PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 187, 296–308 (1988)

Apolipoprotein E-Rich HDL Binding to Liver Plasma Membranes in Copper-Deficient Rats[1] (42668)

CRAIG A. HASSEL,* TIMOTHY P. CARR,* JOHN A. MARCHELLO,*† AND K.Y. LEI*,[2]

*Department of *Nutrition and Food Science and Department of †Animal Science, University of Arizona, Tucson, Arizona 85721*

Abstract. Copper deficiency in rats raises plasma cholesterol concentration while reducing liver cholesterol concentration. One consequence of this cholesterol redistribution is the accumulation of a large high-density lipoprotein (HDL) particle rich in apolipoprotein E (apo E). The purpose of this study was to determine, using an *in vitro* binding assay, if the interaction of apo E-rich HDL with hepatic lipoprotein binding sites may be affected by copper deficiency. Male Sprague–Dawley rats were divided into two dietary treatments (copper-deficient and -adequate) and placed on a dietary regimen for 8 weeks. Subsequent to exsanguination, hepatic plasma membranes were prepared and apo E-rich HDL was isolated from rats of each treatment by ultracentrifugation, agarose column chromatography, and heparin–Sepharose affinity chromatography. Total binding and experimentally derived specific binding of ^{125}I-apo E-rich HDl to hepatic plasma membranes indicated greater binding when lipoproteins and membranes from copper-deficient animals were used in the assay compared to controls. Scatchard analysis of specific binding data indicated that equilibrium binding affinity (K_d) was also affected by copper deficiency. The hepatic binding sites recognizing apo E-rich HDL were not affected by EDTA or pronase, of relatively high capacity, and recognized a variety of other rat lipoproteins.

The association of plasma cholesterol levels with incidence of atherosclerosis has focused interest toward environmental and genetic factors that regulate plasma cholesterol concentration. Current hypotheses implicate dietary factors as fundamental contributors to widespread hypercholesterolemia observed in Western populations. As a result, various macronutrients such as dietary fats, fibers, and proteins have been examined for hypercholesterolemic effects in humans and animal models. However, the degree to which dietary micronutrients, such as trace elements, may contribute to cholesterol and lipoprotein metabolic regulation is, as yet, unclear.

Of the trace minerals, deficiency of dietary copper has been demonstrated to induce hypercholesterolemia in rats (1–11). These observations are significant because although rats are dissimilar to humans in a number of ways regarding cholesterol metabolism, this animal is quite resistant to changes in plasma cholesterol induced by other dietary means (12). The hypercholesterolemic mechanisms operating in this system may possibly be one of many contributing dietary factors influencing cholesterol metabolism.

The first evidence linking dietary copper and cholesterol was a hypercholesterolemia in rats induced by increasing the dietary ratio of zinc to copper (1). Subsequent experiments (2) demonstrated that dietary copper and not the dietary zinc to copper ratio was the factor influencing plasma cholesterol levels. The relationship between dietary copper levels and plasma cholesterol levels in rats has since been documented by a number of investigators (3–10); however, the biochemical role of copper in cholesterol metabolism remains largely unexplained. The first reports attempting to account for this observation showed no changes in hepatic cholesterol synthesis (3), bile acid synthesis (4), or

[1] Aided by funds from USDA Human Nutrition Competitive Grants Program 84-CRCR-1-1397 and 86-CRCR-1-1925 awarded to K. Y. Lei and from the Arizona Agricultural Experiment Station.

[2] To whom all reprint requests should be addressed at Department of Nutrition and Food Science, 309 Shantz Building, University of Arizona, Tucson, AZ 85721.

0037-9727/88 $1.50

biliary sterol excretion (5) as a result of copper deficiency. In a subsequent study, cholesteryl ester, newly synthesized from [2-^{14}C]-mevalonate, cleared the liver faster in copper-deficient rats than in controls (6). Kinetic analyses demonstrated increases in the size and half-life of the rapidly exchangeable cholesterol pool, consisting of tissues equilibrating rapidly with plasma cholesterol, within copper-deficient rats (7). In addition, copper deficiency prolonged the half-life of free and total cholesterol associated with plasma high-density lipoproteins (HDL) (8).

Additional evidence indicates rat plasma lipoprotein profiles are altered as a result of copper deficiency (10, 11). Separation by ultracentrifugation and agarose column chromatography have revealed elevated protein and cholesterol content in low-density lipoproteins (LDL) and HDL (10). Apolipoprotein E (apo E) content of HDL was also increased by copper deficiency (10), and when HDL was partitioned into apo E-rich and apo E-poor subfractions by heparin–Sepharose chromatography, the apo E-rich HDL accounted for most of the observed increases in protein and cholesterol (11).

Recent advances have allowed investigators to distinguish lipoprotein receptors and measure their expression in tissues and cells through *in vitro* binding assays. These studies have established that in rats HDL bind to hepatocytes (13, 14), smooth muscle cells (15), and intestinal mucosal cells (16) and to membranes prepared from liver (17), testis (18), adrenal (19), ovary (20), and kidney (21) tissues. The binding sites observed in these studies recognize apo E-free HDL and possess characteristics distinct from those of the LDL receptor. However, apo E-rich HDL is a ligand which has been shown to recognize multiple hepatic receptors in other animal models (22, 23). Few studies have investigated binding of apo E-rich HDL to liver membranes using a homologous rat system. Therefore, it is not clear which hepatic receptors or binding sites recognize apo E-rich HDL, or how copper deficiency in rats may change its interaction with hepatic binding sites.

The purpose of the present study was to examine the binding of apo E-rich HDL to hepatic plasma membranes using a homologous *in vitro* system. Copper deficiency may affect the binding reaction by altering receptor numbers, receptor properties, and/or ligand properties. In this report we have examined binding reaction characteristics to compare qualitative differences between treatments. We have also compared quantitative binding differences between treatments as a function of free ligand concentration.

Methods. *Animals, diets, tissue sampling, and analysis.* Male weanling Sprague–Dawley rats were equally and randomly divided into two dietary treatments (copper-deficient and -adequate). The basal diet was prepared according to American institute of Nutrition (AIN) specifications (24) except no copper supplement was included in the mineral mix (Table I). The basal (copper-deficient) diet contained 0.7 μg Cu/g diet as measured by atomic absorption spectrophotometry. The control diet was prepared by adding $CuCO_3$ to the basal diet to a final concentration of 8.0 μg Cu/g diet. Rats were housed individually in suspended stainless steel wire cages in a laboratory maintained at 22°C with a 12-hr light cycle. Diet and distilled demineralized H_2O were provided *ad libitum*. After 8 weeks on the dietary regimen, rats were fasted for

TABLE I. COMPARISON OF SEMIPURIFIED RAT DIET

Ingredient	Copper adequate (%)	Copper deficient (%)
Casein	20.0	20.0
DL-Methionine	0.3	0.3
Glucose monohydrate	64.0	65.0
Cellulose	5.0	5.0
Corn oil	5.0	5.0
AIN mineral mix[a]	3.5	3.5
AIN vitamin mix	1.0	1.0
Choline bitartrate	0.2	0.2
Cupric carbonate (1.05 g/kg)	1.0	—
	100.0	100.0
Dietary Cu (ppm)[b]	8.0	0.7

[a] Contains all minerals except copper.
[b] Determined by atomic absorption spectrophotometry.

12 hr and exsanguinated under ether anesthesia. Blood was collected into EDTA (3 m*M* final concentration) and plasma was obtained by low-speed centrifugation. Livers were perfused *in situ,* excised, and placed on ice for subsequent plasma membrane preparation. Duplicate 1-g samples from each liver were dried, digested in nitric acid at 95°C, diluted to 5 ml, and analyzed for hepatic copper content by atomic absorption spectrophotometry. Additional samples from collected tissues were assayed for total cholesterol following chloroform–methanol extraction (25). Aliquots of plasma and isolated lipoprotein fractions were analyzed for total cholesterol using an enzymatic kit (Boehringer-Mannheim, Indianapolis, IN) and for protein by the Lowry method (26). Statistical treatment comparisons were performed using the Student *t* test.

Isolation and iodination of plasma lipoproteins. Plasma lipoproteins were separated and purified by agarose column chromatography following ultracentrifugal floatation (27). Plasma from five rats was pooled, adjusted to *d* 1.225 g/ml with solid KBr, overlayered with *d* 1.225 buffer, and centrifuged in a Ti70 rotor at 30,000 rpm for 24 hr at 15°C in a Beckman L8-80M ultracentrifuge (Beckman Instruments Inc., Fullerton, CA). Floated lipoproteins were removed and applied to a system of two agarose columns in series (2.6 × 85 cm per column). Sepharose CL-4B (Pharmacia Inc., Piscataway, NJ) and Bio-Gel A-1.5m (Bio-Rad, Richmond, CA) were used to pack the first and second column, respectively, maintained and operated at 6°C. Lipoproteins were eluted at 40 ml/hr with 0.15 *M* NaCl, 0.01% EDTA, 0.02% NaAzide, pH 7.4, into 8-ml fractions and monitored for protein concentration at 280 nm. Identification of the eluted peaks was validated by calibrating the columns with VLDL, LDL, and HDL obtained by sequential ultracentrifugation (28) and by 7.5–20% gradient SDS–polyacrylamide gel electrophoresis (29) of eluted fractions. Rat HDL is a heterogeneous mixture of lipoprotein particles (12). For this reason, the HDL peaks were partitioned into three regions of varying apo E and apo A-I content. The largest of these particles was a fraction rich in cholesterol and similar in composition to the HDL_1 isolated by rate zonal ultracentrifugation (30). This fraction (designated HDL-I) was further partitioned by heparin–Sepharose affinity chromatography (31). The HDL were equilibrated overnight in the heparin column (1.0 × 30 cm) at 6°C and eluted the following day using an increasing stepwise NaCl-gradient system at 24 ml/hr. Eluted peaks were dialyzed against 50 m*M* NaCl, 0.01% EDTA, pH 7.4, and concentrated using Amicon (Danvers, MA) CF-25 ultrafiltration cones. Apo E-free HDL were isolated by collecting the nonretained fraction when HDL-III were applied to the heparin column.

The apo E-rich HDL partitioned by heparin–Sepharose chromatography were radiolabeled using the iodine monochloride method (32) as modified by Goldstein *et al.* (33). Iodinated HDL were passed through a Sephadex G-25 column and then dialyzed extensively at pH 7.4 against 150 m*M* NaCl containing 0.24 m*M* EDTA. The radiolabeled HDL preparations were then dialyzed against 300 vol of 50 m*M* Tris, 25 m*M* NaCl, 1 m*M* $CaCl_2$, pH 7.4, for 24 hr. After the final dialysis, iodinated HDL were passed through a 0.45-μm filter (Millipore Corp., Bedford, MA) and stored at 4°C. All radiolabeled HDL preparations contained <2% TCA-soluble radioactivity and <6° lipid-associated radioactivity and were used within 12 days of preparation. Preparations from each treatment were similar in specific activity and in label distribution among apolipoproteins.

Liver plasma membrane preparation. Plasma membranes were prepared from rat liver by employing a modification (34) of the original method of isolation developed by Neville (35). Livers from three rats were pooled, minced, and homogenized by hypotonic buffer (1 m*M* $NaHCO_3$, 0.5 m*M* $CaCl_2$, 1 m*M* PMSF, pH 7.5) using a Polytron homogenizer (three 5-sec bursts, setting No. 4). The homogenized suspension was diluted to 500 ml and filtered through two layers of 200-μm nylon mesh. The filtrate was centrifuged at 1500*g* for 20 min and the supernatant decanted off the surface of the pink gelatinous pellets. The pellets were suspended in 40 ml of homogenation medium and mixed in a dounce homogenizer (about eight gentle strokes). The mixture was di-

luted to 150 ml and centrifuged for 15 min at 1500*g* and the resultant pellets were again gently homogenized. The suspension was centrifuged a third time at 1500*g* for 10 min. The final pellets were taken up with 5.5 vol of a 70.74% sucrose solution to a density of 1.22 g/ml. This suspension was overlayered using a discontinuous sucrose gradient system (36), in which sucrose solutions of 1.18 and 1.16 g/ml are layered successively over the membrane suspension. The overlayered suspensions were centrifuged for 75 min at 25,000 rpm in a SW 28 rotor. After centrifugation, the grayish-white sheet of plasma membranes was collected from the *d* 1.16/1.18 interface, washed free of sucrose, and stored at −85°C until used in binding assays (<2 months). The preparations were monitored for enrichment of plasma membranes through assay of 5′-nucleotidase activity (37), a marker enzyme which in rat liver is primarily associated with plasma membranes.

Binding assays. Preliminary characterization experiments were performed to optimize a set of "standard binding conditions" for subsequent experiments. These studies also investigated chemical and physical properties of the binding site(s). Incubation time, temperature, and pH were altered, ions were added to or deleted from the incubation medium, and the amount of membrane protein added was varied over a wide range to determine conditions which affect bound radioactivity. After optimal binding conditions were selected and standardized, saturation studies were performed in which increasing amounts of labeled lipoprotein were added to a constant amount (110 μg/tube) of membrane protein. These experiments were performed in the absence (total binding) or presence (experimentally determined nonspecific binding) of 50-fold excess unlabeled lipoprotein. Specific binding was determined by subtracting nonspecific from total binding. Displacement studies were performed using a constant amount (10 μg/ml) of labeled lipoprotein and membrane protein (110 μg/tube) with increasing amounts of unlabeled lipoprotein competitors. The incubations for saturation and displacement experiments were performed at 22°C for 60 min in 20 m*M* Tris–HCl, 25 m*M* NaCl, 0.5 m*M* $CaCl_2$, 1% BSA (fatty acid-free), pH 7.5, in a final volume of 100 μl. After incubation, the reaction mixtures were cooled on ice for 5 min and 80-μl aliquots were transferred to siliconized cellulose propionate microfuge tubes (Beckman Instruments, Palo Alto, CA). Tubes were placed in specially designed adaptors and centrifuged in a Ti70 rotor at 100,000*g* for 20 min at 3°C to separate bound from unbound lipoproteins. Supernatant was carefully removed by aspiration, 150 μl cold incubation buffer was gently layered over the pelleted membranes, and the tubes were recentrifuged at 100,000*g* for 10 min at 3°C. After removal of supernatant, the tubes were sliced to obtain the pellets and the bound radioactivity was determined in a gamma counter. Aliquots of the original incubation mixture were counted to assess the total radioactivity added to each tube. Tubes containing radiolabeled lipoproteins without membranes were used as assay blanks. The background binding to the tubes was approximately 0.2% of the total ^{125}I-apo E-rich HDL and was linear over the concentration range studied.

Binding Analysis. Total binding was measured at 16 different label concentrations (0.1–400 μg protein/ml) with each plasma membrane pool, thus producing saturation curves as a function of label concentration. The curves ($n = 4$ per treatment) were compared by two-way analysis of variance (38) to determine treatment differences. Specific binding was measured as the difference between binding in the absence and presence of 50-fold excess unlabeled lipoprotein at 7 different label concentrations (1–250 μg/ml). Specific binding at each concentration was determined for four plasma membrane pools from each dietary treatment and were compared by the Student *t* test. In addition, specific binding data were subjected to Scatchard analysis (39), and the resultant binding parameter estimates (K_d and B_{max}) were compared by the Student *t* test.

Results. Significant reductions in body weight, body weight gain, and feed intake were observed in rats fed the copper-deficient diet (Table II). Elevations in heart weight ($P < 0.001$) and heart to body weight ratio ($P < 0.001$) are indicative of cardiac hypertrophy, a common symptom of copper defi-

TABLE II. EFFECT OF DIETARY TREATMENT ON BODY WEIGHT, ORGAN WEIGHTS, AND LIVER COPPER

Parameter	Dietary treatment		*P* value
	Copper adequate	Copper deficient	
Body weight	324 ± 8.6	236 ± 8.5	<0.001
Body weight gain[a]	279 ± 7.5	189 ± 9.0	<0.001
Feed intake[a]	929 ± 28	771 ± 39	<0.005
Heart weight	1.09 ± 0.03	1.77 ± 0.11	<0.001
Heart/body weight (%)	0.34 ± 0.01	0.75 ± 0.05	<0.001
Liver weight	8.78 ± 0.33	7.32 ± 0.66	<0.001
Liver/body weight (%)	2.71 ± 0.08	3.10 ± 0.19	<0.005
Liver copper (μg/g wet wt)	4.54 ± 0.14	1.00 ± 0.13	<0.001

Note. Values are reported in grams (except for liver copper, as noted) and represent the means ± SEM ($n = 24$). *P* values determined by the Student's *t* test.

[a] g/8 weeks.

ciency in rats (40). Reduction in liver copper content ($P < 0.001$) confirms that rats fed the test diet were copper-deficient.

The effect of copper deficiency on tissue total cholesterol levels is shown in Table III. Although kidney cholesterol concentration was unaffected, both liver cholesterol concentration and total liver cholesterol were significantly reduced in copper-deficient rats, confirming previous reports (3, 4). Heart cholesterol concentration was not affected by dietary treatment. Plasma cholesterol and HDL cholesterol were significantly elevated in copper-deficient rats, also confirming previous reports (10, 11). These data indicate a change in the distribution of cholesterol between liver and plasma as a result of copper deficiency.

The lipoprotein fractions isolated by agarose column chromatography were identified initially by their elution volume through the agarose columns and subsequently by their apolipoprotein profiles as determined by SDS–PAGE. Because high-density lipoproteins comprise a very high percentage of total plasma cholesterol in rats and because rat HDL appears to be a heterogeneous mixture of lipoprotein particles, the HDL peaks were

TABLE III. EFFECT OF COPPER DEFICIENCY ON TISSUE CHOLESTEROL CONCENTRATION

Parameter	Dietary treatment		*P* value
	Copper adequate	Copper deficient	
Plasma			
Total (mg/dl)	94.4 ± 4.1	130.1 ± 8.2	<0.005
HDL[a] (mg/dl)	56.3 ± 3.5	80.8 ± 4.8	<0.01
Kidney			
mg/g	2.96 ± 0.22	3.08 ± 0.18	NS[b]
Liver			
mg/g	2.24 ± 0.05	1.65 ± 0.06	<0.001
mg/total organ	19.7 ± 0.31	12.2 ± 0.40	<0.001
Heart			
mg/g	0.85 ± 0.03	0.87 ± 0.03	NS
mg/total organ	0.93 ± 0.03	1.54 ± 0.05	<0.001

Note. Values represent the means ± SEM. $n = 4$ for plasma measurements and $n = 20$ for kidney, liver, and heart measurements. *P* values determined by the Student *t* test.

[a] HDL were isolated from pooled plasma of six rats (for each observation) by ultracentrifugation and agarose column chromatography.

[b] NS = not significant.

partitioned into three regions, arbitrarily designated HDL-I, HDL-II, and HDL-III. The HDL regions, isolated from 20 ml of plasma, are visualized in Fig. 1. The HDL regions contain apolipoproteins A-I, E, and C's and in the case of HDL-I, apolipoprotein B. Furthermore, these HDL regions differ with respect to the relative amounts of apo E and apo A-I. The ratio of apo E to A-I appears to be highest in the HDL-I fraction, somewhat less in the HDL-II fraction, and quite low in the HDL-III fraction. This pattern was present within HDL from rats of either treatment.

Partitioning the HDL eluted from agarose columns facilitated further subfractionation by heparin–Sepharose affinity chromatography. Elution of HDL-I from the heparin–Sepharose column (Fig. 2) resulted in a reproducible profile of HDL subfractions differing in their apo E content. When HDL-I derived from 20 ml plasma were applied to the column, a retained fraction (region II) was eluted by increasing the ionic strength of the elution buffer to 0.29 *M* NaCl. This HDL subfraction was rich in apo E, contained apo A-I, C's, and relatively little apo B (13% of total protein for controls, 14% for the treatment group) and had a cholesterol/protein ratio of 1.2 for both treatment groups. Apolipoprotein bands were identified by running in parallel purified apolipoprotein standards. Neither the apolipoprotein profile nor the cholesterol/protein ratio of this subfraction was different between treatments. Thus, the particle composition of this lipoprotein subfraction did not appear to be affected by dietary treatment and confirms a previous report (11). This fraction was designated apo E-rich HDL and was utilized in subsequent binding studies.

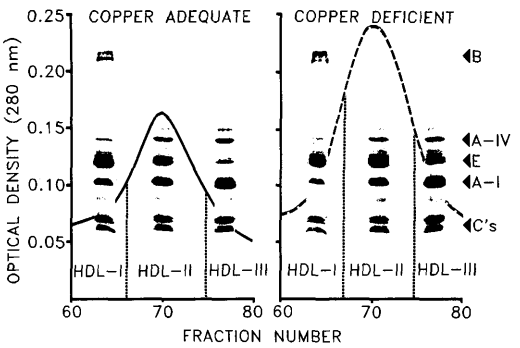

FIG. 1. Separation of rat plasma high-density lipoproteins by agarose column chromatography. Rat lipoproteins ($d < 1.225$) from 20 ml plasma were floated by ultracentrifugation and applied to a system of two agarose columns in series (2.6 × 85 cm per column). Sepharose CL-4B and Bio-Gel A-1.5m were packed in the first and second columns, respectively, maintained and operated at 6°C. Lipoproteins were eluted at 40 ml/hr with 150 m*M* NaCl, 0.01% EDTA, 0.02% NaAzide, pH 7.4, into 8-ml fractions. Apolipoprotein composition was determined by 7.5–20% gradient SDS-PAGE.

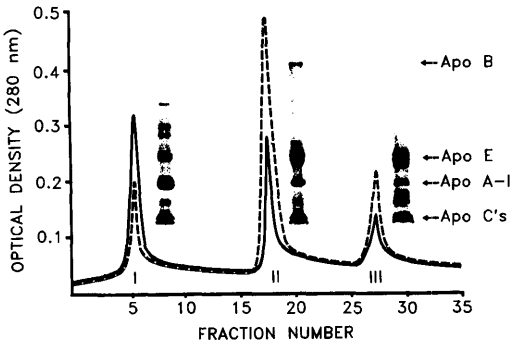

FIG. 2. Heparin–Sepharose affinity chromatography of rat HDL-I. The HDL-I isolated from 20 ml plasma were equilibrated in the heparin column (1.0 × 30 cm) overnight and eluted the following day at 24 ml/hr using an increasing NaCl gradient system. The nonretained fraction (peak I) was eluted by 0.05 *M* NaCl; apo E-rich HDL (peak II) was eluted by 0.29 *M* NaCl; and peak III was eluted by 0.6 *M* NaCl. Eluted fractions containing copper-adequate (—) and copper-deficient (- - -) lipoproteins were monitored for protein at 280 nm and for apolipoprotein composition by 7.5–20% gradient SDS-PAGE.

The enrichment of the liver plasma membrane preparations used in binding assays was monitored by 5′-nucleotidase marker enzyme activity (Table IV). The plasma membrane suspensions were enriched 10- to 15-fold over the starting liver homogenate. Membranes prepared from the livers of copper-deficient rats showed significantly lower 5′-nucleotidase activity compared to membranes prepared from livers of control rats.

Experiments were performed to determine the binding characteristics of apo E-rich HDL and plasma membranes in this *in vitro* system. Results of these experiments are pre-

TABLE IV. EFFECT OF COPPER DEFICIENCY ON 5'-NUCLEOTIDASE MARKER ENZYME ACTIVITY IN PURIFIED LIVER PLASMA MEMBRANE PREPARATTION

Membrane fraction	Dietary treatment		*P* value
	Copper adequate	Copper deficient	
	μmole/hr per mg protein		
Filtered liver homogenate	2.67 ± 0.3	2.48 ± 0.4	NS[a]
Purified plasma membranes	37.8 ± 3.4	25.6 ± 2.7	<0.001

Note. 5'-Nucleotidase activity was measured using the method of Widnell and Unkeless (37). Plasma membranes were purifed following the method of Ray (34) and using the sucrose gradient of Song *et al.* (36). Values represent the means ± SEM (n = 6). P values determined by the Student t test.

[a] NS = not significant.

sented in Table V. The binding observed in both treatments appeared insensitive to the addition of 30 m*M* EDTA and to the addition of 10 μ*M* $CuCl_2$ to the incubation medium. Pretreatment of the plasma membrane preparations with various concentrations of pronase had relatively little effect on total binding in either treatment group. Thus the binding characteristics appeared similar between treatments, suggesting a common binding site. The binding was increased when the incubations were carried out at 37°C, indicating a temperature sensitivity. The higher temperature was not used in these studies since a binding equilibrium could not be demonstrated within 2 hr of incubation. Equilibrium was established within 60 min at 22°C; therefore these conditions were used for all subsequent assays.

TABLE V. EFFECT OF MODIFICATION OF INCUBATION CONDITIONS ON BINDING OF ^{125}I-APO E-RICH HDL TO HEPATIC PLASMA MEMBRANES

Incubation treatment	Copper adequate	Copper deficient
	ng/mg membrane protein	
Standard conditions[a]	450 ± 12	622 ± 21
30 m*M* EDTA	442 ± 16	641 ± 40
10 μ*M* $CuCl_2$	383 ± 20	711 ± 58
Pronase[b] (μg/ml)		
0	380 ± 18	602 ± 19
5	390 ± 31	600 ± 26
25	354 ± 28	663 ± 41
Temperature (°C)		
0	387 ± 21	550 ± 31
37	615 ± 18	841 ± 25
Time (min)		
15	316 ± 19	448 ± 20
30	421 ± 22	578 ± 18
60	450 ± 17	622 ± 19
120	462 ± 18	613 ± 19

Note. All incubations were performed using 10 μg/ml ^{125}I-apo E-rich HDL and 100 μg membrane protein in 100 μl of 20 m*M* Tris–HCl, 25 m*M* NaCl, 0.5 m*M* $CaCl_2$, 1% BSA at pH 7.5. Values represent the means ± SEM (n = 2).

[a] Standard conditions = 60 min incubation at 22°C.

[b] Membranes were treated with the indicated concentration of pronase and incubated for 10 min at 37°C prior to binding. The reaction was stopped by the addition of 2 vol ice-cold 10% BSA.

Numerous binding experiments were conducted to determine if copper status affects the binding interaction of ^{125}I-apo E-rich HDL with liver plasma membrane preparations. For treatment comparisons, "copper deficient" designates experiments in which both lipoproteins and membranes were derived from copper-deficient animals. Similarly, "copper adequate" designates experiments in which lipoproteins and membranes were derived from control rats. Saturation studies were conducted in which an increasing amount of labeled lipoprotein was added to a constant amount of membrane protein. The binding was observed over a wide range of radiolabel concentrations (0.1–400 μg/ml for total binding experiments and 1.0–250 μg/ml for specific binding experiments) in an attempt to approximate near physiologic concentrations.

Total binding data obtained from these experiments were compiled at 16 different label concentrations and then used to construct binding saturation curves. Curve com-

parison by two-way analysis of variance demonstrated a significant copper effect, indicating that the total binding, as a function of ^{125}I-apo E-rich HDL concentration, was greater in the copper-deficient group. The curve means (n = 4) for each treatment are illustrated in Fig. 3.

Specific binding data, experimentally determined as the difference between total binding and binding observed in the presence of 50-fold excess unlabeled lipoprotein, were compiled at seven different label concentrations. These data are shown in Table VI as the average specific binding from four pools of membrane preparations for each treatment. The specific binding was significantly higher in the copper-deficient group throughout the observed concentration range. The specific binding data from all experiments were then plotted on Scatchard coordinates (Fig. 4). Data for each experiment were analyzed by least-squares linear regression to determine estimates of the binding parameters. The data show a linear relationship indicating saturable binding by one class of binding sites. The average K_d and B_{max} estimated for the copper-adequate data were 169 μg/ml and 6.27 μg bound/mg membrane protein, respectively. The average estimates obtained for the copper-deficient group were 76.1 μg/ml and 5.33 μg bound/

TABLE VI. EFFECT OF DIETARY TREATMENT ON SPECIFIC BINDING OF ^{125}I-APO E-RICH HDL TO LIVER PLASMA MEMBRANES

HDL protein concentration (μg/ml)	Dietary treatment: Copper adequate	Dietary treatment: Copper deficient	P value
	ng/mg membrane protein		
1	30 ± 3.3	66 ± 5.5	<0.005
5	153 ± 15	269 ± 24	<0.005
10	290 ± 25	530 ± 34	<0.001
25	653 ± 68	1053 ± 101	<0.01
50	1228 ± 119	1892 ± 113	<0.005
100	2012 ± 202	2886 ± 179	<0.01
250	3131 ± 223	4306 ± 273	<0.01

Note. Bound radioactivity was determined by incubation of 110 μg membrane protein with the indicated concentration of ^{125}I-apo E-rich HDL for 60 min at 22°C in 100 μl of 20 mM Tris–HCl, 25 mM NaCl, 0.5 mM $CaCl_2$, 1% BSA at pH 7.5. Values represent the means ± SEM (n = 4). P values determined by the Student t-test.

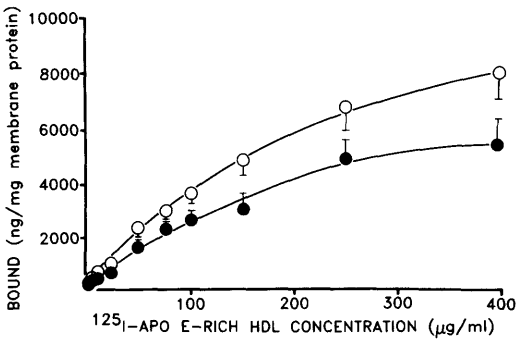

FIG. 3. Saturation curves of total binding as a function of ^{125}I-apo E-rich HDL concentration. Hepatic plasma membranes (110 μg) were incubated with the indicated concentration of ^{125}I-apo E-rich HDL in 100 μl of 20 mM Tris–HCl, 25 mM $NaCl_2$, 0.5 mM $CaCl_2$, 1% BSA (fatty acid-free), pH 7.5, for 60 min at 22°C. Curve averages (n = 4 for each treatment) are shown for the copper-adequate (●) and copper-deficient (○) assays. Curves were analyzed by two-way analysis of variance which demonstrated a highly significant (P < 0.001) copper effect (df = 1).

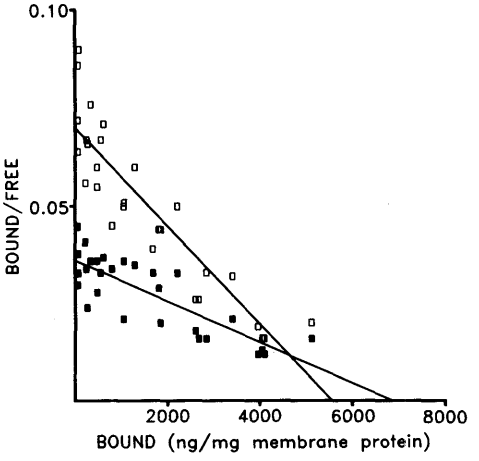

FIG. 4. Scatchard analysis of experimentally derived specific binding data. Specific binding data for all experiments (n = 4 for each treatment) from copper-adequate (■) and copper-deficient (□) assays were subjected to Scatchard analysis. The average K_d and B_{max} for control assays were 169 μg/ml and 6.27 bound/mg membrane protein, respectively. The respective values for copper-deficient assays were 76.1 μg/ml and 5.33 μg bound/mg membrane protein. Binding incubations were performed at 22°C for 60 min in the presence of 50-fold excess unlabeled apo E-rich HDL.

mg membrane protein, respectively. The K_d was significantly lower ($P < 0.005$), indicating higher affinity, and the B_{max} was significantly lower ($P < 0.05$) in the copper-deficient group compared to controls. Therefore, total binding data, specific binding data, and kinetic analysis all demonstrated that the binding was affected by copper deficiency.

The apo E-rich HDL binding specificity was investigated by binding in the presence of increasing concentrations of unlabeled lipoprotein competitors. Two displacement experiments were performed. The first experiment examined binding site specificity in controls; the second experiment investigated dietary treatment differences. These experiments are visualized in Fig. 5. The first of these experiments (Fig. 5A) compared VLDL, LDL, apo E-free HDL, and apo E-rich HDL in their ability to displace labeled apo E-rich HDL from the membranes. All lipoproteins and membranes were obtained from control animals. Although some differences exist, it appears that all of the lipoproteins were capable of competing for the binding site. The relatively nonspecific nature of binding sites recognizing rat HDL has been reported previously (21). In another experiment, the effect of dietary treatment was investigated by displacement studies. Labeled apo E-rich HDL and plasma membranes from copper-deficient rats were incubated with increasing amounts of unlabeled HDL fractions derived from rats of both treatments (Fig. 5B). Apo E-free HDL derived from rats of either treatment effectively displaced the membrane-bound radioactivity. Thus, in both copper-adequate and copper-deficient assays, apo E does not appear to be a primary requisite for recognition by this binding site. The apo E-rich HDL from deficient rats displaced somewhat more label from the binding site than did apo E-rich HDL from controls.

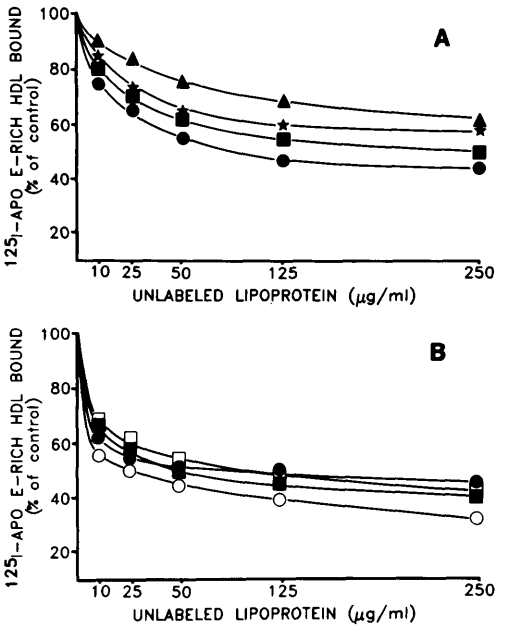

FIG. 5. Competitive displacement experiments. Two separate experiments were performed. (A) Binding site specificity in control assays. Binding of ^{125}I-apo E-rich HDL (10 μg/ml) to plasma membranes (110 μg/tube) in the presence of the indicated concentrations of rat VLDL (▲), LDL (★), apo E-free HDL (■), and apo E-rich HDL (●). All lipoproteins and plasma membranes were obtained from control animals and incubated at 22°C for 60 min. (B) Treatment differences in binding site displacement. Binding of ^{125}I-apo E-rich HDL (10 μg/ml) from copper-deficient rats to plasma membranes (110 μg/tube) from deficient rats in the presence of the indicated concentrations of apo E-free HDL from copper-adequate (■) and -deficient (□) rats and of apo E-rich HDL from copper-adequate (●) and -deficient (○) rats. Incubations were performed at 22°C for 60 min.

Discussion. The Sprague–Dawley rat is known as an "HDL mammal" (41) and possesses a polydispersed and heterogeneous population of HDL particles. For the purpose of this study, we have attempted to isolate and utilize the population of HDL particles most dramatically influenced by copper deficiency. Because these animals lack significant cholesteryl ester transfer protein activity (42), they accumulate appreciable amounts of a large HDL_1 particle rich in cholesteryl ester and apo E, with an isopycnic density of 1.054 g/ml (30, 43). Therefore, rat LDL and HDL_1 are difficult to separate by physical means alone because of their density and size similarities (30, 43). We have found this to hold true when isolating rat lipoproteins by agarose column chromatography. However, by analyzing the eluted fractions for cholesterol content and apolipoprotein composition, the HDL peaks could be selectively partitioned (Fig. 1) into

the desired regions. The HDL-I region was selected from the agarose column because these lipoprotein particles demonstrated an apolipoprotein composition and cholesterol/protein ratio quite similar to rat HDL_1 purified by rate zonal ultracentrifugation (30, 43). Heparin–Sepharose chromatography of HDL-I yielded an apo E-rich HDL subfraction separated by chemical as well as physical properties. The plasma concentration of this HDL subfraction is markedly increased by copper deficiency (11).

The primary objective of this study was to examine the binding of apo E-rich HDL to hepatic plasma membranes using a homologous *in vitro* system. Comparison of the total (Fig. 3) and specific (Table V) binding data show significantly higher binding in the copper-deficient treatment group throughout the range of experimental observations. This increased binding may be attributed to altered ligand and/or binding site properties, or by changes in binding site numbers. The present study investigated binding site properties but was not designed to determine if the quantitative treatment differences in binding were associated with the ligand or the binding site. The apo E-rich particle composition appeared similar between treatments; however, these observations do not preclude the possibility of a change in ligand properties as a result of copper deficiency. The displacement experiments (Fig. 5B) indicate apo E-rich HDL from controls may not compete for the binding site as effectively as apo E-rich HDL from deficient rats.

The results described herein support the existence of a lipoprotein binding site in rat liver which is distinct from the LDL or apo E receptor. Characterization experiments performed at a free ligand concentration of 10 μg/ml (Table V) demonstrate binding properties similar to those reported for apo E-free HDL binding (17, 18, 21). Furthermore, competition experiments (Fig. 5A) indicate other lipoproteins readily compete for the binding site, demonstrating a relatively nonspecific nature. This binding site is not affected by EDTA, is relatively insensitive to pronase, and recognizes a variety of different lipoproteins. These binding characteristics are common between treatments, suggesting this site is present in both control and copper-deficient plasma membrane preparations. The binding observed in this study is not consistent with binding properties of the apo E (22) or LDL (44, 45) receptor. Therefore, it appears that apo E-rich HDL recognize a binding site distinct from the apo E or LDL receptor. Recent reports provide evidence for the existence of a nonspecific lipoprotein binding site in rats with characteristics similar to those reported here. A relatively low affinity, nonspecific binding site was observed in rat hepatocytes (14) and liver membranes (46, 47). Apo E-free HDL bound to hepatocytes with an affinity of 178.6 μg/ml (48), whereas rat IDL bound to membranes with an affinity of 100 μg/ml (46, 47). This site is EDTA insensitive, appears to be of high capacity (8.2 μg/mg membrane protein), and can interact with any lipoprotein (46). The physiological significance of this binding site *in vivo* is unknown, as are the processes governing its regulation. The present results indicate that the expression of this binding site may be affected by copper deficiency.

The kinetic analysis of specific binding indicated that the average B_{max} was significantly higher in the control group, a finding which apparently contradicts the treatment comparisons of observed binding data. However, for the purposes of this experiment, several lines of reasoning suggest discretion in using B_{max} for treatment comparisons. First, maximal binding capacity is a theoretical value obtained by extrapolation of a given model (based on experimental data obtained over relatively narrow free ligand range) to infinite free ligand concentrations. The binding model is assumed to hold true outside the range of experimental observations, although the validity of this assumption cannot be determined (49). Second, these studies were performed using plasma membrane preparations; the differences in marker enzyme recovery suggest that the copper-adequate membrane preparations were enriched in plasma membranes relative to the copper-deficient preparations. These differences were not accounted for in the final B_{max} estimates. Third, loss of plasma membranes during the isolation procedure precludes ob-

taining a legitimate maximal binding capacity for comparison purposes. Because of these considerations, treatment comparisons of B_{max} are not as meaningful as comparison of binding data observed experimentally.

Specific binding data conform to a one-site model (Fig. 4) although it cannot be determined if this model holds true outside the range of experimental data (49). Nevertheless, copper deficiency affects the affinity of the binding reaction within the observed range of label concentrations. When the specific binding data were plotted on Scatchard coordinates (Fig. 4) the treatment effect appears largest at lower free ligand concentrations. This observation suggests the possibility of a higher affinity, lower capacity binding site present in the copper-deficient hepatic membranes which may not be present in control membranes. Such a site could conceivably make a contribution to binding while still being masked by the higher capacity site and thereby escape detection as a distinct binding component by kinetic analysis. The apo E-rich HDL radioligand used in these studies possesses the apolipoproteins necessary for potential interaction with the hepatic apo E receptor (22) as well as the LDL receptor (44, 45). Thus, apo E-rich HDL may interact with these high-affinity receptors (31). Hepatic LDL receptor expression can be affected by dietary factors (50); thus we cannot overlook the possibility that copper deficiency may induce their expression. Normal rats express relatively low levels of hepatic LDL receptors; detectable by immunoassay (51), although closely approaching detection limits of the radioligand binding assay (45, 46). Detection of these receptors as a distinct binding component necessarily entails binding at lower label concentrations. Furthermore, to identify these receptors as such, binding properties should be established at low free ligand concentrations where high-affinity sites would be responsible for a larger component of the observed binding.

Our findings demonstrate that copper deficiency in rats results in increased binding of apo E-rich HDL to hepatic plasma membranes *in vitro*. A recent study reported reduced binding of ^{125}I-lipoproteins ($d < 1.21$) to hepatic membranes from copper-deficient rats compared to controls (52). These investigators used only one concentration level of ligand on which to base their comparisons. Furthermore, binding data were based on marker enzyme activity in order to obtain the reported treatment differences. However, no evidence was provided that copper deficiency does not affect the marker enzyme activity or expression, or the redistribution of enzyme during the liver homogenation procedure. Therefore, comparison of results obtained by these investigators with the present findings are difficult to interpret properly.

The majority of the binding observed in this study appears to be attributable to a high-capacity, relatively nonspecific binding site. The higher binding reported here may be due to elevated expression of this nonspecific, high-capacity site as a result of copper deficiency. Alternatively, subtle changes in lipoprotein structure induced by copper deficiency could enhance recognition by this binding site. The higher binding affinity observed in the copper-deficient group may be due to the contribution of high-affinity receptors present in the membrane preparations but not detectable as a distinct binding component in the assay.

Despite these possibilities, determination of the *in vivo* significance of these *in vitro* observations remains difficult. Binding of lipoproteins to receptors or binding sites is only the first step in a complex series of metabolic events and can by no means be assumed to represent catabolic activity. The events subsequent to binding in this system have not been elucidated, although three possibilities exist: (1) net influx of lipoprotein-bound cholesterol to hepatic cells; (2) exchange of cholesterol between lipoproteins and hepatic cells with no net unidirectional flux; and (3) net efflux of cholesterol from hepatic cells to lipoproteins. The mechanisms by which the current observations contribute to the altered cholesterol metabolism observed in copper-deficient rats require a greater understanding of the metabolic processes subsequent to binding. To this end, studies are being performed to examine binding, uptake, and degradation of HDL subfractions by hepatocytes and Kupffer

cells *in vitro*. These studies will help further resolve the role of dietary copper in the lipoprotein metabolism of rats.

1. Klevay LM. Hypercholesterolemia in rats by an increase in the ratio of zinc to copper ingested. Amer J Clin Nutr **26**:1060–1068, 1973.
2. Murthy L, Petering HG. Effect of dietary zinc and copper interrelationships on blood parameters of the rat. J Agric Food Chem **24**:808–811, 1976.
3. Lei KY. Cholesterol metabolism in copper-deficient rats. Nutr Rep Int **15**:597–605, 1977.
4. Lei KY. Oxidation, excretion and tissue distribution [26-^{14}C]cholesterol in copper-deficient rats. J Nutr **108**:232–237, 1978.
5. Allen KGD, Klevay LM. Copper deficiency and cholesterol metabolism in the rat. Atherosclerosis **31**:259–271, 1978.
6. Shao MTS, Lei KY. Conversion of [2-^{14}C]mevalonate into cholesterol, lanosterol and squalene in copper-deficient rats. J Nutr **110**:859–867, 1980.
7. Lin IM, Lei KY. Cholesterol kinetic analyses in copper-deficient rats. J Nutr **111**:450–457, 1981.
8. Lei KY, Lin IM. Kinetics of high density lipoprotein cholesterol in copper-deficient rats. Nutr Rep Int **24**:1179–1186, 1981.
9. Allen KGD, Klevay LM. Hyperlipoproteinemia in rats due to copper deficiency. Nutr Rep Int **22**:295–299, 1980.
10. Lei KY, Hassel CA, Allen DK. Alterations in plasma lipid, lipoprotein and apolipoprotein concentrations in copper-deficient rats. J Nutr **113**:2178–2183, 1983.
11. Croswell SC, Lei KY. Effect of copper deficiency on the apolipoprotein E-rich high density lipoproteins in rats. J Nutr **115**:473–482, 1985.
12. Mahley RW, Holcombe KS. Alterations of the plasma lipoproteins and apoproteins following cholesterol feeding in the rat. J Lipid Res **18**:314–324, 1977.
13. Ose L, Roken I, Norum KR, Drevon CA, Berg T. The binding of high density lipoproteins to isolated rat hepatocytes. Scand J Clin Lab Invest **41**:63–73, 1981.
14. Van Berkel TJC, Kruijt JK, Van Gent T, Van Tol A. Saturable, high affinity binding, uptake and degradation of rat plasma lipoproteins by isolated parenchymal and non-parenchymal cells from rat liver. Biochim Biophys Acta **665**:22–33, 1981.
15. Innerarity TL, Pitas RE, Mahley RW. Disparities in the interaction of rat and human lipoproteins with cultured fibroblasts and smooth muscle cells: Requirements for homology for receptor binding activity. J Biol Chem **255**:11163–11172, 1980.
16. Kagami A, Fidge N, Suzuki N, Nestel P. Characteristics of the binding of high-density lipoprotein$_3$ by intact cells and membrane preparations of rat intestinal mucosa. Biochim Biophys Acta **795**:179–190, 1984.
17. Chacko GK. Human high density lipoprotein (HDL_3) binding to rat liver plasma membranes. Biochim Biophys Acta **712**:129–141, 1982.
18. Chacko GK. Characteristics of high density lipoprotein binding sites in rat liver and testis membranes. Biochim Biophys Acta **795**:417–426, 1984.
19. Gwynne JT, Strauss JF III. The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. Endocrinol Rev **3**:299–329, 1982.
20. Ghosh DK, Menon KMJ. Identification of gonadotropin inducible, high density lipoprotein receptors in the solubilized membranes from rat ovary. Biochem Biophys Res Commun **134**:1006–1014, 1986.
21. Van Tol A, Dallinga-Thie GW, Van Gent T, Van't Hooft FM. Specific saturable binding of rat high-density lipoproteins to rat kidney membranes. Biochim Biophys Acta **876**:340–351, 1986.
22. Hui DY, Innerarity TL, Mahley RW. Lipoprotein binding to canine hepatic membranes: Metabolically distinct apo E and apo B, E receptors. J Biol Chem **256**:5646–5655, 1981.
23. Mahley RW, Innerarity TL. Lipoprotein receptors and cholesterol homeostasis. Biochim Biophys Acta **737**:197–222. 1983.
24. American Institute of Nutrition. Report of the AIN Ad Hoc Committee on standards for nutritional studies. J Nutr **107**:1340–1348, 1977.
25. De Hoff JL, Davidson LM, Kritchevsky D. An enzymatic assay for determining free and total cholesterol in tissue. Clin Chem **24**:433–435, 1978.
26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **193**:265–275, 1951.
27. Rudel LL, Lee JA, Morris MD, Bragdon JH. Characterization of plasma lipoproteins separated and purified by agarose column chromatography. Biochem J **139**, 89–95, 1974.
28. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest **34**:1345–1353, 1955.
29. Laemmli UK. Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685, 1970.
30. Oschry Y, Eisenberg S. Rat plasma lipoproteins: Re-evaluation of a lipoprotein system in an animal devoid of cholesteryl ester transfer activity. J Lipid Res **23**:1099–1106, 1982.
31. Weisgraber KH, Mahley RW. Subfractionation of human high density lipoproteins by heparin–Sepharose affinity chromatography. J Lipid Res **21**:316–325, 1980.

32. McFarlane AS. Efficient trace-labelling of proteins with iodine. Nature (London) **182**:53, 1958.
33. Goldstein JL, Basu SK, Brown MS. Receptor mediated endocytosis of low density lipoprotein in cultured cells. In: Fleischer S, Fleischer B, Eds. Methods in Enzymology. Orlando, Academic Press, Vol **98**:pp241–260, 1983.
34. Ray TK. A modified method for the isolation of the plasma membrane from rat liver. Biochim Biophys Acta **196**:1–9, 1970.
35. Neville DM Jr. The isolation of a cell membrane fraction from rat liver. J Biophys Biochem Cytol **8**:413–422, 1960.
36. Song CS, Rubin W, Rifkind AB, Kappas A. Plasma membranes of the rat liver: Isolation and enzymatic characterization of a fraction rich in bile canaliculi. J Cell Biol. **41**:124–132, 1969.
37. Widnell CC, Unkeless JC. Partial purification of a lipoprotein with 5′-nucleotidase activity from membranes of rat liver cells. Biochemistry **61**:1050–1057, 1968.
38. Winer BJ. Statistical Principles in Experimental Design, 2nd ed. New York, McGraw-Hill, pp431–513, 1971.
39. Scatchard G. The attractions of proteins for small molecules and ions. Ann NY Acad Sci **51**:660–672, 1949.
40. Hill CH. A role of copper in elastin formation. Nutr Rev **27**:99–100, 1969.
41. Chapman MJ. Comparative analysis of mammalian plasma lipoproteins. In: Segrest JP, Albers JJ, Eds. Methods in Enzymology. Orlando; Academic Press, Vol **128**:pp70–143, 1986.
42. Ha YC, Barter PJ. Differences in plasma cholesteryl ester transfer activity in sixteen vertebrate species. Comp Biochem Physiol **B71**:265–269, 1982.
43. Lusk LT, Walker LF, DuBien LH, Getz GS. Isolation and partial characterization of high density lipoprotein HDL_1 from rat plasma by gradient centrifugation. Biochem J **183**:83–90, 1979.
44. Basu SK, Goldstein JL, Brown MS. Characterization of the low density lipoprotein receptor in membranes prepared from human fibroblasts. J Biol Chem **253**:3852–3856, 1978.
45. Kovanen PT, Brown MS, Goldstein JL. Increased binding of low density lipoprotein to liver membranes from rats treated with 17′-ethinyl estradiol. J Biol Chem **254**:11367–11373, 1979.
46. Brissette L, Roach PD, Noel SP. The effects of liposome-reconstituted apolipoproteins on the binding of rat intermediate density lipoproteins to rat liver membranes. J Biol Chem **261**:11631–11638, 1986.
47. Brissette L, Noel SP. The effects of human low and high density lipoproteins on the binding of rat intermediate density lipoproteins to rat liver membranes. J Biol Chem **261**:6847–6852, 1986.
48. Rifici VA, Eder HA. A hepatocyte receptor for high-density lipoprotein specific for apolipoprotein A-I. J Biol Chem **259**:13814–13818, 1984.
49. Munson PJ, Rodbard D. Number of receptor sites from Scatchard and Klotz graphs: A constructive technique. Science **220**:979–981, 1983.
50. Chao Y, Yamin TT, Alberts AW. Effect of cholestyramine on LDL binding sites on liver membranes from rabbits with endogenous hypercholesterolemia induced by a wheat starch–casein diet. J Biol Chem **257**:3623–3626, 1982.
51. Cooper AD, Nutik R, Chen J. Characterization of the estrogen-induced lipoprotein receptor of rat liver. J Lipid Res **28**:59–68, 1987.
52. Lefevre M, Keen CL, Lonnerdal B, Hurley LS, Schneeman BO. Copper deficiency-induced hypercholesterolemia: Effects on HDL subfractions and hepatic lipoprotein receptor activity in the rat. J Nutr **116**:1735–1746.

Received June 23, 1987. P.S.E.B.M. 1988, Vol. 187.
Accepted November 5, 1987.