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Difference in saturable binding of low density lipoprotein to liver membranes from normocholesterolemic subjects and patients with heterozygous familial hypercholesterolemia

(low density lipoprotein receptor/1,2-cyclohexanedione-modified low density lipoprotein/plasma cholesterol)

KRISTIN HARDERS-SPENGEL*†, CHRISTOPHER B. WOOD‡, GILBERT R. THOMPSON*, NICOLAS B. MYANT*, AND ANNE K. SOUTAR*§

*Medical Research Council Lipid Metabolism Unit, and ‡Department of Surgery, Hammersmith Hospital, DuCane Road, London W12 OHS, England

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ABSTRACT **To investigate the possible role of the low density lipoprotein (LDL) receptor in the catabolism of LDL by the human liver, the binding of ^{125}I-labeled LDL to membrane fractions prepared from human liver biopsies was determined. Biopsy samples taken for routine histology were obtained from seven patients with heterozygous familial hypercholesterolemia, one with nonfamilial hypercholesterolemia, and seven normocholesterolemic subjects. LDL was bound by the membranes from normal subjects in a saturable manner that was inhibited by 56% in the presence of excess LDL. Binding of LDL was also inhibited by modification of the lipoproteins with 1,2-cyclohexanedione. The amount of ^{125}I-labeled LDL bound to membranes from familial hypercholesterolemic livers that could be displaced with excess LDL was significantly less than that bound by normocholesterolemic membranes. These observations suggest that LDL receptors are expressed in normal human liver and are defective in the livers of familial hypercholesterolemic patients.**

A substantial fraction of the low density lipoprotein (LDL) catabolized in the intact animal is degraded in the liver, but the extent to which this process is mediated by hepatic LDL receptors is controversial (1). Membranes prepared from the livers of normal rabbits (2) or estrogen-treated rats (3) have been shown to possess saturable binding sites for LDL similar to the LDL receptors expressed in cultured skin fibroblasts from normal human subjects. Saturable LDL binding sites have also been detected in liver membranes from immature dogs and pigs (4) and from human fetuses (5). However, liver membranes from adult dogs and pigs do not express receptors for LDL, although LDL receptors can be induced in the livers of adult dogs by prolonged fasting or by treating the animals with cholestyramine (6).

Treatment with cholestyramine has also been shown to stimulate LDL catabolism by the LDL receptor pathway in man (7). This suggests that the human liver is capable of expressing LDL receptors, at least when the breakdown of hepatic cholesterol is stimulated. However, there remains the question as to how far hepatic LDL receptors contribute to the catabolism of LDL in man under physiological conditions.

In this investigation, we have measured LDL binding activity in membranes from liver biopsies obtained from eight human subjects with normal or slightly raised cholesterol levels and from seven patients with heterozygous familial hypercholesterolemia (FH), a condition in which only half the normal number of LDL receptors is expressed in cultured fibroblasts (8).

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SUBJECTS AND METHODS

Samples of liver (0.5–1.0 g) were obtained from 15 human subjects. Seven had normal plasma cholesterol concentrations (<6.5 mmol/liter or 250 mg/dl), and one had slight hypercholesterolemia not due to familial hypercholesterolemia (FH). None was known to be suffering from any metabolic disorder known to influence plasma lipoprotein metabolism. The other seven subjects had FH diagnosed on the basis of type IIa or b hyperlipoproteinemia as well as an affected first-degree relative or tendon xanthomata (or both) and were undergoing partial ileal bypass as described (9). Informed consent was obtained from all patients undergoing surgery. Wedge-biopsy of the liver is performed routinely by one of us (C.B.W.) during cholecystectomy and partial ileal bypass, primarily for histological purposes. All samples were obtained between 0800 and 1100 hr after the subject had fasted for at least 8 hr. All but one of the 15 subjects had received no drug known to affect cholesterol metabolism for at least 4 wk before the liver biopsies; one FH subject was receiving cholestyramine (16 g/day). Relevant details of the subjects are shown in Table 1.

Preparation of Liver Membranes. Membranes were prepared from the biopsy samples essentially as described by Kovanen *et al.* (10) within 1 hr of surgery, during which time the liver sample was placed on ice in Dulbecco's phosphate-buffered saline (GIBCO). All subsequent procedures were carried out at 4°C. The liver was minced, suspended in 30 ml of 0.01 M Tris·HCl/0.15 M NaCl/1 mM $CaCl_2$, pH 7.5, and homogenized with 20 strokes of a Teflon/glass homogenizer. After centrifugation for 10 min at 1,000 × g_{avg} followed by centrifugation for 20 min at 8,000 × g_{avg} a membrane pellet was sedimented from the 8,000 × g supernatant by centrifugation for 60 min at 105,000 × g_{avg} in a Beckman 40 rotor. The pellet was suspended in 10 ml of 0.01 M Tris·HCl/0.15 M NaCl/1 mM $CaCl_2$, pH 7.5, and dispersed by 10 strokes of a glass/glass homogenizer. The membranes were resedimented by centrifugation for 60 min at 115,000 × g_{avg} in a Beckman 40.3 rotor. The portions of the tubes containing the membrane pellets (usually two per sample) were stored in liquid N_2 for up to 3 months. No differences were observed between freshly prepared membranes and those stored in liquid N_2.

Abbreviations: FH, familial hypercholesterolemia; LDL, low density lipoprotein; Chd, 1,2-cyclohexanedione; ^{125}I-LDL and ^{125}I-LDL-Chd, ^{125}I-labeled LDL and LDL-Chd, respectively; apoB, apolipoprotein B; apoE, apolipoprotein E.

† Present address: Medizinische Poliklinik der Universität München, Pettenkoferstrasse 8a, D-8000 München 2, Federal Republic of Germany.

§ To whom reprint requests should be addressed.

Table 1. Details of subjects

Subject	Sex	Age, yr	Plasma cholesterol, mmol/liter	Surgical procedure
			FH heterozygotes	
W.P.*	M	62	6.3	Partial ileal bypass
R.S.	M	39	11.2	Partial ileal bypass
D.D.	M	31	9.5	Partial ileal bypass
D.W.	F	40	10.5	Partial ileal bypass
J.K.	M	47	11.1	Partial ileal bypass
T.R.	M	43	13.4	Partial ileal bypass
A.B.	M	36	10.0	Partial ileal bypass
Mean		39.3 ± 5	11.0 ± 1.4	
			Normocholesterolemic controls	
P.W.	F	54	4.8	Partial hepatectomy
B.R.	M	21	3.6	Ileal resection (Crohn disease)
J.O'S.	M	60	5.3	Cholecystectomy
S.O.	F	42	4.6	Cholecystectomy
E.M.	F	55	3.0	Cholecystectomy
A.C.	F	55	5.5	Cholecystectomy
C.G.	F	47	6.3	Cholecystectomy
Mean		47.7 ± 12	4.72 ± 1.05	
			Hypercholesterolemic (non-FH) control	
D.T.	F	57	7.6	Cholecystectomy

* W.P. was receiving cholestyramine at 16 g/day; all other FH subjects had discontinued hypocholesterolemic drugs at least 4 wk before surgery.

Isolation and Labeling of Lipoproteins. Plasma from normal human donors was obtained from freshly drawn blood containing 0.01% EDTA. LDL (density, 1.019–1.055 g/ml) was isolated by differential centrifugation (11) and was labeled with $Na^{125}I$ (Amersham International) by using iodine monochloride (12); in some instances, ^{125}I-labeled LDL (^{125}I-LDL) was treated with 1,2-cyclohexanedione (Chd) as described (13). Labeled and unlabeled lipoproteins were stored sterile in 0.15 M NaCl/1 mM EDTA, pH 7.4, at 4°C for up to 2 wk with no apparent alteration in their properties. Chd-modified LDL was used within 24 hr of preparation. Before each experiment, ^{125}I-LDL and LDL-ChD were dialyzed for 2 hr against 10 mM Tris·HCl (pH 7.5) and the specific radioactivity was determined by using the method of Lowry *et al.* (14) to determine protein concentration, with bovine serum albumin as standard. The sterile labeled lipoprotein solutions were diluted to a concentration of 0.15 mg of LDL protein/ml in 12.5 mM NaCl/0.5 mM $CaCl_2$/50 mM Tris·HCl, pH 7.5, containing bovine serum albumin at 20 mg/ml (Sigma; fraction V, fatty acid free).

Experimental Procedure. On the day of the experiment, the membrane pellets were thawed and resuspended in 0.5 ml of 20 mM Tris·HCl/0.05 M NaCl/1 mM $CaCl_2$, pH 7.5, by flushing through a 25-gauge needle. The suspension was sonicated three times for 10 sec each at 0°C (sonicator peak-to-peak, 18 μm). The protein concentration, determined by the method of Lowry *et al.* (14) with bovine serum albumin as standard, was adjusted with resuspension buffer to 5 mg/ml. Unused sample was stored in liquid N_2 in aliquots of approximately 250 μl and used in subsequent assays after sonication for 10 sec at 0°C. No sample was frozen and thawed more than once after the initial sonication.

The binding assay was carried out essentially as described by Basu *et al.* (15) with the following minor modifications. The labeled lipoproteins were incubated with membranes for 2 hr at 0°C in Beckman 250-μl polyethylene Microfuge tubes. The total assay volume was 60 μl, comprising 10 μl of membrane suspension (5 mg of protein/ml in 0.02 M Tris·HCl/0.05 M NaCl/1 mM $CaCl_2$, pH 7.5) and 50 μl of 0.05 M Tris·HCl/12.5 mM NaCl/0.5 mM $CaCl_2$, pH 7.5, containing bovine serum albumin at 20 mg/ml, the labeled lipoproteins, unlabeled lipoproteins, and EDTA to give the concentrations shown in Figs. 1–3 and Table 2. After incubation, 50 μl of the assay mixture was layered onto 180 μl of fetal calf serum in a Beckman 42.2 rotor tube and centrifuged for 10 min (zero time, maximum speed attained, approximately 7 min) at 42,000 × g_{avg} at 4°C in the 42.2 rotor. The supernatant was aspirated and replaced with 230 μl of fetal calf serum and the centrifugation was repeated. The supernatant was then removed and the whole tube containing the membrane pellet was assayed for radioactivity. With ^{125}I-LDL at a concentration of 25 μg of protein/ml, the amount of LDL bound by membranes from a normocholesterolemic subject was dependent on the amount of membrane protein added in the range 25–100 μg of membrane protein per assay. In all other experiments, the amount of membrane protein per assay was 50 μg. The amount of labeled LDL apparently bound in the absence of membranes was always less than 10% of that bound by 50 μg of membrane protein from a normocholesterolemic subject. In preliminary experiments, no significant differences were found between the results of assays carried out using the Beckman Airfuge as described by Basu *et al.* (15) and by the method described above.

RESULTS

Membranes isolated from liver biopsy samples from a normocholesterolemic subject (P.W.) and a FH heterozygote (J.K.) were incubated with various concentrations of ^{125}I-LDL or ^{125}I-labeled LDL-Chd [a procedure that blocks the recognition sites on LDL for the LDL receptor (16)], and the amount of labeled lipoprotein bound to the membranes was determined (Fig. 1). With membranes from the normocholesterolemic subject, the amount of LDL bound increased in a nonlinear manner as the concentration of LDL in the incubation mixture in-

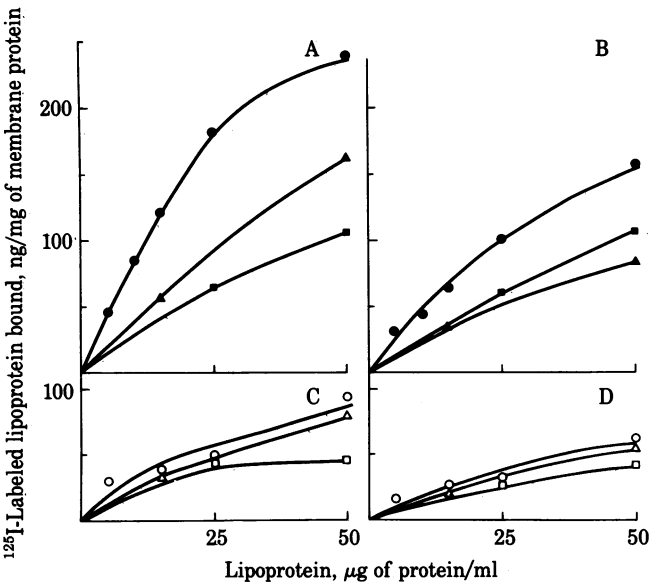

FIG. 1. Binding of ^{125}I-LDL and ^{125}I-LDL-Chd to liver membranes from a normocholesterolemic subject and a FH heterozygote. Membranes from the normal subject (*A* and *C*) or the FH heterozygote (*B* and *D*) (50 μg of protein in a total volume of 60 μl of buffer) were incubated for 2 hr at 0°C with ^{125}I-LDL (*A* and *B*) or ^{125}I-LDL-Chd (*C* and *D*) at various concentrations, and the amount of labeled lipoprotein bound in the presence of no further additions (●, ○), of excess unlabeled LDL (1 mg/ml) (■, □), or of 30 mM Na_2EDTA (▲, △) was determined. The specific activity of the ^{125}I-LDL was 52 cpm/ng and that of the ^{125}I-LDL-Chd was 56 cpm/ng.

creased, suggesting that the LDL was binding to saturable sites on the membranes. When the assay was carried out with excess unlabeled LDL (1 mg of protein/ml) in the incubation mixture, much less ^{125}I-LDL was bound at all concentrations, which confirmed that the binding of LDL was saturable. The addition of 30 mM EDTA also inhibited binding of ^{125}I-LDL to membranes but to a lesser extent than unlabeled LDL. In the presence of both EDTA and unlabeled LDL, the amount of ^{125}I-LDL bound by the membranes was slightly lower than that bound in the presence of unlabeled LDL alone but it was not reduced to the level that would be expected if the effects of EDTA and unlabeled LDL were additive.

Liver membranes from the normocholesterolemic subject were also able to bind ^{125}I-LDL-Chd, although less than one-third as much LDL-Chd was bound at any concentration tested. Neither the addition of EDTA (30 mM) nor the addition of unlabeled LDL (1 mg/ml) had any marked effect on the binding of LDL-Chd.

The amount of ^{125}I-LDL bound by membranes from the FH heterozygote was lower at all concentrations of ^{125}I-LDL in the incubation mixture than that bound by membranes from the normocholesterolemic subject, and the addition of unlabeled LDL (1 mg of protein/ml) reduced the amount of labeled LDL bound to a lesser extent. With membranes from the FH subject, the effect of EDTA (30 mM) on binding of ^{125}I-LDL was similar to that of unlabeled LDL. The amount of LDL-Chd bound by membranes from the FH subject was lower than the amount of LDL bound and was similar to the amount of LDL-Chd bound by membranes from the normocholesterolemic subject. Neither EDTA (30 mM) nor unlabeled LDL (1 mg of protein/ml) had any significant effect on the binding of LDL-Chd to membranes from the FH subject.

Subtraction of the amount of ^{125}I-LDL bound in the presence from that bound in the absence of excess unlabeled LDL provided an estimate of the amount of LDL bound at any given concentration of labeled LDL to saturable binding sites (saturable binding); similarly, the amount of LDL bound to sites inhibited in the presence of EDTA (EDTA-sensitive binding) was calculated by subtracting the amount of ^{125}I-LDL bound in the presence of 30 mM EDTA from that bound in its absence. At the highest concentration of ^{125}I-LDL tested (50 μg of protein/ml) saturable binding of LDL by the membranes from the normocholesterolemic subject (P.W.) was more than 3 times that by the membranes from the FH heterozygote (J.K.) (135 ng compared with 39 ng/mg of membrane protein). Similar binding curves were obtained with membranes from a second normocholesterolemic subject (A.C.) and a second FH heterozygote (A.B.); saturable binding of LDL at 50 μg of protein/ml to membranes from A.C. was approximately 50% higher than binding to membranes from A.B. (108 ng compared with 74 ng/mg of membrane protein). Saturable and EDTA-sensitive binding of ^{125}I-LDL-Chd was negligible with membranes from all four subjects.

A limited study of the effects of pH and ionic strength on LDL binding was made with the small amounts of biopsy material that were available. The results are shown in Table 2. Saturable binding was higher at pH 7.5 than at pH 7.2 or pH 8.0 and was partially inhibited by increasing the NaCl concentration in the medium from 12.5 mM to 150 mM. EDTA-sensitive binding was higher at pH 8.0 than at pH 7.5 or pH 7.2 and was partially inhibited when the NaCl concentration was increased to 150 mM.

The ability of liver membranes from six heterozygous FH subjects and seven normocholesterolemic subjects to bind LDL to saturable or EDTA-sensitive binding sites was compared by using a concentration of ^{125}I-LDL of 25 μg of protein/ml in the incubation mixture. The amount of LDL bound to saturable

Table 2. Effects of pH and ionic strength of the incubation medium on binding of LDL to liver membranes from normocholesterolemic subjects

	pH			NaCl	
Binding	7.2	7.5	8.0	12.5 mM	150 mM
Total	128	200	160	186	175
Saturable	86	142	110	109	77
EDTA sensitive	44	68	90	63	44

Membranes were incubated with ^{125}I-LDL (25 μg of protein/ml) as described in *Subjects and Methods* except that either the pH of the Tris·HCl buffer or the NaCl concentration in the medium was varied. The amount of labeled LDL bound in the presence or absence of excess unlabeled LDL (1 mg/ml) or EDTA (30 mM) was determined at each pH or NaCl concentration. Values for saturable and EDTA-sensitive binding are expressed as ng of ^{125}I-LDL bound/mg of membrane protein and are means of duplicate assays.

binding sites by membranes from the normocholesterolemic subjects was 82.3 ± 28.7 (mean ± SD) μg/mg of membrane protein and was nearly twice the value for membranes from the FH subjects (42.7 ± 19.8 μg/mg of membrane protein) (Fig. 2); the difference between normocholesterolemic and FH subjects was statistically significant ($P < 0.02$). However, there was no difference in the amount of LDL bound to EDTA-sensitive binding sites at pH 7.5. Two subjects were excluded from the comparison in Fig. 2: one subject with mild nonfamilial hypercholesterolemia (D.T.) and one FH subject who was taking 16 g of cholestyramine/day (W.P.). These subjects are included in the data shown in Fig. 3, in which the saturable binding of LDL

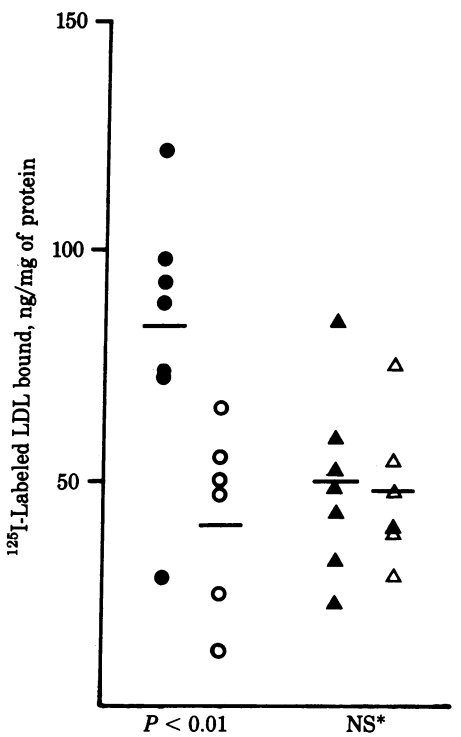

FIG. 2. Comparison of binding of ^{125}I-LDL to liver membranes from six normocholesterolemic subjects with that to liver membranes from six heterozygous FH subjects. Saturable binding (●, ○) and EDTA-sensitive binding (▲, △) of ^{125}I-labeled normal human LDL at a concentration of 25 μg of LDL protein/ml of incubation mixture by liver membranes from normal (●, ▲) and FH heterozygous (○, △) subjects was determined. The specific activity of the ^{125}I-LDL was 75 cpm/ng. Each point is the mean of triplicate determinations for membranes from a single subject. *Significance level was determined by Student's *t* test. NS, not significant.

to liver membranes was compared with the plasma cholesterol level of each subject. There was a highly significant inverse correlation ($r = -0.7186$, $P < 0.02$) between these two variables for the whole group. However, there was no correlation between plasma cholesterol concentration and saturable binding of LDL to liver membranes within either of the two groups of subjects.

DISCUSSION

We have shown that membranes from fresh human liver have saturable binding sites for LDL that are completely inhibited by Chd modification of the LDL. This suggests that adult human liver in the unstimulated state expresses LDL receptors similar to the LDL [apolipoprotein B (apoB), apolipoprotein E (apoE)] receptors identified by Mahley *et al.* (6) in the livers of immature dogs. Our finding that saturable binding of LDL by liver membranes of FH subjects is about half that by membranes from normal subjects provides strong evidence that the receptors responsible for this binding are the same gene products as the LDL receptors present on human fibroblasts in culture (8). However, it is not easy to explain why binding to the saturable binding sites was only partially inhibited by 30 mM EDTA, since the LDL receptor in adrenal cortex membranes is Ca^{2+} dependent (10) and binding of LDL to normal rabbit liver membranes is inhibited by much lower concentrations of EDTA than we used, while that to liver membranes from the WHHL rabbit, which lacks LDL receptors, is unaffected (17). It is possible that the assay conditions that were optimal for determination of saturable binding of LDL to the liver membranes were not optimal for the determination of EDTA-sensitive binding (Table 2), and thus we have placed greater emphasis on the results for saturable binding. Further experiments will be necessary to clarify the significance of the apparent lack of EDTA sensitivity of LDL binding to human liver membranes.

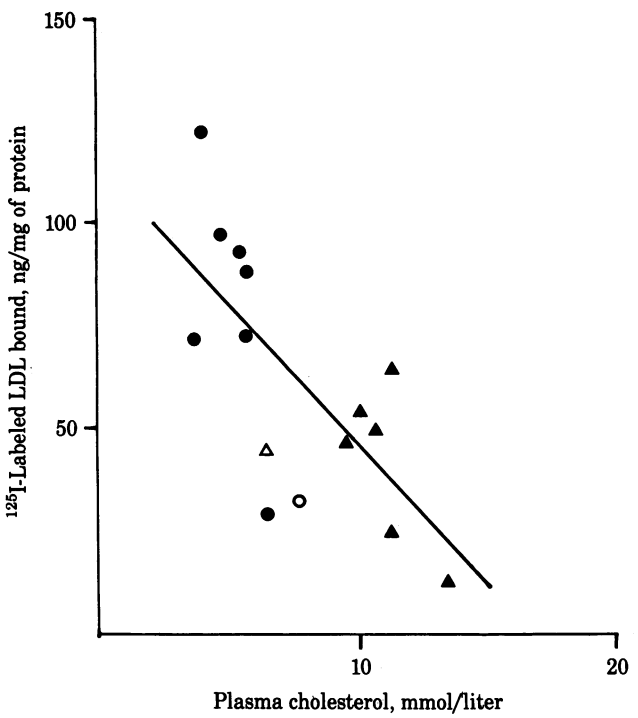

FIG. 3. Relationship between saturable binding of ^{125}I-LDL to liver membranes from normal subjects and from patients with heterozygous FH and plasma cholesterol concentration. Saturable binding of ^{125}I-LDL at a concentration of 25 μg of LDL protein/ml of incubation mixture by liver membranes from non-FH subjects (●) and FH heterozygotes (▲) was determined as described in Figs. 1 and 2. The specific activity of the LDL was 75 cpm/ng of protein. Each point is the mean of triplicate determinations of binding by membranes from a single subject determined in two separate experiments. The data include two subjects excluded from the data shown in Fig. 2 because one had mild nonfamilial hypercholesterolemia (○) and the other was a FH subject receiving cholestyramine (△). Significance ($P < 0.02$, $r = -0.7186$) was determined by Student's t test.

Mahley *et al.* (6) were unable to demonstrate significant binding of LDL to membranes from the livers of three human adults, although these membranes expressed receptors for apoE-containing lipoproteins having properties similar to the receptors on dog liver membranes, which recognize apoE but not apoB (apoE receptors) (4). This discrepancy is difficult to explain, but it should be noted that, in our study, the membranes were prepared from fresh tissue within 1 hr of surgery whereas the membranes used by Mahley *et al.* (6) were prepared from tissues frozen 72 hr previously. Also, the LDL receptors in dog liver are markedly influenced by nutritional and other factors (6) and it is possible that differences in the physiological states of the subjects at the time of operation contributed to the differences in the results of the two studies.

We cannot exclude the possibility that small amounts of apoE-containing lipoproteins were present in our LDL preparations and that the saturable binding observed was, in fact, due to binding by the apoE receptors present in human adult liver. However, this seems unlikely for several reasons. First, apoE was not detectable by $NaDodSO_4$/polyacrylamide gel electrophoresis in the LDL preparation used in this study. Second, the binding of apoE-containing lipoproteins by the apoE receptor is independent of the salt concentration in the medium but is very sensitive to pH (4) whereas the saturable binding of LDL by human liver membranes was reduced by less than 25% when the pH was changed from 7.5 to 8.0. Finally, it is difficult to explain the difference between the binding of LDL to normocholesterolemic and FH membranes in terms of the apoE receptor; Mahley *et al.* (6) found no evidence that the activity of the apoE receptor was subject to metabolic control by changes in cholesterol metabolism, and there is no evidence that FH homozygotes are unable to clear chylomicron remnants from the circulation to the extent that would be expected if their apoE receptors are defective.

In view of the strong inverse correlation between maximum saturable binding of LDL to hepatic membranes from an individual and his or her plasma cholesterol concentration, the possibility exists that the reduced binding of LDL to FH membranes is a consequence of the high plasma cholesterol concentration rather than a reflection of a defect in the LDL receptor in hepatic membranes. In this context, it was of some interest that the two non-FH subjects who had the highest plasma cholesterol levels (C.G. and D.T.) also showed the lowest binding of LDL to saturable sites on the membranes, although there were not sufficient hypercholesterolemic non-FH subjects to determine the significance of this observation.

The extent to which an LDL receptor-mediated pathway in the liver contributes to the catabolism of LDL in man under physiological conditions cannot, of course, be determined from our study of the behavior of liver membranes *in vitro*. However, the present work suggests that man resembles the rabbit (2), and possibly the rat (3), in expressing functional hepatic LDL receptors in the mature state *in vivo*.

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