Characterization of chylomicron remnant binding to rat liver membranes

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Abstract The binding of chylomicron remnants to rat liver membranes was investigated using radioiodinated lipoproteins. The specific activity of binding increased in parallel with increased enrichment in plasma membrane markers. The yield of receptor activity, however, decreased with enrichment. Accordingly, a partially purified plasma membrane preparation was used for routine studies. Binding was saturable, with half maximal binding achieved at 4.6 μg tetramethylurea-precipitable protein per ml. The rate of binding was time- and temperature-dependent. It could be inhibited only moderately by 10 mM EDTA. Chylomicron remnants appeared to bind to the membrane as a unit. The bound particle was richer in apoproteins of 20,000–50,000 molecular weight relative to low molecular weight apoproteins than the particles that were not bound. Lipoprotein particles containing only human apoB did not bind to liver membranes nor did they compete for the remnant binding site. Rat lipoproteins of d 1.019–1.063 g/ml did compete for remnant binding. When they were separated into apoB-rich (LDL) or apoE-rich (HDL_c) fractions by block electrophoresis, the apoE-rich fraction was a more potent competitor. ApoE purified and reconstituted into dimyristoyl phosphatidylcholine vesicles was a potent competitor for the remnant binding site. Vesicles containing ^{125}I-labeled apoE bound to the membranes, and they could be displaced by unlabeled remnants. Dimyristoyl phosphatidylcholine vesicles themselves did not compete with either remnants or apoE-phospholipid vesicles. These results offer strong support for the hypothesis that the liver membrane chylomicron remnant receptor recognizes apoE with a high affinity, and this initiates the rapid removal of lipoproteins that contain this apoprotein.—**Cooper, A. D., S. K. Erickson, R. Nutik, and M. A. Shrewsbury.** Characterization of chylomicron remnant binding to rat liver membranes. *J. Lipid Res.* 1982. **23:** 42–52.

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The liver plays a central role in the metabolism of lipoproteins. Early investigations focused on its role in the synthesis and secretion of lipids and lipoproteins. More recently there has been considerable interest in its role as a site of lipoprotein catabolism. The liver appears to have a limited role in high density lipoprotein catabolism (1). It is, however, now believed to be a quantitatively important site of low density lipoprotein catabolism. Recent estimates suggest that up to 50% of LDL is removed and catabolized by the liver (2). The cellular mechanism of LDL removal and degradation has been studied extensively by Brown and Goldstein (3) using cultured fibroblasts as a model. The details of how this mechanism applies to liver are not fully understood. The rate of LDL removal and catabolism by rat liver is, in fact, relatively slow (4) as compared to the removal and catabolism of a more recently described class of lipoproteins, the chylomicron remnant (5, 6). This latter class of lipoproteins seems to be the principle source of exogenous cholesterol for the liver (7) and may be an important source of fatty acids as well (8).

Work from this laboratory over the last several years has been directed toward studying the mechanism of chylomicron remnant metabolism (5, 8–10) in the rat. The rapid, saturable, and specific uptake of chylomicron remnants by liver suggested that this organ might have a cell surface receptor that recognizes some constituent of these particles. In a previous publication from this laboratory (10), high affinity binding of chylomicron remnants to rat liver plasma membranes was described. The binding appeared to be specific and saturable and had other features compatible with the interaction of a receptor and its ligand. Furthermore, in a preliminary report we suggested that apoE was a principle determinant of remnant binding (11). Several other recent reports are in agreement with this suggestion (12–14).

Other work suggests that lipoproteins containing only apoB are normally removed slowly by liver (15) and bind poorly to liver membranes (16). Treatment of animals with lipid-lowering agents increases both rate of removal and binding of the lipoproteins (15–17), possibly by inducing a second lipoprotein receptor (18). The present studies provide additional insights into the nature of the lipoprotein-plasma membrane interaction.

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; DMPC, dimyristoyl phosphatidylcholine; TMU, tetramethylurea; SDS, sodium dodecylsulfate; INT, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-2H-tetrazolium chloride.

MATERIALS AND METHODS

Animals

Sprague-Dawley strain rats (Simonson Laboratories, Gilroy, CA) were housed in a windowless room with a controlled illumination schedule. Animals were fed a standard chow diet and weighed between 200 and 300 g. Lymph donors weighed 300 g and were treated as previously described (19).

Preparation of lipoproteins

Mesenteric lymph was collected through an in-dwelling silastic catheter. Lymph was collected during intragastric egg infusion: 1 egg per 125 ml of 0.9% NaCl infused at a rate of 2 ml/hr. EDTA (2 mg) and gentamycin (4 mg) were added to the 125-ml collection flasks. Chylomicrons were prepared as previously described (4). Chylomicron remnants were prepared in rats eviscerated by a modification of the technique of Redgrave (20) as previously described (5, 19). The lipoprotein classes were separated by the method of Havel, Eder, and Bragdon (21). The precise densities of the lipoproteins are given with the individual experiments. All lipoprotein fractions were further purified by chromatography on agarose. This removed the contaminating proteins including albumin. This step was omitted for only one experiment. The composition of chylomicrons was 90.6% triglyceride, 1% cholesteryl ester, 0.2% free cholesterol, 7.7% phospholipid, and 0.6% protein while that of remnants was 79.5% triglyceride, 6.5% cholesteryl ester, 1.3% free cholesterol, 10.5% phospholipid, and 2.2% protein. Rat HDL_c was prepared from the serum of rats fed an atherogenic diet (14), by ultracentrifugation at a density of 1.020–1.065 g/ml. The HDL_c was then separated from LDL by block electrophoresis as described by Mahley and Weisgraber (22), except that only Pevikon was used for preparation of the block. Lipoproteins were radioiodinated exactly as described previously (19).

Characterization of the lipoproteins

The amount of apoB in the lipoproteins was estimated by multiplying the percent of radioactivity which was TMU-precipitable (23) by the total protein content as estimated by the method of Lowry et al. (24). The TMU precipitation was carried out by adding the radiolabeled lipoproteins to 200 μl of human serum, which acts as a carrier. Four hundred μl of TMU was added and the mixture was incubated in the cold overnight. After centrifugation, the clear phase, which contains the TMU-soluble protein, was removed and the cloudy phase was extracted with 1–2 ml of ethanol–diethyl ether 3:1 (v:v). This was mixed and vortexed. The supernatant which contained the lipid was removed. The pellet contained apoB. This approach assumes that the radiolabel is uniformly distributed on the apoproteins. Since apoE labels poorly by the McFarlane (25) method, some error is introduced by this assumption. By this method, apoB was estimated to comprise 31 ± 4% (SE, n = 9) of the protein mass of the remnant. This was somewhat higher than the estimates of apoB which we obtained from protein measurements of TMU-soluble and -insoluble apoproteins by the method of Lowry et al. (24) which showed that 24% of the protein was TMU-insoluble. However, such direct mass measurement may represent an underestimate (26); therefore, using TMU-precipitable ^{125}I counts appears to provide a reasonable value for the mass of apoB. In all classes less than 10% of the radioactivity was extracted with the lipid.

Purification and reconstitution of apoE

The lipoprotein fraction was isolated from the serum of hypercholesterolemic rats by centrifugation at d 1.21 g/ml. Lipids were moved by the method of Shore and Shore (27) using sequential extraction with mixtures of ethanol and ether. The lipid-free protein mixture was dialyzed against a buffer containing 5 mM urea, 2 mM sodium phosphate (pH 7.4), and 0.05 mM NaCl. The urea-soluble proteins were then fractionated as described by Shelburne and Quarfordt (28) on a heparin-Sepharose affinity column. The apoprotein composition of the fraction was monitored by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (see below). Those fractions containing only apoE were combined, concentrated, and used for reconstitution with phospholipid.

Reconstitution was carried out by the method of Innerarity, Pitas, and Mahley (29). Dimyristoyl phosphatidylcholine (DMPC) dissolved in organic solvent was dried under N_2 and then lyophilized. Buffered saline was added and the solution was sonicated at 30°C under N_2 in 30-sec bursts for 15 min. ApoE in buffered saline was then added to the solution to give a lipid to protein ratio of 4:1. This mixture was vortexed briefly and incubated overnight at 26°C.

Membrane preparations

Partially purified plasma membranes were prepared as previously described according to the method of Pohl, Birnbaumer, and Rodbell (30). In some experiments further purification was carried out by the method of Neville (31).

Cell membranes were prepared as described by Kovanen et al. (32). Rat livers were washed with ice cold saline. They were placed in buffer containing 150 mM

NaCl, 1 mM $CaCl_2$, 10 mM Tris (pH 7.5), and homogenized with two 10-sec pulses of a polytron homogenizer. The homogenate was centrifuged at 500 *g* for 5 min. The supernatant was centrifuged at 100,000 *g* for 1 hr. The pellet was washed by resuspension and centrifugation at 100,000 *g* for one hr. This pellet was frozen under liquid N_2 and was resuspended directly before use.

Polyacrylamide gel electrophoresis

For the iodinated lipoprotein to plasma membrane binding studies, polyacrylamide gel electrophoresis in urea was performed as described by Kane (33) and SDS gel electrophoresis was performed by the method of Wycoff, Rodbard, and Crambach (34). These gels were in tubes. For routine visualization of apoproteins, SDS disc gel electrophoresis in slabs was performed by the method of Laemeli (35) with 10–12% polyacrylamide. After electrophoresis, gels were fixed by the method of Kane (33) and stained with Coomassie Brilliant Blue R250.

Binding of lipoproteins to liver membranes

Binding of lipoproteins or reconstituted apoE preparations to plasma membranes was carried out by incubating the lipoprotein and plama membranes in siliconized Beckman microfuge tubes. The incubation buffer was 0.2 M sodium phosphate, pH 7.5. The standard assay contained 50 μg of membrane protein. The amount of lipoprotein present is described with each experiment. Routinely, incubation was for 40 min at 37°C followed by centrifugation for 2.5 min in a Beckman Microfuge. The pellet was washed twice with 10% sucrose and its radioactivity was measured in a Beckman gamma counter. In all experiments, control preparations containing no membranes were run.

Binding of remnants to cell membranes was carried out as described by Kovanen et al. (32) in a buffer containing 100 mM NaCl, 0.5 mM $CaCl_2$, 50 mM Tris HCl, and 20 mg/ml bovine serum albumin. One hundred micrograms of membrane and the indicated amount of lipoprotein were incubated at room temperature for 60 min. Membrane-bound lipoprotein was separated from unbound by centrifugation through fetal calf serum at 100,000 *g* in a Beckman Airfuge. The pellet was washed once and the ^{125}I counted.

In vivo wheat germ agglutinin binding to plasma membranes was assessed by the method of Chang, Bennett, and Cuatrecasas (36). ^{125}I-Labeled wheat germ agglutinin dissolved in Krebs-Ringer bicarbonate buffer was injected into the portal vein of anesthetized rats. Five minutes later the liver was flushed with 20 ml of ice cold buffer without wheat germ agglutinin. The liver was then removed and divided in half for the preparation of plasma membranes and of cell membranes.

Glucagon binding was studied by the method of Rodbell et al. (37). ^{125}I-Labeled glucagon was incbuated with plasma membranes and the bound and free glucagon were separated by centrifugation in a Microfuge. An Airfuge was used to separate the glucagon bound to cell membranes from unbound glucagon.

Other assays

Protein was determined by the method of Lowry et al. (24). In triglyceride-rich samples, the turbidity was removed by chloroform extraction after color formation (38). Cholesterol was determined by gas–liquid chromatography (19), and triglyceride was determined by the glycerol kinase method after alkaline hydrolysis (39). Glucose-6-phosphatase (40), succinic INT dehydrogenase (41) and 5′-nucleotidase (42) were assayed to estimate the relative purity of the plasma membrane preparations.

RESULTS

Characterization of liver membrane preparations

The liver plasma membrane is a complex and heterogeneous organelle. Different methods of liver membrane preparation provide material with different characteristics (43). It is worthwhile considering these characteristics when interpreting studies of ligand binding to membranes. Several types of membrane preparations were characterized by a variety of criteria. The preparations studied were the cell membrane preparation utilized by Kovanen et al. (32) for LDL binding studies, the partially purified plasma membranes utilized by Pohl et al. (30) for glucagon binding studies and by us previously (10) for remnant binding studies, and the purified plasma membrane preparation of Neville (31). Marker enzyme analysis (**Table 1**) demonstrated that the cell membranes were enriched about twofold in the plasma membrane marker, 5′-nucleotidase, and threefold in the endoplasmic reticulum marker, glucose-6-phosphatase. The partially purified plasma membranes were 16-fold enriched in 5′-nucleotidase and depleted to 2% of the glucose-6-phosphatase activity. The fully purified plasma membranes had no detectable glucose-6-phosphatase and a 32-fold enrichment of 5′-nucleotidase. There was significant contamination of both cell and partially purified plasma membranes with mitochondria. The purity of the plasma membrane preparation was confirmed by electron microscopy.

To assess whether any preparation was particularly

TABLE 1. Characterization of membrane preparations

	Succinate INT Dehydrogenase	Glucose-6-Phosphatase	5'-Nucleotidase		Wheat Germ Agglutinin		Glucagon	
				Rec[a]		Rec		Rec
				%		%		%
Homogenate	1.0	1.0	1.0	100	1.0	100	1.0	100
Cell membranes	1.1 ± 0.1	2.1 ± 0.6	2.5 ± 0.3	35 ± 11	2.1 ± 0.3	39 ± 13	1.5 ± 0.3	22 ± 2
Partially purified plasma membranes	1.8 ± 0.3	0.1 ± 0.1	17 ± 2	6.6 ± 0.5	4.0 ± 0.1	1.3 ± 0.2	74 ± 25	39 ± 1
Fully purified plasma membranes	0.2 ± 0.1	N.D.[b]	32.6	<1%				

[a] Recovery.
[b] None detectable.

Whole cell homogenates, cell membranes (32), partially purified plasma membranes (30), and fully purified plasma membranes (31) were prepared as described in Methods. They were then characterized with respect to membrane marker enzymes, their ability to bind glucagon (37), or their content of I^{125}-labeled wheat germ agglutinin (WGA) which had been injected into the portal vein 5 min before the animal was killed (36). Each activity is expressed as relative to the homogenate as 1.0. The activities of marker enzymes in the homogenate were succinate INT dehydrogenase = 94 ± 4 nmol INT reduced hr^{-1}·mg $protein^{-1}$; glucose-6-phosphatase = 0.8 ± 0.1 nmol Pi released hr^{-1}·mg $protein^{-1}$; 5'-nucleotidase = 0.58 ± 0.10 nmol Pi released hr^{-1}· mg $protein^{-1}$. The wheat germ agglutinin recovery in the whole homogenate was 1.3×10^5 cpm·mg $protein^{-1}$, and the glucagon binding to the whole homogenate was 5.9×10^3 cpm·mg $protein^{-1}$. The recoveries of 5'-nucleotidase, WGA, and glucagon binding capacity were also included. Each value is the mean and SE of at least three determinations, except for the fully purified membrane preparation which was prepared twice.

enriched in that portion of the membrane that is exposed to the blood flow (blood sinusoidal membrane), the technique of Chang et al. (36) was utilized. ^{125}I-Labeled wheat germ agglutinin was injected into the portal vein and 5 min later the liver was flushed with saline, and membranes were prepared. The amount of ^{125}I recovered is reflective of the amount of plasma membrane that is exposed to the blood. None of the membrane preparations were highly enriched in this marker (Table I). As expected, the recovery of ^{125}I-labeled wheat germ agglutinin was substantially greater in the cell membrane preparation than in the plasma membrane preparations.

To further characterize the membranes in terms of cell surface receptor activity, glucagon binding to the membrane preparations was studied. Glucagon binding was chosen because it represents a well-characterized receptor localized on the liver plasma membrane. (30, 37). The partially purified plasma membrane preparation was very highly enriched in ability to bind glucagon (Table 1). The cell membrane preparation provided only slight enrichment over the whole homogenate. The yield of glucagon receptors was actually greater with plasma membranes (Table 1).

Taken together these results point out the advantages and disadvantages of different liver membrane preparations. None of the preparations provide the ideal of a high yield of high specific activity material from the region of the membrane that is exposed to normal blood flow. The cell membrane preparation provides a good yield of cell surface markers, but it also contains a large amount of intracellular membranes so that binding specific activity is only moderate. The plasma membrane preparations are highly enriched in the organelle with receptor activity and are relatively free of intracellular membrane contamination, but the yield of material is not high.

Remnant binding to the various membrane preparations

The ability of chylomicron remnants to bind specifically to each of these membrane preparations was assessed. Binding conditions for each type of preparation were chosen to maximize specific binding and have been validated previously (10, 32). There was tenfold more binding to cell membranes than to whole homogenate on a per milligram protein basis. There was a sevenfold further enhancement of specific binding to partially purified plasma membranes compared to cell membranes. Yield of receptors was greater with cell membranes (57%) than with plasma membranes (16%). Further purification of the plasma membranes resulted in a commensurate increase in the specific binding activity. For example, at 0.075 mg of apoB/ml the specific binding was twice as great with the fully purified preparation as with the partially purified preparation but the receptor yield was one-fifth to one-tenth as great. Neither sonication nor freezing and thawing of the membranes increased the amount of remnant binding, suggesting that the vesicles were not inside out. These results provide very strong evidence that the observed binding of chylomicron remnants is a specific property of the plasma membrane. In the studies below, the partially purified plasma membrane preparation of Pohl et al. (30) was used because it combined an adequate yield with relatively low contamination by intracellular membranes and a high specific activity.

Binding constants for remnants

Remnant binding was saturable and had both specific and nonspecific components, as assessed by use of 100-fold excess of unlabeled lipoprotein. In the representative experiment shown in **Fig. 1,** half maximal binding as assessed by the Scatchard method (44) was achieved at a lipoprotein concentration of 4.6 μg of apoB/ml. Maximal occupancy was 6 μg of apoB/mg of membrane protein. In this and several subsequent experiments, lipoprotein concentration is expressed in terms of apoB. This was chosen because it is generally believed (45) that the amount of apoB per lipoprotein particle is the same in the various lipoprotein classes.Thus, the mass of apoB is an accurate reflection of the number of lipoprotein particles present and is probably the most useful parameter in comparing classes.

The results reported here are comparable with those obtained with particles labeled in vivo with [^{3}H]cholesterol (10). This suggests that the iodination process did not substantially alter the capability of the lipoproteins to bind to the receptor.

Effect of time and temperature on remnant binding

The amount of remnant binding was dependent on both time and temperature. At each time point up to 40 min, more remnants were bound at 37°C than at 20°C (**Fig. 2**). By one hour, binding appeared to be complete and was the same at the two temperatures. At 4°C, binding was substantially lower than at the higher temperatures. It increased slowly with time but did not approach

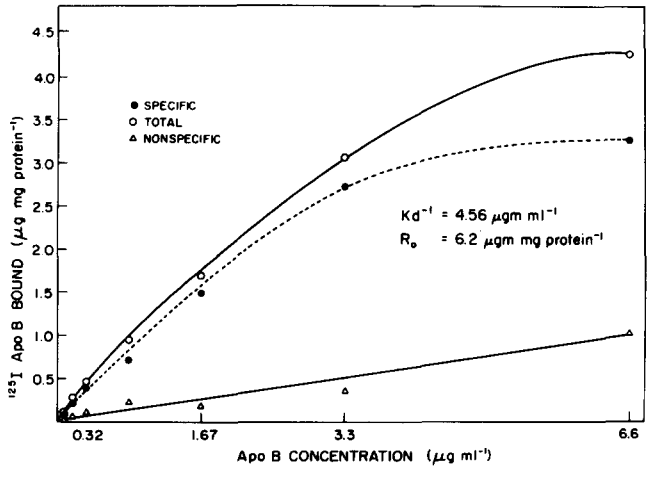

Fig. 1. Binding constants for remnants. The binding of remnants to partially purified plasma membranes as a function of apoB concentration in the incubation mixture was determined. Total binding (○) is the total number of ^{125}I counts bound. Nonspecific (△) is number of ^{125}I counts bound in the presence of 100-fold excess of unlabeled remnants. The specific binding (●) was calculated by subtracting the nonspecific binding from the total binding. Each incubation contained 50 μg of membrane protein in 0.3 ml total volume. The constants were calculated by the method of Scatchard (44).

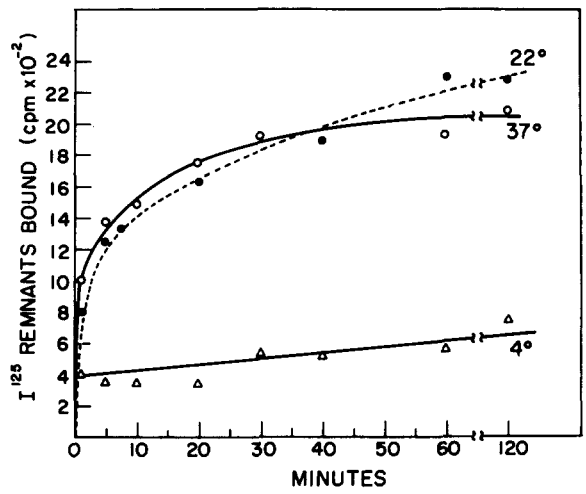

Fig. 2. Remnant binding as a function of time and temperature. Specific remnant binding assays were carried out at 4°C (△), 22°C (●), and 37°C (○). The mixture was incubated for various times before separating bound from unbound particles. Each incubation contained chylomicron remnants at a final concentration of 0.15 μg apoB ml^{-1} and membranes at a final concentration of 150 μg ml^{-1}.

a plateau during the first hour. The reason for this is unclear, but it has been observed with other membrane receptors (46) although not with LDL binding to fibroblast membranes (3). It may be due to agglutination of the triglyceride-rich lipoprotein.

Binding of various lipoprotein classes to liver plasma membrane

By use of lipoproteins labeled on the protein moiety it was possible to compare the binding of intact chylomicrons, chylomicron remnants, and low density lipoproteins directly in terms of apoB concentration.

The absolute amount of remnant bound varied both

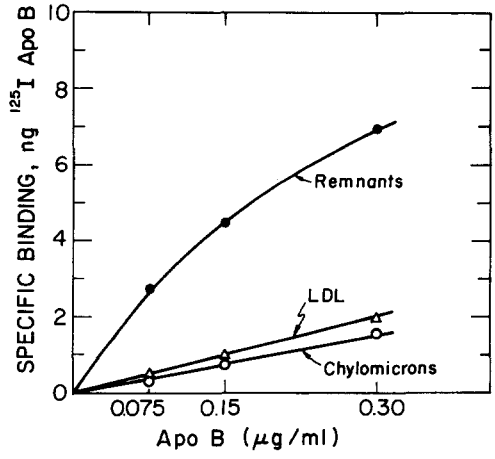

Fig. 3. Comparison of remnant binding with binding of other classes of lipoprotein. The specific binding of lipoproteins to partially purified liver plasma membrane was measured as described in Fig. 2. Specific binding was calculated from total and nonspecific binding as described in Fig. 1.

TABLE 2. Effect of EDTA on remnant binding

EDTA	Total ^{125}I Bound
mM	*dpm*
0.1	3844
5.0	3247
10.0	3026

The specific binding of remnants to plasma membranes was measured as described in Methods, except that the incubation medium contained the concentrations of EDTA shown. Each number is the average of two determinations.

with the particular preparation of remnants used and with the age of the membrane preparation. However, remnant binding was always saturable and generally more than 75% of the binding could be displaced by 100-fold excess of unlabeled remnants. In contrast, chylomicron binding was considerably lower than remnant binding, although specificity could be demonstrated (**Fig. 3**).

The binding of human LDL to these membranes was also tested. Human LDL has been found to bind to membranes of a number of tissues from several species (3, 32, 47). Moreover, human LDL was used because it contains only a single apoprotein (apoB) while a comparable fraction of rat LDL is difficult to obtain. Binding of these particles was consistently quantitatively less than remnants and saturability and specificity were difficult to demonstrate (Fig. 3).

Effects of EDTA, Concanavalin A, and Triton WR 1339 on remnant binding

Remnant binding to liver plasma membranes was only moderately sensitive to EDTA. Binding was decreased by about 30% at 10 mM EDTA (**Table 2**).

Concanavalin A caused a dramatic increase in remnant binding (**Fig. 4**) probably by creating a new, nonspecific class for glycoproteins. A similar phenomenon has been reported for LDL (3).

The detergent Triton WR 1339, which is known to coat lipoproteins and thus prevent their catabolism (48) blocked remnant binding (Fig. 4). Treatment of remnants with this detergent also prevents their removal by perfused liver.[1]

Gel electrophoresis patterns of ^{125}I-labeled chylomicron remnants before and after binding to membranes

In order to gain insight into the nature of the particle that was binding to the membrane, a series of studies were carried out utilizing polyacrylamide gel electrophoresis. Electrophoresis was performed on the radioiodinated lipoprotein directly as well as on the membrane-lipoprotein complex after binding. The gels were then sliced into 1-mm sections and ^{125}I radioactivity was determined.

In one set of experiments, the contaminating serum albumin was deliberately not removed from the iodinated remnants. Analysis by SDS polyacrylamide gel electrophoresis revealed a large peak of radioactivity in the albumin region before binding. After binding to plasma membrane, very little radioactivity was observed in the albumin region while that associated with the apoproteins remained. This result suggested that the observed binding did not merely represent trapping of material in the incubation mixture. Furthermore, presence of most of the apoprotein peaks in the bound particle is consistent with the concept that the particle binds as a unit (4, 6, 9). Similarly, the same percent of radioactivity was extracted with lipid before and after binding.

In another set of experiments using TMU-urea polyacrylamide gel electrophoresis (30), the radioactivity was localized in three regions both before and after binding (**Fig. 5**). The pattern was generally similar before and after binding except that the ratio of 20–60,000 dalton molecular weight apoproteins (Zone 3), probably apoA-I and apoE, to small molecular weight apoproteins (Zone 4), presumably C apoproteins and apoA-II, was higher in the bound particle than in the unbound. The ratio of radioactivity in Zone 3 relative to Zone 4 was 1.1 before binding and 2.7 after binding. This result supports the concept that true remnants are poor in the C apoproteins and rich in apoprotein E.

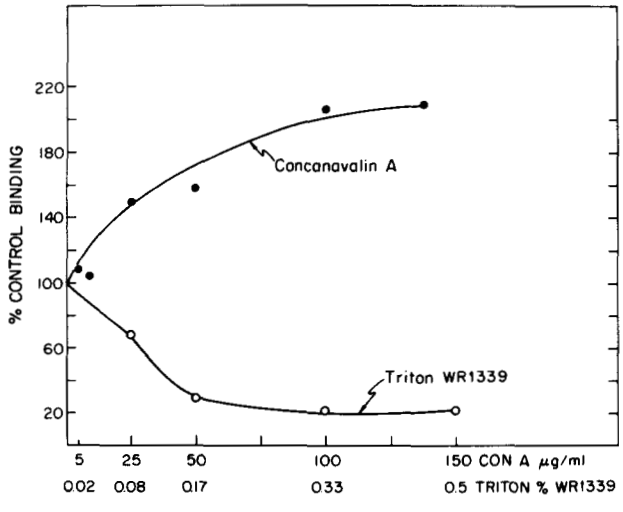

Fig. 4. Effect of Concanavalin A and Triton WR 1339 on remnant binding. Remnant binding assays were carried out in the presence of different amounts of Concanavalin A or Triton WR 1339. Concentrations of remnant and membrane were as described in Fig. 2. Total binding is shown.

[1] Cooper, A. D. Unpublished observations.

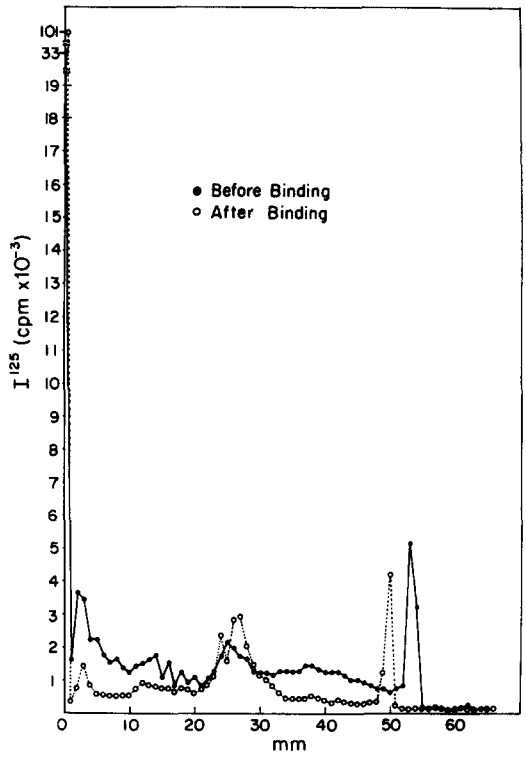

Fig. 5. Comparison of remnant particles before and after binding to plasma membranes. ^{125}I-Labeled chylomicron remnants were prepared as described in Methods. One aliquot was subjected to TMU-urea polyacrylamide gel electrophoresis as described by Kane (33). A second aliquot was incubated with partially purified plasma membranes as described in Fig. 2. After removal of the unbound particles, the entire plasma membrane-particle complex was also subjected to gel electrophoresis. After electrophoresis the gels were sliced into 1-mm sections and the amount of I^{125} in each section was determined. (— ● —), Before binding; (— O —), after binding. Zone I = 0–15 mm, Zone II = 15–30 mm, Zone III = 30–40 mm, and zone IV = 45–60 mm.

Competitive inhibition of ^{125}I remnant binding by other rat lipoproteins

To further determine which constituent of the remnant was responsible for the binding, competitive inhibition studies were carried out. In our previous report (10), it was found that human HDL and rat chylomicrons could compete with rat chylomicron remnants, but neither human LDL nor human VLDL competed effectively. Consistent with this is the present finding that human LDL binds poorly to the membranes (Fig. 3). This could be due either to a species difference in the LDL's or to the fact that the remnant receptor primarily recognizes a ligand other than apoB.

To differentiate between these possibilities it would be desirable to use a rat lipoprotein that contained only apoB. As an approach to this problem, lipoproteins of d 1.020–1.063 g/ml from the serum of cholesterol-fed hypothyroid rats were utilized. These lipoproteins did compete with radiolabeled remnants for the membrane binding site. On a protein basis, they were less potent competitors than unlabeled remnants (**Fig. 6**).

This density class contains two distinct lipoprotein types, LDL which contains apoB as its principal apoprotein, and HDL_c which contains apoE as its principal apoprotein. These lipoproteins were separated by block electrophoresis (22) and the ability of the two subclasses to compete for the remnant receptor was studied. The LDL fraction which always contained some apoE (**Fig. 7**) was a relatively poor competitor, while the HDL_c fraction was at least as potent a competitor for the binding site as the remnants themselves (Fig. 6). These results suggest that apoE is a primary determinant of remnant binding.

In other experiments, LDL from normal or hyperlipemic rats showed varying ability to displace remnants (**Fig. 8**). Despite purification on Pevikon blocks, these lipoproteins always had a trace of apoE visible by SDS polyacrylamide gel electrophoresis and the HDL_c always had some apoB present. Thus these experiments were not definitive in proving that apoE was responsible for the binding and displacement.

Displacement by apoE-reconstituted into vesicles

In order to assure that apoE itself was a determinant of displacement, this apoprotein was purified and reconstituted into vesicles composed of DMPC.

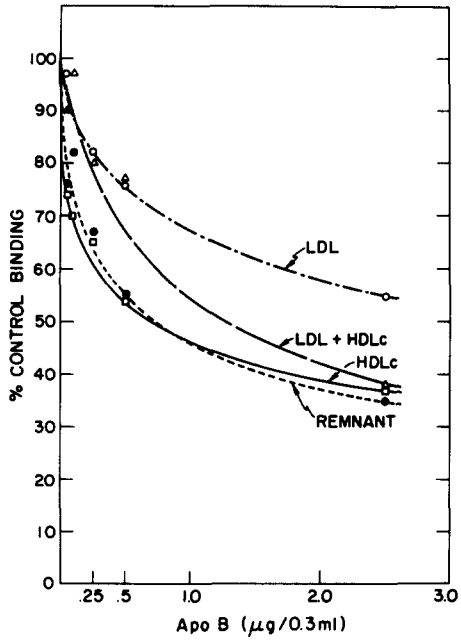

Fig. 6. Displacement of chylomicron remnants by lipoproteins from hyperlipemic rats. The ability of lipoproteins of d 1.019–1.063 g/ml from hyperlipemic rats to compete for the chylomicron remnant binding site was studied. Each incubation contained 50 μg of plasma membrane, ^{125}I-labeled chylomicron remnants containing 0.025 μg of apoB and various amounts of unlabeled lipoproteins. Total ^{125}I binding was measured. The lipoproteins tested were unlabeled remnants (— ● —), the unfractionated d 1.019–1.063 g/ml called LDL + HDL_c (△), the LDL fraction (— O —), and the HDL_c (— □ —) fraction. The LDL and HDL_c were separated by Pevikon block electrophoresis (22).

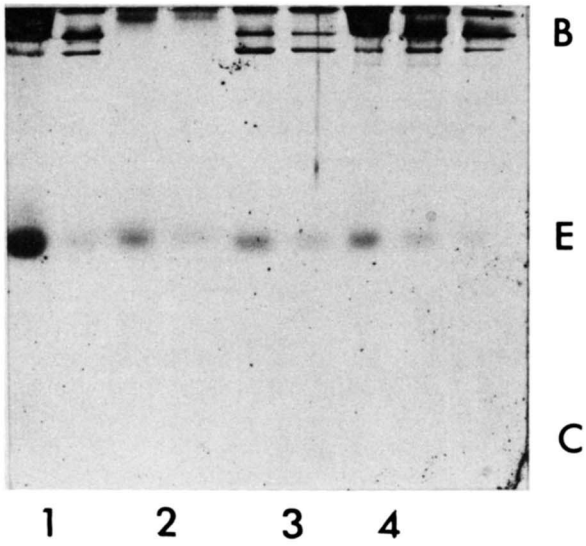

Fig. 7. SDS polyacrylamide gel electrophoresis of lipoproteins. SDS polyacrylamide gel electrophoresis of the lipoproteins was carried out by the method of Laemmli (35). Two aliquots of each sample, 10 μg and 20 μg of protein were analyzed. 1, Lipoproteins of d 1.019–1.063 g/ml from hypercholeserolemic animals. 2, HDL_c fractions of this lipoprotein after Pevikon block electrophoresis. 3, Intermediate fraction from the Pevikon block. 4, LDL fraction from the Pevikon block. B, E, and C refer to the area where the B, E, and C apoproteins migrate.

The apoE-containing vesicles were very effective competitors for the remnant binding site (**Fig. 9a**). In fact, they were generally more effective than unlabeled remnants themselves. Pure DMPC did not affect remnant binding. In a complementary experiment (Fig. 9b), vesicles containing pure ^{125}I-labeled apoE were prepared. These bound specifically to membranes. Their binding could be displaced by vesicles containing unlabeled apoE or by chylomicron remnants, but not by DMPC vesicles without apoE (Fig. 9b). Taken together, these experiments provide further evidence that the binding of chylomicron remnants to plasma membranes is mediated by the presence of apoE in the lipoprotein particle.

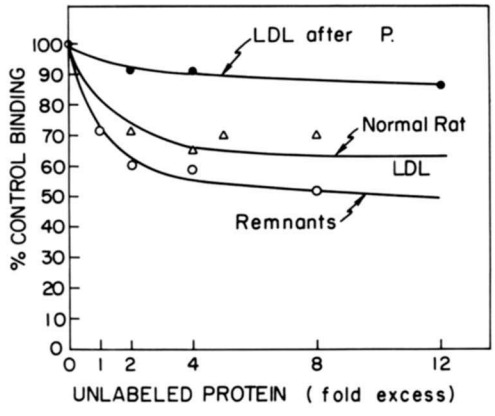

Fig. 8. Displacement of chylomicron remnants by LDL from normal rats. The experiment was performed exactly as described in Fig. 6 except that LDL from normal rats was used before (△) and after (●) Pevikon block electrophoresis. The results of displacement with unlabeled remnants (○) are also shown.

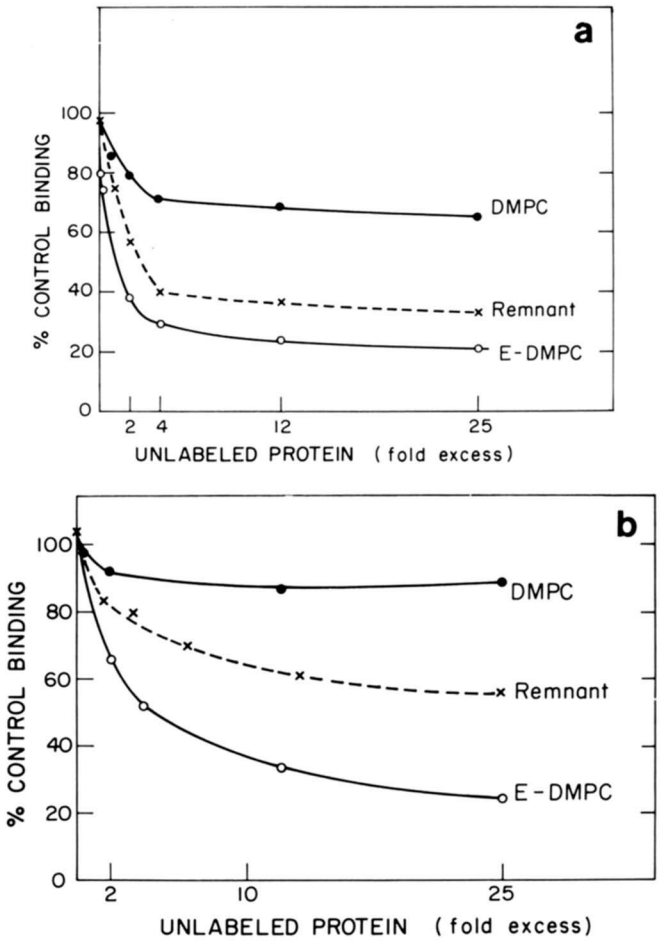

Fig. 9. Use of pure apoE:dimyristoyl phosphatidylcholine (DMPC) vesicles in binding studies. A), Membrane binding studies were carried out as described in Fig. 6 using a trace of labeled remnants and varying amounts of unlabeled remnants (×), DMPC vesicles (●), or DMPC vesicles containing pure apoE (○). B), 0.025 μg of I^{125}-labeled apoE in DMPC vesicles was incubated with membranes in the absence or presence of unlabeled remnants (×), DMPC vesicles (●), or DMPC vesicles with apoE (○).

DISCUSSION

The results of the present study confirm and extend those of previous investigations from this and other laboratories. The specific goal of this study was to characterize in detail the binding of chylomicron remnants to liver plasma membranes. One concern about the use of membranes for such studies is whether they accurately reflect events on the cell surface. Several types of membrane preparations are available for binding studies. The preparation chosen for use in these studies is highly enriched in several markers characteristic of the cell surface (5′-nucleotidase and glucagon receptor) and is essentially

free of contamination with intracellular organelles. As the preparation is progressively enriched in plasma membrane markers the specific activity of remnant binding increases. Moreover, the binding could not be displaced or prevented by non-lipoproteins such as DMPC vesicles, DMPC:cholesterol vesicles, Lipomul, or albumin (10). Taken together, these observations provide strong support for the contention that a plasma membrane lipoprotein receptor is being studied. This is distinct from the non-protein mediated LDL binding to plasma membranes described by others (49, 50). The use of a preparation with high specific activity and little contamination should have advantages for biochemical studies. However, the poor yield may detract from the usefulness of this preparation for studying the physiologic regulation of receptors because changes in total number of receptors per unit liver weight could be obscured by changes in yield. An alternative approach has been taken by Kovanen and colleagues (32). They use a cell membrane preparation in which the yield of receptor is high. However, the preparation is also enriched in intracellular organelles and thus the presence of binding sites on organelles such as the Golgi and endoplasmic reticulum are potentially a concern. Despite this, the results obtained with the two approaches were generally in agreement.

By using lipoproteins with the radiolabel on the protein moiety, it was possible to compare quantitatively the binding of remnants with that of other lipoproteins. The assumption made in doing this is that the amount of apoB per lipoprotein particle is the same in the various classes. Hence by comparing apoB concentrations one is actually comparing particle numbers. This has been validated for VLDL and LDL and seems to be true for chylomicrons and their remnants (45). A direct comparison of the amount of apoB per chylomicron with that per VLDL has not been made. An additional potential problem with this is the recent description of heterogeneity of apoB (51, 52), as well as controversy about the amount of apoB per particle. Despite this, the use of apoB as determined by TMU precipitation as a standard of comparison is likely to provide more accurate information than the use of total protein, and certainly more than the use of one of the lipid constituents. It must be kept in mind that when methodology for apoB quantification improves, the absolute kinetic parameters may require redetermination. Utilizing this approach, it was clear that, at equal concentrations of apoB, far more chylomicron remnants bound to liver plasma membranes than did LDL or intact chylomicrons. This is consistent with several physiologic observations. First, LDL removal by liver is a slow process even at low concentrations of LDL (44). Second, at comparable concentrations, the liver discriminates between chylomicrons and chylomicron remnants. Finally, the K_m for removal by liver and the K_d for binding are comparable (5, 6, 10).

The conclusion reached in this study, that apoE is responsible for remnant binding to normal liver membrane, has physiologic correlates as well, particularly in the perfused liver studies of Sherrill, Innerarity, and Mahley (12), Windler, Chao, and Havel (14), and Shelbourne et al. (13). Thus it seems reasonable to conclude that remnant removal is initiated by a liver plasma membrane receptor that has a high affinity for apoE. This is true both for chylomicron and VLDL remnants (14, 15, 17). The two classes appear to share the same receptor site and have a similar affinity per particle for it.[2] It has been suggested that the C apoproteins have a role in inhibiting remnant or apoE removal by perfused livers (13, 14). The mechanism of the C effect is not known.

The role of apoB in this system is somewhat less clear. In both this and the study of Windler et al. (17), human LDL bound poorly to the membranes and did not compete with remnants for the remnant binding site. On the other hand, in the normal state, rat LDL does bind to cell membranes but to a limited extent (17), and in our hands rat LDL was a weak competitor of remnant binding. In contrast, following ethinyl estradiol treatment, human and rat LDL bind well to rat liver membranes (16, 17). Thus although there are species differences in the binding of human and rat apoB to human and rat tissues (53, 54), these can not account for all of the current data. There are several possible explanations. This receptor, like the human LDL receptor may have a far greater affinity for apoE than apoB. Thus under normal circumstances, the acquisition of apoE by a lipoprotein would accelerate its removal. This explanation has been proposed by Windler et al. (17) to explain how ethinyl estradiol enhances the rate of LDL removal by liver since the drug induced an increase in binding of both E and B containing lipoproteins. A second possibility is that there are two distinct receptors; one, which recognizes only apoE, is abundant in liver, is not regulated by cholesterol (5, 6, 19), and is physiologically responsible for remnant removal. The other is analogous to the human LDL receptor, recognizes both apoB and apoE, and is the one that is stimulated by ethinyl estradiol. After the submission of this manuscript, Hui, Innerarity, and Mahley (18) published evidence that there are two distinct receptors on dog liver and that only one is stimulated by hypolipemia. Either of these hypotheses explains the slow but significant removal of LDL by liver and the fact that, when chylomicrons are converted to remnants, the concomitant acquisition of apoE markedly accelerates the rate of lipoprotein removal.

[2] Cooper, A. D., M. A. Shrewsbury, and S. K. Erickson. Unpublished observations.

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