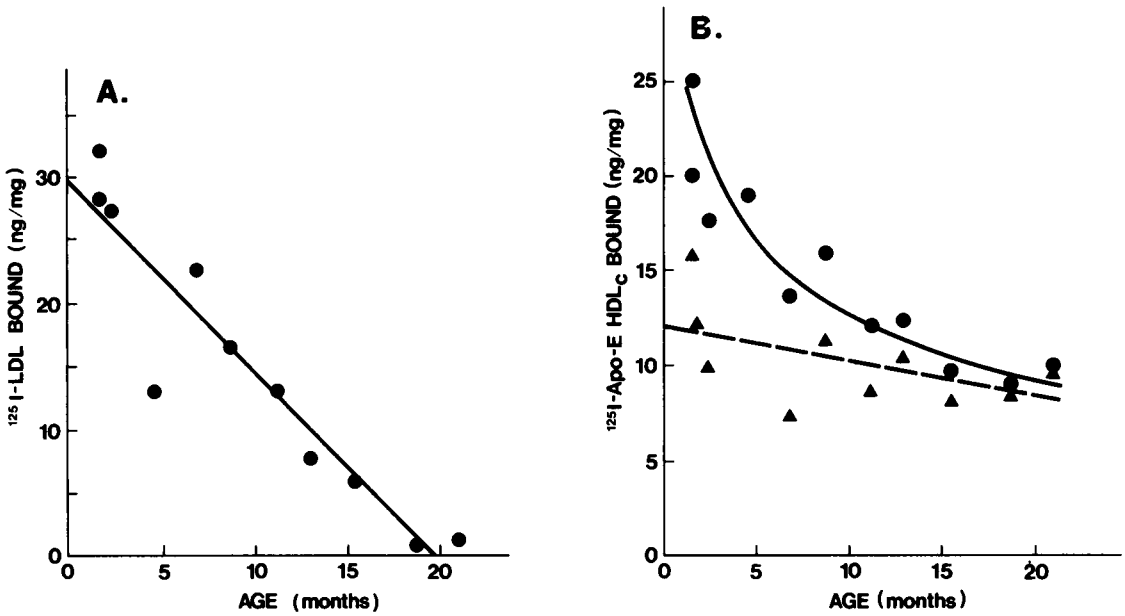

FIGURE 1 Binding of canine ^{125}I-LDL (A) or canine ^{125}I-apo-E HDL$_c$ (B) to dog liver membranes as a function of age. Liver membranes were prepared from male beagles of different ages as indicated. Each incubation tube contained 160 μg of membrane protein and 18.7 μg/ml of ^{125}I-LDL (335 cpm/ng) or 0.9 μg/ml of ^{125}I-apo-E HDL$_c$ (687 cpm/ng) in the presence or absence of 60 mM EDTA. After incubation for 60 min at 0°C, the amount of iodinated lipoprotein bound to the membranes was determined. The data shown (●) represent results of specific binding which were calculated by subtracting the amount of lipoprotein bound in the presence of EDTA from that bound in the absence of EDTA. The binding of ^{125}I-apo-E HDL$_c$ to the apo-E receptors (dashed line) (▲) was calculated by subtracting the amount bound to the apo-B,E receptors from the total specific apo-E HDL$_c$ bound. The amount of ^{125}I-apo-E HDL$_c$ binding to the apo-B,E receptor was derived from the assumption that this receptor bound LDL and apo-E HDL$_c$ at a ratio of 3.5:1.

centration (1). The LDL binding to the apo-B,E receptor was inhibited by ~75% at a NaCl concentration of 150 mM as compared with results obtained at 25 mM NaCl (data not shown). On the other hand, the binding of ^{125}I-apo-E HDL$_c$ to the apo-E receptor was independent of ionic strength (1). When we compared the binding activities of apo-E HDL$_c$ to liver membranes at 25 mM and 150 mM NaCl, the number of apo-B,E receptors and apo-E receptors could be calculated. The binding of ^{125}I-apo-E HDL$_c$ to apo-E receptors was equal to the total amount bound minus the amount bound to the apo-B,E receptors. The binding of the apo-E HDL$_c$ to the apo-B,E receptors was determined by taking the difference of the binding activity of this lipoprotein at 25 and 150 mM NaCl and correcting this value for lack of complete inhibition of the apo-B,E receptor binding activity at 150 mM NaCl (assuming that apo-E HDL$_c$ binding to the apo-B,E receptors was inhibited 75% at a NaCl concentration of 150 mM).[2]

[2] Binding of ^{125}I-apo-E HDL$_c$ to apo-E receptor = A − ([A − B]/0.75), where A = ^{125}I-apo-E HDL$_c$ binding at 25 mM NaCl; B = ^{125}I-apo-E HDL$_c$ binding at 150 mM NaCl; 0.75 = 75% inhibition of binding to apo-B,E receptor at 150 mM NaCl.

When the liver membranes obtained from the dogs of different ages, shown in Fig. 1, were assayed for apo-E HDL$_c$ binding at 25 and 150 mM NaCl, the calculated mean value for ^{125}I-apo-E HDL$_c$ binding to the apo-E receptor was 11.2 ng of apo-E HDL$_c$ protein per milligram of membrane protein (±5.0 ng/mg; SD) regardless of the age of the animal. This value is in good agreement with the level of apo-E receptor binding activity calculated as shown in Fig. 1B.

The in vitro observation that adult dogs lack, or have very low levels of hepatic apo-B,E receptors, led us to reason that these animals would catabolize LDL at a slower rate than young dogs that possess hepatic apo-B,E receptors. To test this, we compared the turnover of canine LDL in immature dogs and mature adult dogs. As shown in Fig. 2, for a representative pair of dogs, canine LDL were cleared from the plasma of the young immature dog more rapidly than from the plasma of the adult foxhound. The fractional catabolic rate (FCR) for the clearance of canine LDL in a series of experiments was greater by ~50% in the immature dogs as compared with the values obtained in the older animals. The results from 12 animals are shown in Table II. In these studies, dogs with similar plasma

TABLE I

Binding of Canine ^{125}I-Apo-E HDL$_c$ and ^{125}I-LDL to Canine Liver Membranes

Canine liver membranes	Age	n	^{125}I-apo-E HDL$_c$		^{125}I-LDL	
			K_d*	B_{max}*	K_d*	B_{max}*
		n	*nM*	*ng/mg*	*nM*	*ng/mg*
Beagle puppies	7–12 wk	5	0.27	22.6 (19–26)§	11.0	52.3 (32–64)§
Beagle adults	19 & 21 mo	2	0.31	11.5 (11, 12)§	ND‡	<1
Foxhound puppy	7 wk	1	0.30	22	16	59
Foxhound adults	24–30 mo	8	0.23	12 (10–15)§	ND‡	<1
Fasted adult foxhound**						
A	30 mo	1	0.31	19	8.3	14
B	24 mo	1	0.29	166	19.0	306
C	24 mo	1	0.27	127	18.9	313
Beagle puppies —cholesterol-fed	8 wk	2	0.27	23 (12, 34)§	ND‡	0
Beagle adults —cholesterol-fed	18, 19 mo	2	0.42	12.5 (11, 14)§	ND‡	0

* The equilibrium dissociation constants (K_d) and the maximum binding (B_{max}) were calculated from the Scatchard plots of the binding data as described under Methods. The values represent the average of *n* experiments. All Scatchard plots were linear with correlation coefficients between 0.91 and 0.97.

‡ ND, not detectable.

§ Values in parentheses represent the ranges.

** The marked difference between the response of dog A vs. dogs B and C remains unexplained. However, the ages of the dogs were different as were their plasma cholesterol levels (A, 234 mg/dl; B, 160 mg/dl; C, 108 mg/dl).

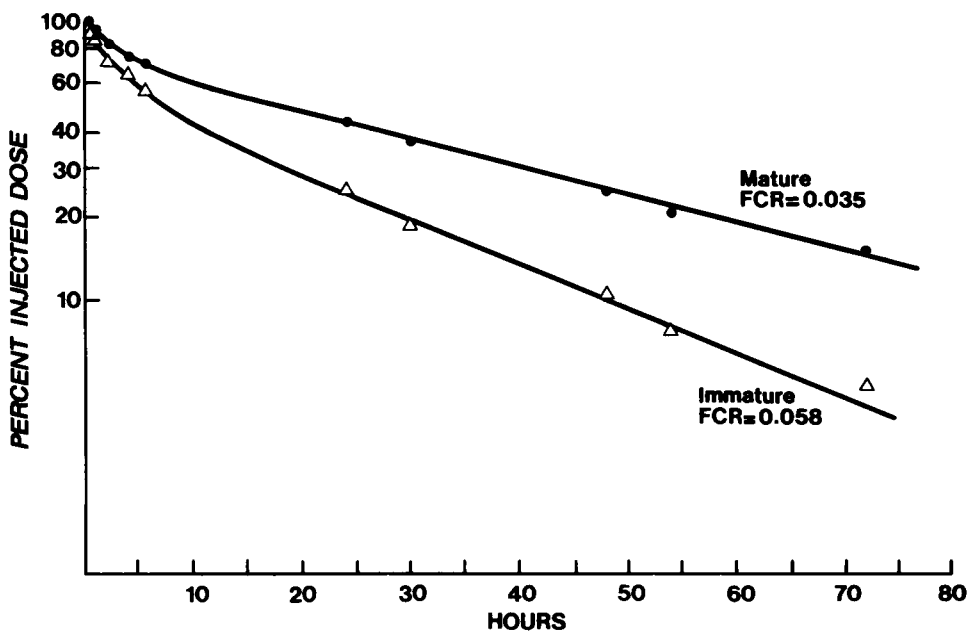

FIGURE 2 Percent of the 5-min plasma sample of canine ^{125}I-LDL that remained in the plasma of mature (●) and immature (△) dogs as a function of time after intravenous injection of 500 μg of lipoprotein protein.

TABLE II
Turnover of Canine ^{125}I or ^{131}I-Labeled LDL in Immature and Mature Dogs

Study	Mature dog n	Mature dog FCR	Immature dog n	Immature dog FCR	Percent difference
A*	3‡	0.053§ (±0.004)	3‡	0.073§ (±0.010)	40%
B*	3	0.034 (±0.005)	3	0.054 (±0.004)	57%

* Two different preparations (A and B) of canine LDL were used in these studies. Furthermore, the plasma cholesterol levels were matched in the mature and immature dogs and were different for the two studies: A, ~123 mg/dl; B, ~140 mg/dl. Because of these two variables between studies, the results are compiled separately. Previously, we have determined that the LDL levels in mature and immature dogs are not significantly different within the range of plasma cholesterols described in these studies. The LDL protein concentration in nine adult and seven immature dogs was 9.2±4.8 and 11.3±5.1 mg/dl (mean±SD), respectively. These values include LDL determinations on four of the adults and three of the immature dogs used in the present turnover studies.

‡ Number of dogs studied.

§ FCR, pools per hour (±SD)

cholesterol levels and LDL pool size were compared. The more rapid clearance rate observed for LDL in the immature dogs cannot be explained by a difference in LDL pool size between the immature and mature dogs (see footnote, Table II).

We demonstrated previously with the in vitro hepatic binding assay that the apo-B,E receptor could be induced in mature adult dogs by treating the animals with the hypocholesterolemic drug cholestyramine (1). Furthermore, LDL binding could be induced in adult foxhounds by prolonged fasting with deprivation of all food for 3.5 d (Table I). In two of the fasted animals (B and C; Table I), both the LDL and apo-E HDL_c binding were markedly increased, reflecting an enhanced number of both apo-B,E and apo-E receptors. In an additional experiment utilizing the sensitivity of the apo-B,E receptor to inhibition by high salt concentrations, it was possible to quantitate the relative increase in the apo-B,E and apo-E receptors using ^{125}I-apo-E HDL_c. The binding of apo-E HDL_c to the apo-E receptor increased from a mean of 11.2 ng/mg in control adult dogs to levels of 88–122 ng/mg (~ a 10-fold increase) in these two fasted dogs (Table III). On the other hand, the binding of apo-E HDL_c to the apo-B,E receptors increased from a value of <1 ng/mg in control adult foxhounds to levels of 61–70 ng/mg (at least a 60- to 70-fold increase induced by the prolonged fasting in dogs B and C) (Table III). Furthermore, a comparison of the values obtained for LDL binding to the apo-B,E receptors (Table I, dogs B and C) with those obtained for HDL_c binding to the apo-B,E receptors (Table III) revealed a ratio between LDL and HDL_c binding of ~4.5:1. This is in reasonable agreement with the 4:1 ratio described for the binding of LDL and HDL_c to the apo-B,E receptors of fibroblasts, as previously described (21). These results demonstrate the capability of the adult liver to express large numbers of apo-B,E receptors under certain metabolic conditions.

In addition to the inducibility of the apo-B,E receptors in adult dogs, it was of interest to determine if the binding activity of the apo-B,E receptor in the liver of immature dogs could be suppressed or regulated by diets high in fat and cholesterol. Two immature beagle puppies (8 wk of age at start of the study) and two adult beagles (18 and 19 mo of age) were fed a semi-

TABLE III
Determination of ^{125}I-Apo-E HDL_c Binding to Apo-B,E Receptors and Apo-E Receptors in Fasted Canine Liver Membranes by Ionic Strength Differences

	Age	^{125}I-Apo-E HDL_c bound* 25 mM NaCl A	^{125}I-Apo-E HDL_c bound* 150 mM NaCl B	NaCl-sensitive binding* A minus B	Apo-B,E receptor binding‡ $\frac{A \text{ minus } B}{0.75}$	Apo-E receptor binding‡ A minus $\left(\frac{A-B}{0.75}\right)$
	mo	*ng/mg*	*ng/mg*	*ng/mg*	*ng/mg*	*ng/mg*
Dog B	24	191.6	138.9	52.7	70.1	121.5
Dog C	24	149.4	103.4	46.1	61.3	88.1

* Binding of ^{125}I-apo-E HDL_c to the liver membranes was performed with 1.0 μg/ml of ^{125}I-apo-E HDL_c protein.

‡ Binding of ^{125}I-apo-E HDL_c to the apo-B,E receptor = (A − B)/0.75. Binding of ^{125}I-apo-E HDL_c to the apo-E receptor = A − ((A − B)/0.75): A = ^{125}I-apo-E HDL_c binding at 25 mM NaCl; B = ^{125}I-apo-E HDL_c binding at 150 mM NaCl; 0.75 = correction factor to account for the partial inhibition of apo-B,E receptor binding activity at 150 mM NaCl.

TABLE IV

Binding of Canine ^{125}I-Apo-E HDL$_c$ and Swine ^{125}I-LDL to Swine Liver Membranes

Swine liver membranes	Age		^{125}I-apo-E HDL$_c$		^{125}I-LDL	
			K_d*	B_{max}*	K_d*	B_{max}*
		n	*nM*	*ng/mg*	*nM*	*ng/mg*
Fetal Pig	Fetal	3	0.72	16.0	14	34.1
Adult-1	1 yr	1	0.77	6.0	ND‡	<1
Adult-2	10 yrs	1	0.92	5.6	ND‡	0

* The K_d and the B_{max} were determined from the Scatchard plots of the binding data as described in Methods and in the legend to Table I.
‡ ND, not detectable.

synthetic diet containing 16% hydrogenated coconut oil and 5% cholesterol for 30 d. The dogs developed a mild to moderate hypercholesterolemia (puppies, 306 and 154 mg/dl; adults, 408 and 360 mg/dl). The dietary cholesterol and/or the hypercholesterolemia totally repressed the expression of the apo-B,E receptors in the immature dogs (Table I). The apo-E HDL$_c$ binding to the hepatic membranes from both cholesterol-fed puppies and adults remained high (23 and 12.5 ng of apo-E HDL$_c$ protein per milligram of membrane protein, respectively; Table I). It should be noted that the binding activity of the apo-E receptors was not markedly different after cholesterol feeding. Specifically, the apo-E HDL$_c$ binding to the hepatic membranes of the adult control and cholesterol-fed dogs was identical (11–14 ng/mg). One of the cholesterol-fed puppies, which developed a very mild hypercholesterolemia (154 mg/dl), appeared to have an increase in apo-E receptor binding activity (34 ng/mg). The resistance to hypercholesterolemia may reflect the increase in the activity of this receptor; however, this remains to be determined in more detailed studies.

It was of interest to determine if other apo-E-containing HDL would bind to the hepatic membrane receptors of the adult dog. Canine HDL$_1$, normolipidemic lipoproteins that contain both the E and A-I apoproteins (~50% apo-E) (10), bound to the adult liver membranes with a K_d of 13.5 nM and a maximum binding of 20.6 ng of HDL$_1$ protein per milligram of membrane protein.

The observations in the dog concerning the existence of two independent hepatic receptors were extended to the swine. Livers were obtained from fetuses of sows during the last 2 wk of gestation by cesarian section. Adult liver was obtained from a 1-yr-old, domestic mixed-breed swine and from a 10-yr-old miniature swine. As shown in Table IV, the fetal liver of swine bound both swine LDL and canine apo-E HDL$_c$, whereas the membranes from the adult livers bound only the apo-E HDL$_c$. These data suggested the age-dependent expression of the apo-B,E receptor in the swine, similar to the results obtained in dogs.

The finding of an absence of significant LDL binding to the liver membranes from adult dogs and swine has been extended to adult human liver membranes. As shown in Table V, human hepatic membranes bound little, if any, human LDL. This was determined using LDL concentrations of 10 and 20 μg of LDL protein per milliliter. By comparison, apo-E HDL$_c$ bound to the human hepatic membranes with a K_d of 0.8 ng. The maximum amount bound was 12–14 ng of apo-E HDL$_c$ protein per milligram of membrane protein (Table V). Furthermore, it was possible to demonstrate that adult human liver membranes would bind other apo-E-

TABLE V

Binding of Canine ^{125}I-Apo-E HDL$_c$, Human ^{125}I-LDL, and ^{125}I-Chylomicron Remnants to Human Liver Membranes

Human liver membranes	Sex	Age	^{125}I-Apo-E HDL$_c$		^{125}I-LDL bound‡		^{125}I-Chylomicron remnants**	
			K_d*	B_{max}*	at 10 μg/ml§	at 20 μg/ml§	K_d*	B_{max}*
			nM	*ng/mg*	*ng/mg*	*ng/mg*	*μg cholesterol per ml*	*μg cholesterol per ml protein*
Adult-1	M	67 yr	0.42	12.0	0.12	0.86	4.00	35.4
Adult-2	F	23 yr	0.88	14.3	0	0.18	—	—
Adult-3	F	47 yr	0.83	12.4	0.10	0.91	6.67	21.2

* The K_d and B_{max} for ^{125}I-apo-E HDL$_c$ binding were determined from the Scatchard plots of ^{125}I-apo-E HDL$_c$ binding to liver membranes as described in the legend to Table I.
‡ Values of <1 ng/mg of membrane protein represent insignificant binding and should be considered as undetectable.
§ Binding of ^{125}I-LDL to the liver membranes at the concentrations indicated.
** Chylomicron remnant binding was performed at 23°C to minimize the high level of nonspecific (calcium-independent) binding observed when studies were conducted at 4°C. The high affinity specific binding of apo-E HDL$_c$ observed in studies conducted at 23°C was essentially identical to data obtained at 4°C. Chylomicrons did not bind to the hepatic membranes.

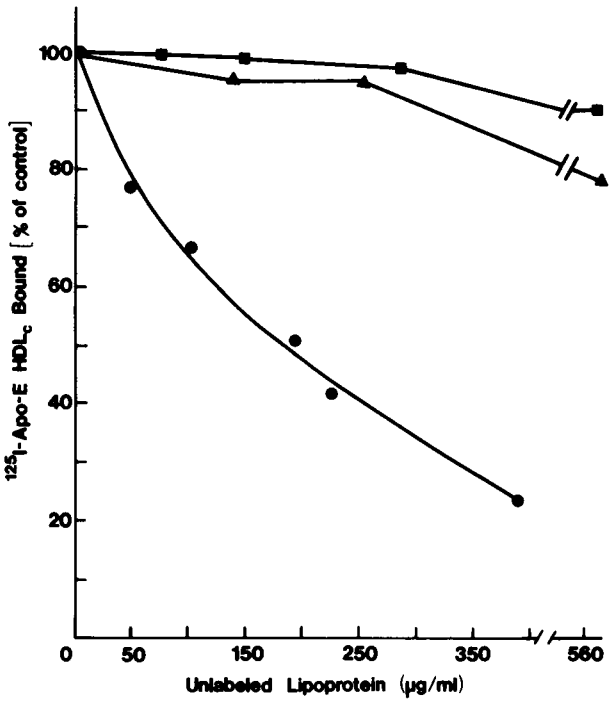

FIGURE 3 Competitive displacement of ^{125}I-apo-E HDL$_c$ from human liver membranes by unlabeled human lipoproteins. Human liver membranes (250 μg protein) were incubated with 0.175 μg/ml of ^{125}I-apo-E HDL$_c$ (687 cpm/ng) and human HDL containing apoprotein E (●), human HDL-without apo-E (■), or human LDL (▲) for 1 h at 4°C. Binding of ^{125}I-apo-E HDL$_c$ to the membranes in the absence of human HDL containing apo-E was 3.58 ng/mg of protein.

containing lipoproteins. We prepared human HDL-with apo-E from a normal subject using heparin-Sepharose affinity chromatography (9). In a competitive binding study using ^{125}I-apo-E HDL$_c$, it was shown that the human HDL-with apo-E would displace the iodinated lipoprotein (Fig. 3). Human HDL-with apo-E, which contain the E apoprotein as a minor constituent (~5% of the total protein), represent a subclass of HDL (d = 1.063–1.21) (22). By comparison, human HDL containing no apo-E and human LDL failed to compete significantly with ^{125}I-apo-E HDL$_c$ for binding to the human liver membranes (Fig. 3). We have reported that HDL that lack apo-E do not displace the apo-E HDL$_c$ (1). Thus, it appeared that the adult human liver possessed the apo-E receptors and lacked detectable apo-B,E receptors, at least under the metabolic conditions represented by these human liver specimens obtained at surgery. Furthermore, it was shown that the human liver membranes bound chylomicron remnants, but not chylomicrons, with very high affinity (Table V). Chylomicron remnants were generated by incubating the chylomicron fraction from a patient with type V hyperlipoproteinemia with plasma containing post-heparin lipolytic activity.

DISCUSSION

The results of this study extend previous observations describing the existence of two distinct receptors on hepatic membranes, an apo-B,E receptor and an apo-E receptor (1). The apo-B,E receptor is expressed on the hepatic membranes of immature dogs but not in adult dogs. In fact, binding activity of LDL to the apo-B,E receptor correlates with age (r = 0.88) and decreases to the point that LDL binding is not detectable (<1 ng of LDL protein per milligram of membrane protein). Only the apo-E receptor, which binds canine apo-E HDL$_c$ and canine HDL$_1$, is detected on the hepatic membranes of adult dogs. Furthermore, the binding of apo-E HDL$_c$ specifically to the apo-E receptor of dogs remains relatively constant over the range of ages studied (10–12 ng/mg; derived in Fig. 1B).

In an attempt to determine the physiological significance of a reduced expression of the apo-B,E receptors on adult liver membranes, the in vivo turnover of LDL in mature and immature dogs was compared. The FCR of LDL in the immature dogs is faster (~50%) than in the mature dog, which may be due to the presence of apo-B,E receptors in the liver of immature dogs. Recently, Kovanen et al. (6), using 32 dogs, demonstrated an excellent correlation (r = 0.87) between hepatic LDL binding activity determined by the membrane binding assay and FCR of human LDL in vivo. Although they did not use adult dogs in their study, extrapolation of their FCR vs. LDL binding data to zero LDL binding gives an FCR of 0.93/d or 0.039/h, which is in excellent agreement with the average FCR (0.044/h) reported in this study. Another possibility to explain our results is that there may be a generalized increase in peripheral apo-B,E receptor activity to supply LDL for the cholesterol needs of these rapidly growing, immature animals. To resolve the two possible interpretations of these results, measurements of direct liver uptake of LDL will be required.

We have shown that hepatic apo-B,E receptors capable of LDL binding can be induced in adult dogs by treatment with the hypocholesterolemic agent cholestyramine (1). In this study, the apo-B,E receptor is also induced in adult dogs by prolonged fasting. In agreement with this observation, Quarfordt et al. (23), have reported that the livers from fasted rats accumulated significantly more triglyceride than did the livers of fed rats when perfused with apo-E containing triglyceride emulsions. In addition, expression of the hepatic apo-B,E receptor in the membranes of immature dogs can be repressed. Following cholesterol feeding in young dogs, the binding activity of LDL to liver membranes is markedly reduced to an undetectable level. This down regulation of the apo-B,E receptor may result from the hypercholesterolemia and/or from the level of cholesterol delivered to the liver. Kovanen et al. (24),

demonstrated that feeding cholesterol to rabbits led to a 60% reduction of β-VLDL (very low density lipoproteins) binding to the liver membranes (24). In addition, the data from the cholesterol-feeding study indicate that the apo-E receptor is not significantly suppressed by this dietary perturbation. It remains to be determined if there are metabolic or hormonal conditions that regulate the apo-E receptor.

The finding that membranes from adult human livers bind canine apo-E HDL_c, human HDL with apo-E, and chylomicron remnants, but do not significantly bind human LDL, indicates that man has an hepatic apo-E receptor. It remains to be determined if livers from children express the apo-B,E receptors in a manner analogous to the expression of the receptor in immature dogs. In this regard, Brown et al. (25) have shown that hepatic membranes from 16- to 20-wk-old human fetuses bind normal human LDL, presumably via the apo-B,E receptors. The existence of a unique apo-E receptor, distinct from the apo-B,E receptor in adult human liver is of potential importance in understanding human lipoprotein metabolism. It has been previously shown in the rat that chylomicron remnants are cleared by a receptor-mediated process specific for the E apoprotein (5). It is reasonable to speculate that the existence of the apo-E receptor in adult human liver may be responsible for the clearance of chylomicron remnants. This hypothesis is supported by observations concerning lipoprotein metabolism in patients with type II hyperlipoproteinemia (homozygous familial hypercholesterolemia). Patients with this genetic disorder lack functional LDL (apo-B,E) receptors in all tissues examined and have greatly elevated LDL levels without major alterations in the levels of other lipoproteins (26). If the apo-B,E receptors in the liver were of major importance in the clearance of apo-E-containing plasma lipoproteins, including chylomicron remnants, then one might expect that subjects with familial hypercholesterolemia would have abnormal remnant clearance. Since they do not have an abnormality of chylomicron remnant clearance, it is reasonable to speculate that the apo-E receptor is functional in these patients. The further extension of these observations requires more detailed studies with human liver.

Consideration was given to whether the number of apo-E receptors demonstrated by the in vitro assays would be adequate to account for the clearance of chylomicron remnants in vivo. Based on the binding data performed with crude membrane fractions isolated by centrifugation between 8,000 and 100,000 *g*, and taking into account that only 60% of the receptor binding activity of the whole liver homogenates can be recovered in this fraction (unpublished observation), we calculated that there are 6×10^{14} receptors in the liver of an adult dog. Assuming that each receptor can internalize a chylomicron remnant particle every 5 min, this would result in the clearance of 1.7×10^{17} particles of chylomicron remnants per day. By using a value of 5×10^{-18} g of cholesterol per particle for chylomicron remnants (5), and based on the above measurements and assumptions, we calculated that the liver could take up ~1 g of cholesterol per day via the hepatic apo-E receptors. This value compares favorably with 1–1.5 g/d of absorbed cholesterol delivered to the liver of dogs fed various levels of dietary cholesterol as determined previously in sterol balance studies (27–29).

ACKNOWLEDGMENTS

We thank Drs. Bo Angelin and Staffan Ewerth for providing the liver biopsies, Dr. Thomas P. Bersot for obtaining the post-heparin plasma and chylomicrons, and Dr. Donald L. Fry for providing the miniature swine liver. We thank Mr. Walter Brecht for his technical assistance. We thank Mrs. Mary Hamman, Mrs. Karalee Forsling, and Mr. Richard Wolfe for typing the manuscript and Mr. Joe F. Andres for his editorial assistance.

REFERENCES

1. Hui, D. Y., T. L. Innerarity, and R. W. Mahley. 1981. Lipoprotein binding to canine hepatic membranes. Metabolically distinct apo-E and apo-B,E receptors. *J. Biol. Chem.* **256**: 5646–5655.
2. Mahley, R. W., T. L. Innerarity, K. H. Weisgraber, and S. Y. Oh. 1979. Altered metabolism (in vivo and in vitro) of plasma lipoproteins after selective chemical modification of lysine residues of the apoproteins. *J. Clin. Invest.* **64**: 743–750.
3. Shelburne, F., J. Hanks, W. Meyers, and S. Quarfordt. 1980. Effect of apoproteins on hepatic uptake of triglyceride emulsions in the rat. *J. Clin. Invest.* **65**: 652–658.
4. Windler, E., Y. S. Chao, and R. J. Havel. 1980. Regulation of the hepatic uptake of triglyceride-rich lipoproteins in the rat (opposing effects of homologous apolipoprotein E and individual C apoproteins). *J. Biol. Chem.* **255**: 8303–8307.
5. Sherrill, B. C., T. L. Innerarity, and R. W. Mahley. 1980. Rapid hepatic clearance of the canine lipoproteins containing only the E apoprotein by a high affinity receptor (identity with the chylomicron remnant transport process). *J. Biol. Chem.* **255**: 1804–1807.
6. Kovanen, P. T., D. W. Bilheimer, J. L. Goldstein, J. J. Jaramillo, and M. S. Brown. 1981. Regulatory role for hepatic low density lipoprotein receptors in vivo in the dog. *Proc. Natl. Acad. Sci. U. S. A.* **78**: 1194–1198.
7. Beisiegel, U., T. Kita, R. G. W. Anderson, W. J. Schneider, M. S. Brown, and J. L. Goldstein. 1981. Immunologic cross-reactivity of the low density lipoprotein receptor from bovine adrenal cortex human fibroblasts, canine liver and adrenal gland, and rat liver. *J. Biol. Chem.* **256**: 4071–4078.
8. Mahley, R. W., T. L. Innerarity, K. H. Weisgraber, and D. L. Fry. 1977. Canine hyperlipoproteinemia and atherosclerosis (accumulation of lipid by aortic medial cells in vivo and in vitro). *Am. J. Pathol.* **87**: 205–225.
9. Weisgraber, K. H. and R. W. Mahley. 1980. Subfractionation of human high density lipoproteins by heparin-Sepharose affinity chromatography. *J. Lipid Res.* **21**: 316–325.

10. Mahley, R. W. and K. H. Weisgraber. 1974. Canine lipoproteins and atherosclerosis. I. Isolation and characterization of plasma lipoproteins from control dogs. *Circ. Res.* **35:** 713–721.
11. Innerarity, T. L., R. E. Pitas, and R. W. Mahley. 1979. Binding of arginine-rich (E) apoprotein after recombination with phospholipid vesicles to the low density lipoprotein receptors of fibroblasts. *J. Biol. Chem.* **254:** 4186–4190.
12. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–221.
13. Ahlberg, J., B. Angelin, I. Björkhem, K. Einarsson, and B. Leijd. 1979. Hepatic cholesterol metabolism in normo- and hyperlipidemic patients with cholesterol gallstones. *J. Lipid Res.* **20:** 107–115.
14. Kovanen, P. T., S. K. Basu, J. L. Goldstein, and M. S. Brown. 1979. Low density lipoprotein receptors in bovine adrenal cortex. II. Low density lipoprotein binding to membranes prepared from fresh tissue. *Endocrinology.* **104:** 610–616.
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
16. Basu, S. K., J. L. Goldstein, and M. S. Brown. 1978. Characterization of the low density lipoprotein receptor in membranes prepared from human fibroblasts. *J. Biol. Chem.* **253:** 3852–3856.
17. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51:** 660–672.
18. Innerarity, T. L., and R. W. Mahley. 1978. Enhanced binding by cultured human fibroblasts of apo-E-containing lipoproteins as compared with low density lipoproteins. *Biochemistry.* **17:** 1440–1447.
19. Matthews, C. M. E. 1957. The theory of tracer experiments with ^{131}I-labelled plasma proteins. *Phys. Med. Biol.* **2:** 36–53.
20. Florén, C. H., J. J. Albers, B. J. Kudchodkar, and E. L. Bierman. 1981. Receptor-dependent uptake of human chylomicron remnants by cultured skin fibroblasts. *J. Biol. Chem.* **256:** 425–433.
21. Pitas, R. E., T. L. Innerarity, K. S. Arnold, and R. W. Mahley. 1979. Rate and equilibrium constants for binding of apo-E HDL (a cholesterol-induced lipoprotein) and low density lipoproteins to human fibroblasts: Evidence for multiple receptor binding of apo-E HDL$_c$. *Proc. Natl. Acad. Sci. U. S. A.* **76:** 2311–2315.
22. Innerarity, T. L., R. W. Mahley, K. H. Weisgraber, and T. P. Bersot. 1978. Apoprotein (E–A-II) complex of human plasma lipoproteins. II. Receptor binding activity of a high density lipoprotein subfraction modulated by the apo(E–A-II) complex. *J. Biol. Chem.* **253:** 6289–6295.
23. Quarfordt, S. H., F. A. Shelburne, W. Meyers, L. Jakoi, and J. Hanks. 1981. Effect of apolipoproteins on the induction of hepatic steatosis in rats. *Gastroenterology.* **80:** 149–153.
24. Kovanen, P. T., M. S. Brown, S. K. Basu, D. W. Bilheimer, and J. L. Goldstein. 1981. Saturation and suppression of hepatic lipoprotein receptors: A mechanism for the hypercholesterolemia of cholesterol-fed rabbits. *Proc. Natl. Acad. Sci. U. S. A.* **78:** 1396–1400.
25. Brown, M. S., P. T. Kovanen, and J. L. Goldstein. 1979. Receptor-mediated uptake of lipoprotein-cholesterol and its utilization for sterol synthesis in the adrenal cortex. *Recent Prog. Horm. Res.* **35:** 215–257.
26. Fredrickson, D. S., J. L. Goldstein, and M. S. Brown. 1978. The familial hyperlipoproteinemias. *In The Metabolic Basis of Inherited Disease.* J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. 4th ed. McGraw-Hill Book Co., Inc., New York. 604–655.
27. Wojciech, R., H. M. Janecek, and A. C. Ivy. 1960. Endogenous excretion and intestinal capacity for absorption of cholesterol in the dog. *Am. J. Physiol.* **201:** 190–193.
28. Dietschy, J. M., and J. D. Wilson. 1970. Regulation of cholesterol metabolism. *N. Engl. J. Med.* **282:** 1128–1248.
29. Pertsemlidis, D., E. H. Kirchman, and E. H. Ahrens, Jr. 1973. Regulation of cholesterol metabolism in the dog. I. Effects of complete bile diversion and of cholesterol feeding on absorption, synthesis, accumulation, and excretion rates measured during life. *J. Clin. Invest.* **52:** 2353–2367.