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References

Brandes, J. F. (1967) in *Thermobiology* (Rosa, A. H., Ed.) Academic Press, New York, N.Y.
Camejo, G., Mateu, L., Lalaguna, F., Padrón, R., Waich, S., Acquatella, H., & Vegas, H. (1976a) *Artery 2*, 79.
Camejo, G., Waich, S., Mateu, L., Acquatella, H., Lalaguna, F., Quintero, G., & Berrizbeitia, M. L. (1976b) *Ann. N.Y. Acad. Sci. 275*, 153.
Deckelbaum, R. J., Shipley, G. G., Small, P. M., Lees, R. S., & George, P. K. (1975) *Science 190*, 392.
Janiak, M. J., Loomis, C. R., Shipley, G. G., & Small, D. M. (1974) *J. Mol. Biol. 86*, 325.
Lake, J. A. (1967) *Acta Crystallogr. 23*, 191.
Loomis, L. H., Janiak, M. J., Small, D. M., & Shipley, G. G. (1974) *J. Mol. Biol. 86*, 309.
Luzzati, V., Tardieu, A., Mateu, L., & Stuhrmann, H. G. (1976) *J. Mol. Biol. 101*, 115.
Mateu, L., Tardieu, A., Luzzati, V., Aggerbeck, L., & Scanu, A. M. (1972) *J. Mol. Biol. 70*, 105.
Mateu, L., Kirchhausen, T., & Camejo, G. (1977) *Biochim. Biophys. Acta 487*, 243.
Mateu, L., Kirchhausen, T., Padrón, R., & Camejo, G. (1978) *J. Supramol. Struct.* (in press).
Mazur, P. (1970) *Science 168*, 939.
Polge, C., Smith, A. U., & Parkes, A. S. (1949) *Nature (London) 164*, 666.
Pollard, H., Scanu, A. M., & Taylor, E. W. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64*, 304.
Tardieu, A., Mateu, L., Sardet, C., Weiss, B., Luzzati, V., Aggerbeck, L., & Scanu, A. M. (1976) *J. Mol. Biol. 101*, 129.

Enhanced Binding by Cultured Human Fibroblasts of Apo-E-Containing Lipoproteins as Compared with Low Density Lipoproteins†

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ABSTRACT: Specificity for lipoprotein binding to the high affinity cell surface receptors of fibroblasts has been shown to be mediated by the B or arginine-rich (apo-E) apoproteins. The occurrence of a lipoprotein (the HDL_c) in cholesterol-fed dogs, distinguished by the presence of the apo-E as the only detectable protein constituent but with other characteristics similar to those of the apo-B-containing low density lipoproteins (LDL), allowed for a direct comparison of the binding activity of the B vs. E apoproteins. The apo-E HDL_c were found to possess an enhanced binding activity which ranged from 10- to 100-fold greater for the HDL_c by comparison with human or canine LDL as determined by competitive binding assays performed at 37 and 4 °C, respectively. Furthermore, the enhanced binding activity of the apo-E HDL_c resulted in an increased rate of cholesterol esterification as measured by [^{14}C]oleate incorporation into intracellular cholesteryl esters. The increased potency of the HDL_c was apparent regardless of whether the data were compared on the basis of lipoprotein protein, cholesterol, or molar ratios and appeared to be related directly to inherent differences in the reactivity of the B vs. E apoproteins. To establish that the differences in the binding activities were not due to differences in the lipid composition or particle size, the human LDL and canine HDL_c were partially delipidated and their reactivities compared before and after such treatment. Partial delipidation, which removed more than three-fourths of the total lipid and converted the spherical particles to disks, did not alter the reactivity of the LDL or HDL_c with the high affinity receptor sites and, furthermore, the inherent differences between the LDL and HDL_c remained apparent. In addition, the increased potency of the E apoprotein appeared to account for all or most of the high affinity receptor binding by the typical HDL (d = 1.09–1.21) of the swine. A subfraction of the HDL, representing 15% of the total protein in the d = 1.09–1.21 fraction, was isolated by heparin precipitation and was found to possess all of the binding activity. This active subfraction was distinguished from the remaining 85% of the HDL by the increased content of the E apoprotein. The enhanced binding activity of the E apoprotein could modulate HDL binding and competitive displacement of LDL from the cell receptors with relatively minor changes in the E apoprotein content of these plasma lipoproteins.

The binding of specific plasma lipoproteins to high affinity receptors on the cell surfaces of cultured fibroblasts initiates a series of intracellular events which regulate cellular cholesterol metabolism. The bound lipoproteins are internalized by endocytosis and degraded by lysosomal hydrolysis of the protein and cholesteryl esters. Free cholesterol released from the lipoproteins regulates endogenous cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and by stimulating acyl-CoA:cholesterol acyltransferase (ACAT)[1] activity, causing a reesterification of the lipoprotein cholesterol (Goldstein & Brown, 1976). The cell surface receptors, initially shown to bind human low density lipoproteins (LDL), also bind a special class of lipoproteins

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[1] Abbreviations used: LDL, low density lipoproteins; HDL, high density lipoproteins; apo-E, arginine-rich apoprotein; ACAT, acyl-CoA:cholesterol acyltransferase; $NaDodSO_4$, sodium dodecyl sulfate; DME media, Dulbecco's modified Eagle's media; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

referred to as HDL_c (Assmann et al., 1975; Bersot et al., 1976; Mahley & Innerarity, 1978).

The HDL_c are cholesterol-rich, α_2-migrating lipoproteins which are induced by cholesterol feeding in swine and dogs. They are similar to LDL in lipid composition, particle size, and flotation density but have a distinct apoprotein content. The protein portion of the LDL is composed almost exclusively of apo-B, whereas HDL_c lack apo-B and contain the E (arginine-rich) apoprotein and a variable amount of the A-I and C apoproteins (Mahley et al., 1974, 1975; Mahley, 1978). Particularly distinctive HDL_c which contain apo-E as the only detectable apoprotein (to be referred to as apo-E HDL_c) can be isolated from the plasma of dogs fed a coconut oil–cholesterol diet (Mahley et al., 1977a). The typical HDL of dogs and swine are distinguished from HDL_c by their α_1 mobility, smaller particle size, and prominence of the A-I apoprotein.

Our studies indicate that specificity for cell surface receptor binding resides with specific apoproteins and that either the apo-B of LDL or the apo-E of HDL_c can interact with the same receptor sites (Mahley & Innerarity, 1977). Furthermore, a selective modification of approximately half of the arginyl residues of either human LDL or canine HDL_c totally abolishes their binding activity, suggesting that the recognition sites on these apolipoproteins are similar (Mahley et al., 1977b). In earlier studies (Mahley et al., 1977b), it appeared that HDL_c might be more potent than LDL in displacing iodinated LDL from fibroblast receptor sites. The present studies were designed to compare directly the binding properties of apo-E HDL_c, which contain exclusively the E apoprotein, with those of apo-B-containing LDL. In addition, the binding activity of typical swine HDL, i.e., the 100-Å A-I-rich lipoprotein in the density range 1.09 to 1.21, will be correlated with the presence of a small amount of apo-E in a potent subfraction of the HDL.

Materials and Methods

Materials. Dulbecco's phosphate-buffered saline (cat. No. K-13), Dulbecco's modified Eagle's (DME) media (cat. No. H-21), fetal calf serum, trypsin-EDTA solution, potassium penicillin G, and streptomycin sulfate were purchased from GIBCO (Grand Island, N.Y.). Sodium [^{125}I]iodide (carrier-free) in NaOH was obtained from Amersham/Searle (Arlington Heights, Ill). Bovine serum albumin was obtained from Calbiochem (San Diego, Calif.). Tissue culture flasks (75 cm^2, 250 mL) and Petri dishes (60 × 15 mm) were obtained from Falcon Plastics. Heparin (sodium salt, grade 1) was obtained from Sigma Chemical. Other analytical grade reagents were purchased from Fisher Scientific.

Plasma Lipoprotein Isolation and Characterization. Canine LDL were prepared by sequential ultracentrifugation of plasma in a 60 Ti rotor (Beckman Instruments, Spinco Division, Palo Alto, Calif.) at 59 000 rpm for 18 h at d = 1.02–1.063. The LDL were purified by Geon-Pevikon block electrophoresis as previously reported (Mahley & Weisgraber, 1974). Apo-E HDL_c (d = 1.006–1.02) and HDL_c (d = 1.02–1.063) were isolated from the plasma of foxhounds fed a semisynthetic diet containing coconut oil and cholesterol (Mahley et al., 1977a). The d = 1.006–1.02 and the d = 1.02–1.063 ultracentrifugal fractions contained LDL and HDL_c which were separated by Geon-Pevikon electrophoresis. Swine HDL (d = 1.09–1.21) were isolated by centrifugation at 59 000 rpm for 48 h and then washed by recentrifugation at 59 000 rpm for 24 h. Human LDL (d = 1.02–1.05) were isolated from the plasma of a fasted healthy male subject by centrifugation at 59 000 rpm in a 60 Ti rotor for 18 h. The LDL were then washed at d = 1.05 by centrifugation for 16 h at 59 000 rpm. The purity of the lipoproteins was determined by polyacrylamide gel electrophoresis and Ouchterlony double immunodiffusion. Antisera to both swine and canine apo-B, tested with serial dilutions of LDL from the respective species, were sensitive enough to show that the B apoprotein, if present, comprised less than 1% of the total HDL or HDL_c protein. Human lipoprotein-deficient serum was prepared by centrifugation at d = 1.215 at 59 000 rpm for 48 h as described (Mahley & Innerarity, 1977). The cholesterol content of the lipoprotein-deficient serum as determined by gas–liquid chromatography was less than 2 μg/mL.

Lipoproteins were characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, paper electrophoresis, and negative staining electron microscopy as described (Mahley et al., 1974, 1975). Cholesterol (Abell et al., 1952), triglyceride (Fletcher, 1968), phospholipid (Bartlett, 1959), and protein (Lowry et al., 1951) contents were determined. For quantitation of the phospholipid classes of LDL and HDL_c, the lipids were extracted (Kates, 1972) and the phospholipids isolated by two-dimensional thin layer chromatography and quantitated as described by Nelson (1975). Cholesteryl esters were isolated by thin-layer chromatography on silica gel G developed with hexane:diethyl ether:acetic acid (80:20:2; v/v). Methyl esters were prepared and analyzed by gas–liquid chromatography as described (Pitas et al., submitted).

Human LDL and HDL_3 (d = 1.125–1.21) and canine ^{125}I-labeled apo-E HDL_c were chromatographed on 6% agarose (Bio-Gel A-5m, 200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.) for molecular weight determinations. The column was eluted with 0.15 M NaCl, 0.01% EDTA, pH 7, and 3-mL fractions were collected. The iodinated apo-E HDL_c were applied to the column in trace amounts and the elution profile was determined by measurement of the radioactivity in the fractions. Elution of the LDL and HDL_3 was determined by absorbance at 280 nm. The molecular weight of apo-E HDL_c was estimated as described by Rudel et al. (1977) using human LDL as a standard (molecular weight of 3 × 10^6; Margolis, 1967).

Iodination. The lipoproteins were iodinated using the iodine monochloride method of McFarlane as modified by Bilheimer et al. (1972). The ^{125}I-labeled lipoproteins were exhaustively dialyzed against 0.15 M NaCl, 0.01% EDTA, pH 7.0, and sterilized by filtration through a 0.45-μm Millipore filter. Less than 2.5% of the radioactivity in the human [^{125}I]LDL and 6.0% of the radioactivity in the canine [^{125}I]LDL were extractable into chloroform–methanol (2:1; v/v). The iodinated sterile lipoproteins were used for up to 4 weeks after iodination but were redialyzed the day before each experiment.

Cells in Culture. Human fibroblasts were derived from a preputial specimen from a normal infant. The cells were grown routinely in Dulbecco's modified Eagle's (DME) media supplemented with 10% fetal calf serum, 3.7 mg/mL of $NaHCO_3$, 100 units of potassium penicillin G, and 100 μg/mL of streptomycin sulfate. The cultures were maintained in a humidified atmosphere of 5–8% CO_2 in air at 37 °C. Confluent monolayers were harvested with a solution of 0.5% trypsin, 0.02% EDTA, in Hank's base and reseeded in 75-cm^2 flasks for cell maintenance or in 60-mm Petri dishes (9×10^4 or 1×10^5 cells per dish) for use in experiments. All cultures were routinely tested for and found free of mycoplasma contamination (Barile, 1973).

Five or six days after the cells had been plated into Petri dishes they were washed twice with DME media containing 5% (v/v) lipoprotein-deficient serum, and 2 mL of DME media containing 10% lipoprotein-deficient serum was added. The experiments were begun on day seven after the cells had grown

for either 24 or 48 h in media containing 10% liprotein-deficient serum. Human or canine lipoprotein-deficient serum was used, respectively, with human or canine iodinated lipoproteins.

Assays for Binding, Internalization, and Degradation. The procedures of Goldstein & Brown (1974), with minor modifications (Mahley & Innerarity, 1977), were used for binding and degradation assays performed at 37 °C. The assays were performed by the addition of 2 mL of DME media containing 10% lipoprotein deficient serum, 5 μg/mL [^{125}I]LDL, and varying amounts of unlabeled lipoproteins to cells growing in 60-mm Petri dishes. The media, [^{125}I]LDL, and lipoproteins were warmed to 25–35 °C and mixed together immediately before addition to the cells. The incubations were performed in a CO_2 incubator for 5 h during which time the dishes of cells were gently oscillated (60 oscillations/min). High affinity binding (receptor bound and internalized) was determined after washing the cell monolayers at 4 °C with three 3-mL aliquots of cold phosphate buffered saline containing 2 mg/mL bovine serum albumin. Each dish was then incubated twice for 10 min at 4 °C with the same albumin-buffered saline solution followed by one rapid wash with the phosphate buffered saline without albumin. The cells were removed from the dishes by digestion with three 0.5-mL aliquots of 0.1 N NaOH. After the total 1.5 mL was counted in a γ counter, duplicate aliquots were taken for protein determination. Proteolytic degradation was determined by analysis of the incubation media for [^{125}I]monotyrosine. The media was treated with trichloroacetic acid and hydrogen peroxide and then extracted with chloroform to remove free iodine.

Binding studies at 4 °C were performed as described by Goldstein et al. (1976), except for minor modifications (Mahley et al., 1977b). The DME media contained 25 mM Hepes (pH 7.4) instead of bicarbonate. All solutions were prechilled to 4 °C prior to addition to the cells, and the incubations were performed with dishes on ice.

Assay for the Incorporation of [^{14}C]Oleate into Cholesteryl Esters. [1-^{14}C]Oleate incorporation into cholesteryl esters by the cell monolayers was measured as previously described (Goldstein et al., 1974, 1975). The [1-^{14}C]oleate was complexed to albumin as described by Van Harken et al. (1969). On day six, after the cells had grown for 24 h in media containing lipoprotein-deficient serum, the sterile lipoproteins were added to 2 mL of new 10% lipoprotein-deficient media in the amounts indicated. After 15 h, 20 μL of 10 mM [^{14}C]oleate bound to albumin was added; the monolayers were harvested 2 h later. After aliquots were taken for protein determination, [1,2-^{3}H]cholesterol was added as an internal standard, the cells were extracted with chloroform–methanol (2:1 v/v), and the ^{14}C-labeled cholesteryl ester content was determined following isolation by thin-layer chromatography.

Heparin Precipitation of Lipoproteins. The lipoproteins were dialyzed against 0.15 M NaCl, 0.01% EDTA, pH 7.0, and adjusted to a protein concentration of 3 mg/mL. Heparin (33 mg/mL in H_2O) and 1 M $MnCl_2$ were added in a ratio of 1 mg of heparin to 4.8 mg of $MnCl_2$. The remainder of the procedure was carried out as previously described (Mahley & Innerarity, 1977).

Partial Delipidation of HDL_c and LDL. HDL_c (d = 1.02–1.063) and human LDL (d = 1.02–1.05) were partially delipidated with diethyl ether. Diethyl ether (250 mL; 4 °C) was layered on top of 10 mg of LDL or HDL_c in 25 mL of 0.05 M borate buffer, pH 8.1, similar to the procedure of Levy et al. (1966). The lipoproteins in the aqueous layer were very gently stirred for 18–20 h at 4 °C, and then the ether layer was removed and the surface of the aqueous phase was washed with four 100-mL aliquots of diethyl ether. Traces of ether which remained in the aqueous phase were removed under vacuum. In addition, human LDL were partially delipidated by the procedure of Chan & Knowles (1976). To 10 mg of LDL in 1.5 mL of 0.15 M NaCl, 0.01% EDTA (pH 7.0), 10 mL of 1-butanol:diisopropyl ether (15:85, v/v) was added. This was gently shaken for 1 h at room temperature and then the organic phase was removed and the aqueous phase washed five times with 3-mL aliquots of diisopropyl ether. The partially delipidated LDL and HDL_c were dialyzed against 0.15 M NaCl, 0.01% EDTA, for 24 h at 4 °C.

Antisera Preparation and Radioimmunoassay. Antisera to swine apo-E were prepared in New Zealand white rabbits. The primary injection consisted of 350 μg of purified apo-E protein in 1 mL of saline emulsified with an equal volume of Freund's complete adjuvant. The antigen was injected into two footpads and intradermally in multiple sites on the back. Three and six weeks after the initial injection the rabbits received booster injections (350 μg of protein) emulsified in Freund's incomplete adjuvant. The rabbits were exsanguinated 9 days after the last injection. The antisera did not react with swine albumin or the swine A-I, B, or C apoproteins. The antisera were monospecific for apo-E.

Swine apo-E was labeled with sodium [^{125}I]iodide by the lactoperoxidase method (Marchalonis, 1969). A detailed description and validation of the radioimmunoassay procedure, a modification of the method of Fainaru et al. (1977), will be reported elsewhere. The assay tubes contained the following: swine [^{125}I]apo-E (~20 000 cpm), anti-apo-E (diluted 1:1200), Triton X-100 (100 μL of 0.1%), sodium dodecyl sulfate (50 μL of 50 mM), and phosphate-buffered saline (pH 7.4) to bring the volume to 1 mL. After 48 h of incubation at 4 °C, goat anti-rabbit IgG and carrier rabbit IgG were added, and the incubation was continued for an additional 18 h at 4 °C. The iodinated swine apo-E bound to the antiserum was separated from the unbound apo-E by centrifugation.

Results

Canine [^{125}I]LDL as well as human [^{125}I]LDL have been shown to bind to the high affinity receptors of human fibroblasts and to be internalized and degraded (Goldstein & Brown, 1974; Mahley & Innerarity, 1977). At a concentration of 5 μg/mL of [^{125}I]LDL protein, more than 90% of the [^{125}I]LDL bound to the high affinity receptors can be displaced by the addition of unlabeled LDL. Moreover, any other lipoprotein that binds to the receptors would displace [^{125}I]LDL, resulting in less [^{125}I]LDL bound, internalized, and degraded. Figure 1 shows a composite of the results of six different competitive binding and degradation experiments which compared the effectiveness of three different preparations of canine LDL and five preparations of apo-E HDL_c to compete with [^{125}I]LDL. Addition of 20 μg/mL of unlabeled canine LDL protein to the media reduced the binding of [^{125}I]LDL by 50%. Apo-E HDL_c were much more effective in displacing [^{125}I]LDL from the receptors, with 50% displacement occurring at 2–2.5 μg/mL of HDL_c protein. As shown in Figure 1B, the results of the competitive degradation assays confirmed that apo-E HDL_c had a greater affinity for the receptors than canine LDL and displaced more [^{125}I]LDL. Furthermore, the enhanced binding activity of apo-E HDL_c was also established when human [^{125}I]LDL and unlabeled human LDL were used in the competitive binding and degradation studies for comparison with the apo-E HDL. The apo-E HDL_c were approximately ten times more effective per microgram of lipoprotein protein in displacing human [^{125}I]LDL from the high affinity binding sites than the human LDL. The apo-B and

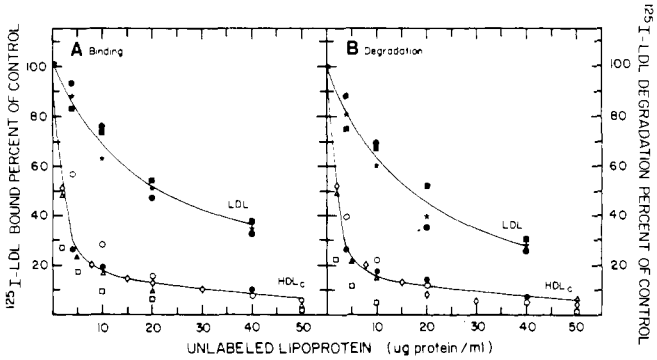

FIGURE 1: Comparison of the ability of canine LDL (★, ●, ■) and apo-E HDL_c (○, ◇, ⊙, □, △) (d = 1.006–1.02) to compete with canine [^{125}I]-LDL for (A) binding and internalization and (B) degradation. The human fibroblast monolayers were grown for 24 h in media containing 10% canine lipoprotein-deficient serum. On day 7, the media was replaced with fresh media containing 10% canine lipoprotein-deficient serum, 5 μg/mL (○, ◇, ⊙, △, ★, ●) or 2 μg/mL (□, ■) of canine [^{125}I]LDL, and the unlabeled LDL or apo-E HDL_c at the concentrations indicated. After incubating at 37 °C for 5 h, the binding, internalization, and degradation were determined as described. The specific activity of the canine [^{125}I]LDL ranged from 21 to 177 cpm/ng of lipoprotein protein.

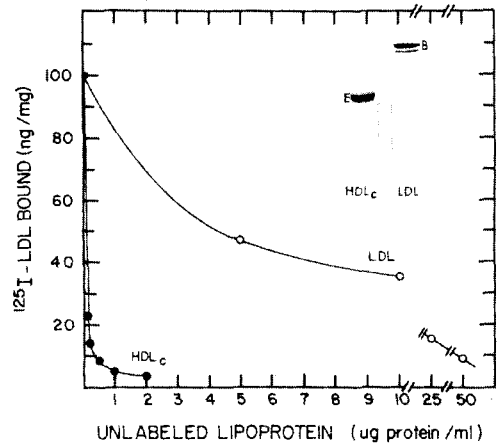

FIGURE 2: A comparison of the competitive displacement of human [^{125}I]LDL from the cell surface receptors by unlabeled human LDL and canine apo-E HDL_c at 4 °C. On day 7, the media was replaced with fresh media containing 25 mM Hepes, 10% human lipoprotein-deficient serum, 2 μg/mL of human [^{125}I]LDL (164 cpm/ng), and the unlabeled LDL or apo-E HDL_c at the concentrations indicated. After incubating at 4 °C for 2 h, the binding assay was performed as described. The inset shows the $NaDodSO_4$-polyacrylamide gels of the human LDL and canine apo-E HDL_c that were used in this experiment.

apo-E were the only protein constituents of the canine or human LDL and canine HDL_c, respectively. Other physical and chemical properties of LDL and HDL_c were not strikingly different (to be discussed below).

In the above experiments, a concentration of 5 μg/mL of [^{125}I]LDL protein, which was chosen to minimize nonspecific binding sites for LDL (half-saturation occurred at 20 to 25 μg/mL), would, in fact, underestimate the differences between LDL and HDL_c. Therefore, in an additional study, 25 μg/mL of [^{125}I]LDL protein was used to allow more of the total available receptors to be occupied initially and to allow a more accurate estimate of total displacement. The study, identical with that reported above except for the use of 25 μg of [^{125}I]LDL, revealed that the binding activity of apo-E HDL_c was enhanced 14- to 17-fold as compared with human LDL.

To take advantage of the higher affinity of the LDL for the receptor, which results in increased sensitivity of the assay system, and to measure binding in the absence of internalization (Goldstein et al., 1976), competitive displacement studies were performed with the human fibroblasts at 4 °C. Human [^{125}I]LDL (2 μg/mL of protein) were displaced from the binding sites by the addition of increasing concentrations of human LDL or canine apo-E HDL_c (Figure 2). For the LDL, 50% displacement occurred at a concentration of 4 μg/mL of protein. The apo-E HDL_c were 100 times more effective with a 50% reduction of [^{125}I]LDL binding at a concentration of 0.04 μg/mL of HDL_c protein. The apoprotein content of the LDL and HDL_c is shown (inset, Figure 2).

To determine if the enhanced binding activity of apo-E HDL_c had a direct modulating effect on one of the regulated intracellular processes, we compared the cholesteryl ester synthetic activity of cultured fibroblasts in response to increasing concentrations of LDL and HDL_c. Cholesterol esterification as determined by the measurement of [^{14}C]oleate incorporation into cellular cholesteryl esters has been shown to be one of the metabolic processes regulated by high affinity binding, internalization, and degradation (Goldstein et al., 1974). With both LDL and apo-E HDL_c, cholesteryl ester synthetic activity as determined by [^{14}C]oleate incorporation increased as the concentration of LDL or HDL_c was increased to saturation levels in the culture media. However, as shown in Figure 3, apo-E HDL_c were much more effective than

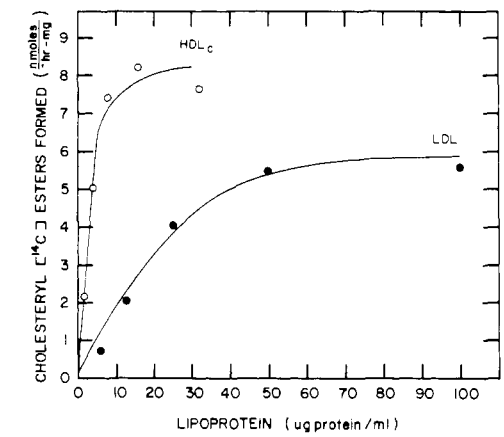

FIGURE 3: Incorporation of [^{14}C]oleate into cholesteryl esters of human fibroblasts as a function of LDL (●) or apo-E HDL_c (○) concentration. On day 6, normal human fibroblasts were incubated with the indicated amount of lipoprotein for 15 h and then pulse labeled with [^{14}C]oleate (0.1 mM; 40 000 cpm/nmol) for 2 h. The cell monolayers were harvested and the ^{14}C-labeled cholesteryl esters determined.

human LDL in stimulating cholesteryl ester formation. The half-maximal activity for cholesterol esterification occurred at an LDL concentration of 18 μg/mL of protein, which is similar to the LDL concentration giving half-maximal binding (~20 μg/mL). The apo-E HDL_c caused half-maximal stimulation of cholesteryl [^{14}C]oleate synthesis at a much lower concentration (3 μg/mL of HDL_c protein) and achieved a higher maximal level of cholesteryl ester synthesis than did the LDL. When the results were calculated on the basis of micrograms of LDL or HDL_c cholesterol added to the cultured fibroblasts, the increase in cholesteryl ester synthesis after addition of the lipoprotein cholesterol was still seen to be significantly higher with HDL_c than with LDL (approximately a fourfold increase in [^{14}C]oleate incorporation by apo-E HDL_c).

Table I summarizes the physical and chemical properties of human and canine LDL and canine apo-E HDL_c. Although the properties of the LDL and apo-E HDL_c were not strikingly different, there were differences in the lipid composition. To establish that the enhanced binding activity of the apo-E HDL_c

TABLE I: Physical and Chemical Properties of Human and Canine LDL and Canine Apo-E HDL_c.

	Apo-E HDL_c	Human LDL	Canine LDL
Density (g/mL)	1.006-1.02	1.02-1.05	1.02-1.063
Electrophoretic mobility	α_2	β	β
Heparin precipitability[a]	+	+	+
Mol wt[b]	3.6×10^6	3×10^6	3×10^6
Protein mol wt	0.54×10^6	0.63×10^6 (0.55×10^6)[c]	0.60×10^6[d]
Size, mean diameter (Å)	~240	~200	~200
Protein content	Apo-E	Apo-B	Apo-B
Chemical composition			
Protein	15.3[e]	21.1[e]	20.8[f]
T. cholesterol	50.8	45.5	45.9
Phospholipid	31.4	26.8	27.0
Triglyceride	2.3	6.6	6.3
Fatty acid composition of cholesteryl esters[g]			
Linoleate	20.8	53.4	9.4
Oleate	30.7	16.5	50.1
Palmitate	5.0	10.3	6.7
Palmitoleate	25.4	3.7	21.1
Others	18.1	16.1	12.7
Phospholipid classes[h]			
Phosphatidylcholine	80.5	65.4	78.0
Sphingomyelin	16.5	28.9	17.0
Others	3.4	5.7	5.0

[a] See Mahley & Innerarity, 1977. [b] Determined by A-5m agarose column chromatography based on the method of Rudel et al. (1977). [c] Based on literature value (Miller et al., 1977a). [d] Based on the assumption that the molecular weight of canine LDL is the same as for human LDL since they both are the same size and have similar compositions. [e] Average of two determinations. [f] A single determination on LDL from coconut oil-cholesterol fed dog. [g] Triplicate determinations (% by weight). [h] Triplicate determinations (mol %).

TABLE II: Lipids Removed from LDL and HDL_c by Partial Delipidation.

	% of total removed		
	LDL[a]	LDL[b]	HDL_c
Total cholesterol			
Free	98	94	95
Esterified	97	98	89
Phospholipid	50	31	34
Triglyceride	95	ND[c]	ND

[a] Diisopropyl ether:butanol. [b] Diethyl ether. [c] ND, not determined.

was not directly related to the lipid content, the lipid contents of the LDL and HDL_c were markedly altered by partial delipidation, and then their binding activities were compared. Partial delipidation of human LDL with diethyl ether or diisopropyl ether:butanol removed more than 90% of the total cholesterol and 31 to 50% of the phospholipid (Table II). As shown in Figure 4, the ether-extracted LDL exhibited a binding activity essentially identical with that obtained with the untreated LDL, and a 50% displacement occurred at 25 μg of protein/mL. It is noteworthy that the delipidated LDL did not stimulate cholesteryl ester synthesis (Figure 4, inset). The diisopropyl ether:butanol delipidated LDL gave identical results. Likewise, partial delipidation of canine HDL_c (d = 1.02-1.063) removed greater than 90% of the total cholesterol and 34% of the phospholipid (Table II) and converted the particles to disk and amorphous forms (Figure 5, inset). However, the apoprotein patterns of the untreated and delipidated HDL_c (d = 1.02-1.063) revealed the presence of the A-I and E apoproteins and were unchanged by the treatment. Despite the markedly altered lipid content and particle morphology, the partially delipidated HDL_c were as effective as the untreated HDL_c in displacing the iodinated canine LDL (Figure 5). Thus, partial delipidation with removal of significant amounts of lipid did not alter receptor binding activity. These data indicated that the differences in lipid composition between LDL and HDL_c did not account for the differences in the binding activity.

However, the importance of the lipid was noted, because total delipidation of canine HDL_c with chloroform:methanol abolished all binding activity despite the fact that the protein remained soluble in the culture media. In addition, human apo-E purified by Sephadex G-200 chromatography in guanidine (Mahley et al., 1975, 1976) failed to displace human [^{125}I]LDL from the receptors. These results indicated that lipid was required to preserve the protein conformation necessary for binding to the receptor and that total delipidation completely altered the recognition site on the apoprotein.

Because of the enhanced binding capability of apo-E, a small quantity of apo-E in a minor subfraction of typical HDL (d = 1.09-1.21) of swine and man could account for the observed binding activity of HDL. Carew et al. (1976) and Miller et al. (1977b) have shown that normal HDL (d = 1.09-1.21) did displace [^{125}I]LDL from the cell surface receptors at concentrations much higher than required for comparable displacement by unlabeled LDL. We have repeated these experiments and have observed similar results. As shown in Figure 6, swine HDL at a concentration of 200 μg/mL of protein displaced 30% of the [^{125}I]LDL (5 μg/mL). Previously we have shown that the cholesterol-induced HDL_c of dogs and swine could be fractionated into several subpopulations by heparin/manganese (Mn) precipitation (Mahley & Innerarity, 1977). The fractions which precipitated with heparin/Mn were the most effective in competing with [^{125}I]LDL for binding, internalization, and degradation. The most striking characteristic of these active lipoproteins was an increased concentration of the E apoprotein. To determine if the binding activity of the typical HDL was due to a minor subfraction of the HDL which contained the apo-E, swine HDL were subjected to the heparin/Mn precipitation procedure and the binding activities of the precipitable and nonprecipitable fractions were compared. The precipitable fraction, which accounted for only 15.8% of the total HDL protein, possessed most of the binding activity. The supernatant fraction, which contained most of the protein (84.2%), had little or no capacity to displace the [^{125}I]LDL from the receptor sites on human fibroblasts (Figure 6). The content of apo-E was measured in the original HDL, the precipitate, and the supernatant fraction by radioimmunoassay. The percentage of the total lipoprotein protein represented by the apo-E was 1.2% in the original HDL, 4.2% in the precipitate, and 0.62% in the supernatant. Previously, we have reported that a swine HDL_c subfraction with an apo-E content of 4.5% displaced approximately 50% of the [^{125}I]LDL from the cell receptors at a protein concentration of 200 μg/mL in the culture media (Mahley & Innerarity, 1977). Similar results were observed with the precipitated subfraction of the typical HDL (Figure 6). It was possible to convert the micrograms of lipoprotein protein to micrograms of apo-E at each concentration of the HDL and its subfractions and to replot the data in Figure 6 to compare the concentration of apo-E with [^{125}I]LDL displacement (Figure 7). The binding activity correlated with the apo-E concentration. No other

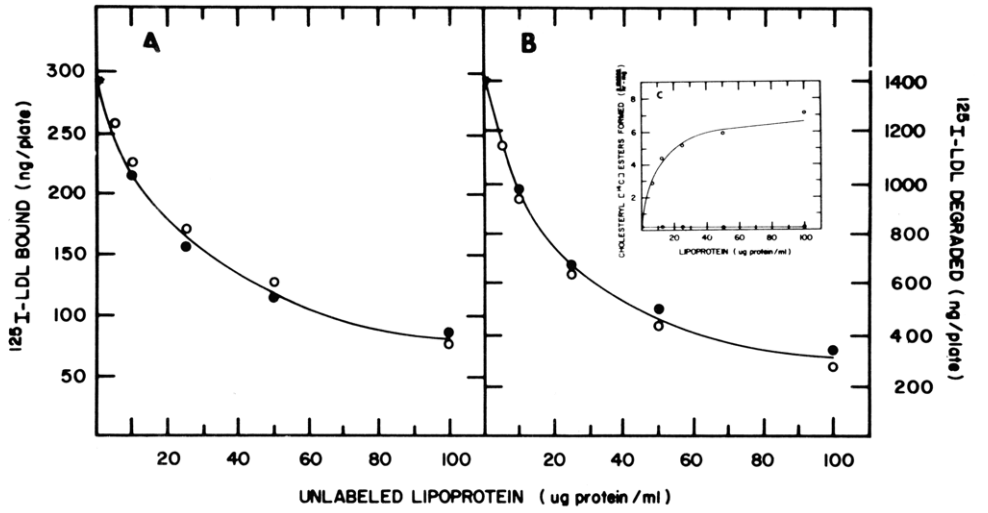

FIGURE 4: Ability of human LDL (○) and partially delipidated LDL (●) to compete with human [^{125}I]LDL for binding, internalization, and degradation. On day 7, the media was replaced with new media containing 10% human lipoprotein-deficient serum, 5 μg/mL of human [^{125}I]LDL (147 cpm/ng), and the unlabeled human LDL or partially delipidated LDL at the protein concentrations indicated. After incubation at 37 °C for 5 h, the binding, internalization, and degradation assay was performed as described in Materials and Methods. The inset compares the ability of increasing concentrations of the same LDL (○) and partially delipidated LDL (●) to stimulate the incorporation of [^{14}C]oleate into cholesteryl esters. The experimental procedure was as described in Figure 3.

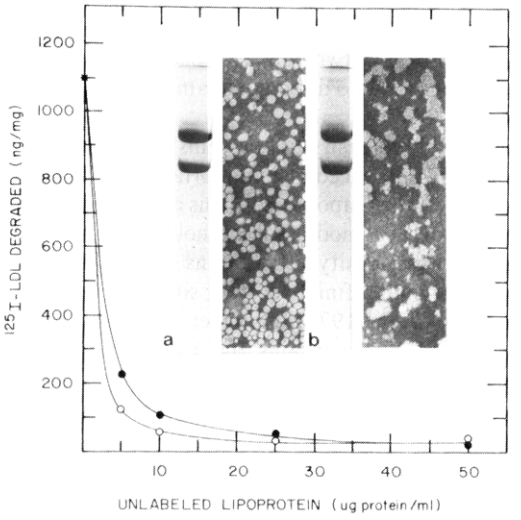

FIGURE 5: Comparison of the ability of HDL_c (○) and partially delipidated HDL_c (●) to compete with canine [^{125}I]LDL for high affinity proteolytic degradation. The human fibroblasts were grown for 24 h in media containing 10% canine lipoprotein-deficient serum. On day 7, the media was replaced with fresh media containing 10% canine lipoprotein-deficient serum, 5 μg/mL of canine [^{125}I]LDL (128 cpm/ng), and the unlabeled native HDL_c or partially delipidated HDL_c at the concentrations indicated. After incubation for 5 h, the binding, internalization, and degradation were determined as described in the Materials and Methods (only the degradation is shown). The insets show the $NaDodSO_4$-polyacrylamide gel patterns and negative staining electron micrographs of native HDL_c (a) and partially delipidated HDL_c (b).

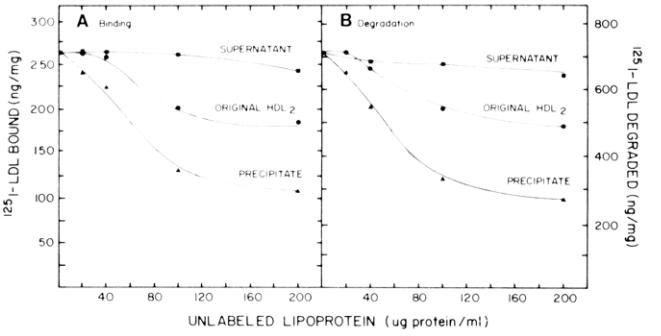

FIGURE 6: Ability of swine HDL (d = 1.09–1.21) (●), heparin/manganese precipitated HDL (▲), and the HDL that remained in the supernatant (■) to compete with swine [^{125}I]LDL for binding and degradation in normal human fibroblasts. Swine [^{125}I]LDL (5 μg/mL, 274 cpm/ng) and the indicated amounts of unlabeled fractions were added to the cells. After a 5-h incubation at 37 °C, the binding, internalization, and degradation of [^{125}I]LDL were determined.

chemical or physical properties were notably different when the precipitate and supernatant fractions were compared. These data suggested that small quantities of the highly reactive apoprotein E account for most, if not all, of the high affinity receptor binding activity of HDL.

Discussion

Lipoproteins which have been shown to bind to the high affinity cell surface receptors of fibroblasts and smooth muscle cells include LDL, which contain the B apoprotein, and HDL_c, which contain the E (arginine-rich) apoprotein (Goldstein & Brown, 1977; Mahley & Innerarity, 1978). It has been established that the apo-B- and apo-E-containing lipoproteins bind to the same cell surface receptors and that they regulate intracellular cholesterol metabolism (Bersot et al., 1976; Mahley & Innerarity, 1977; Mahley et al., 1977b). Isolation of an HDL_c which contained the E apoprotein as the exclusive protein constituent provided an opportunity for comparison of these apo-E HDL_c with the apo-B-containing LDL (Mahley et al., 1977a). Selective modification of the B and E apoproteins produced evidence that the protein was the important determinant for specific high affinity binding and that the recognition sites on the two different apoproteins responsible for their binding to the same receptor might be structurally similar. After treatment of the lipoproteins with cyclohexanedione, a reagent specific for the amino acid arginine, the binding activity of both the LDL and HDL_c was abolished (Mahley et al., 1977b). It was therefore concluded that there were functionally important arginyl residues in or near structurally similar recognition sites of both the B and E apolipoproteins.

The present studies demonstrate that there is a significant difference between the binding activities of the apo-E HDL_c and the LDL. The activity of the apo-E HDL_c is greater by a

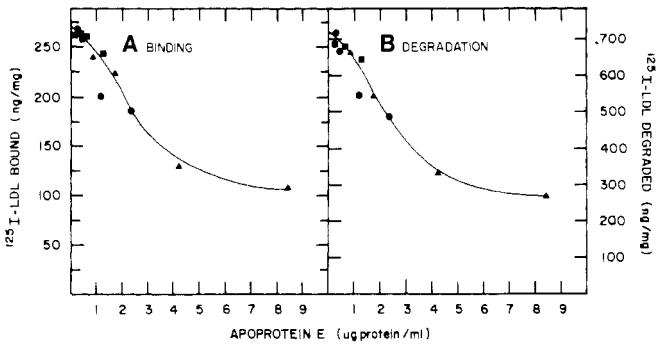

FIGURE 7: A comparison of the concentration of apoprotein E in swine HDL (●), precipitated HDL (▲), and supernatant HDL (■) vs. the binding, internalization, and degradation of 5 μg/mL of swine [^{125}I]LDL. The percent of apoprotein E in each fraction was determined by radioimmunoassay and the apoprotein E concentration for each protein concentration was calculated. The binding and degradation data shown on the ordinate of Figure 6 were then replotted against apoprotein E concentration (on the abscissa) for each lipoprotein fraction.

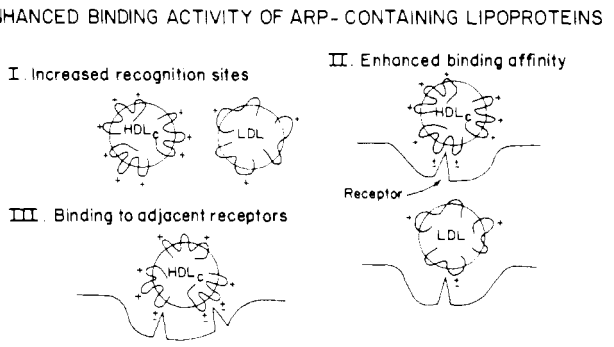

FIGURE 8: Schematic diagram showing possible mechanisms to explain the enhanced binding of apo-E-containing lipoproteins.

factor of 10 to 17 than the activity of LDL in competitive binding assays performed at 37 °C and 100 times greater in assays performed at 4 °C. The increased sensitivity of the 4 °C competitive binding assays has been reported (Goldstein et al., 1976). Whether the comparisons are based on lipoprotein protein, lipoprotein cholesterol, estimates of particle number, or molar ratios (see below), HDL$_c$ are several-fold more active than LDL. Furthermore, the greater binding activity of HDL$_c$ is directly reflected in the increase in cholesteryl ester synthesis by the cells. Less apo-E HDL$_c$ than LDL is needed for half-maximal stimulation of cholesteryl ester synthesis, which confirms by a biological assay the results of the binding assay. The absolute quantity of cholesteryl ester formed during the 2-h pulse labeling with [^{14}C]oleate for HDL$_c$ is approximately 1.3–1.4 times that of human LDL. Using the molecular weight and chemical composition of HDL$_c$ (Table I), the molar ratio of the total cholesterol in the apo-E HDL$_c$ is 1.34 times that of the human LDL. It has been shown that the increased availability of cholesterol to the microsomal ACAT results in increased enzymatic activity (Hashimoto & Dayton, 1978). It is reasonable to assume that the increased delivery of cholesterol by apo-E HDL$_c$ may account for these observations.

To demonstrate that the minor differences in chemical composition and particle size between HDL$_c$ and the canine and human LDL are not the factors responsible for the observed differences in binding activities, the activities were compared on the basis of the molar ratios of the protein in these lipoproteins. Table I shows the protein content of the HDL$_c$ to be somewhat less than that determined for canine and human LDL. However, because of the increased particle size and molecular weight of the HDL$_c$, the molar ratios of the LDL and HDL$_c$ protein are very similar (Table I). Therefore, at any given protein concentration, approximately the same number of LDL and HDL$_c$ particles would be added to the fibroblasts. Furthermore, the results obtained with the partially delipidated lipoproteins support the contention that the difference in binding activities of LDL and HDL$_c$ are not due to differences in chemical composition. Partial delipidation, which markedly alters the lipid content and particle morphology, does not significantly alter the binding activity of either LDL or HDL$_c$.

It is reasonable to speculate that the enhanced binding activity of the HDL$_c$ might be related to inherent differences between the B and E apoproteins with respect to their interaction with the cell surface receptors. As shown in a highly schematic diagram (Figure 8), it is possible (I) that the enhanced binding activity of the E apoprotein may be related to a greater number of recognition sites (more arginyl residues, clusters of positive charges, or active sites per mole) available on apo-E HDL$_c$ than on LDL. An increased number of recognition sites would increase the likelihood of apo-E interacting with the receptors as compared with apo-B. Another possibility (II) is that the receptor binding or affinity constant for the apo-E is greater than for the apo-B due to a "positive cooperativity" by which adjacent recognition sites on the apo-E may enhance the binding of these lipoproteins to a single receptor. On the other hand (III), a single apo-E-containing lipoprotein particle might bind to two adjacent receptor sites. Additional studies are necessary to distinguish among these or alternate mechanisms.

Binding of high density lipoproteins to the cell surface receptors may be influenced in an important way by the potency of the binding of the E apoprotein. This may furthermore affect the role of HDL as a modulator of cholesterol metabolism. In general, the high density lipoproteins of man interact minimally with the high affinity receptor site, as previously shown (Brown & Goldstein, 1974). However, a minor subfraction of human HDL, which contains the E apoprotein, possesses considerably high affinity binding activity (Innerarity, T. L., Mahley, R. W., Weisgraber, K. H., and Bersot, T. P., submitted for publication). Likewise, as shown in this paper, swine HDL (d = 1.09–1.21) contain a minor subfraction which possesses most or all of the binding activity of this class. Addition of heparin/manganese to the d = 1.09–1.21 fraction precipitates this active subclass, which accounts for less than 15% of the HDL protein of the fraction. The most striking difference between the relatively inactive HDL which remain in the supernatant and the active subclass which precipitates is the presence of the E apoprotein. Previously, we have shown that heparin precipitability correlates directly with receptor binding activity and with the E apoprotein content of various HDL fractions (Mahley & Innerarity, 1977). Because of its enhanced binding activity, the E apoprotein, present in relatively small quantities in a subclass of the HDL, could confer considerable binding ability on these lipoproteins, and furthermore HDL binding could be modulated by relatively small changes in the E apoprotein content of the HDL. One factor known to alter the E apoprotein content of lipoproteins is cholesterol feeding which is associated with HDL$_c$ production by several different animal species (Mahley, 1978). The importance of dietary cholesterol or other factors which alter the HDL apolipoproteins and thereby affect cholesterol metabolism remains to be determined.

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References

Abell, L. L., Levy, B. B., Brodie, B. B., & Kendall, F. E. (1952) *J. Biol. Chem. 195,* 357.

Assmann, G., Brown, B. G., & Mahley, R. W. (1975) *Biochemistry 14,* 3996.

Barile, M. F. (1973) in *Contamination in Tissue Culture* (Fogh, J., Ed.), p 132, Academic Press, New York, N.Y.

Bartlett, G. R. (1959) *J. Biol. Chem. 234,* 466.

Bersot, T. P., Mahley, R. W., Brown, M. S., & Goldstein, J. L. (1976) *J. Biol. Chem. 251,* 2395.

Bilheimer, D. W., Eisenberg, S., & Levy, R. I. (1972) *Biochim. Biophys. Acta 260,* 212.

Brown, M. S., & Goldstein, J. L. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 788.

Carew, T. E., Koschinsky, T., Hayes, S. B., & Steinberg, D. (1976) *Lancet 1,* 1315.

Chan, B. E., & Knowles, B. R. (1976) *J. Lipid Res. 17,* 176.

Fainaru, M., Havel, R. J., & Imaizumi, K. (1977) *Biochim. Biophys. Acta 490,* 144.

Fletcher, M. J. (1968) *Clin. Chim. Acta 22,* 393.

Goldstein, J. L., & Brown, M. S. (1974) *J. Biol. Chem. 249,* 1974.

Goldstein, J. L., & Brown, M. S. (1976) *Curr. Top. Cell. Regul. 11,* 147.

Goldstein, J. L., & Brown, M. S. (1977) *Annu. Rev. Biochem. 46,* 897.

Goldstein, J. L., Dana, S. E., & Brown, M. S. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 4288.

Goldstein, J. L., Dana, S. E., Brunschede, G. Y., & Brown, M. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 1092.

Goldstein, J. L., Basu, S. K., Brunschede, G. Y., & Brown, M. S. (1976) *Cell 7,* 85.

Hashimoto, S., & Dayton, S. (1978) *Atherosclerosis* (in press).

Kates, M. (1972) *Techniques of Lipidology*, p 351, Elsevier, New York, N.Y.

Levy, R. I., Lees, R. S., & Frederickson, D. S. (1966) *J. Clin. Invest. 45,* 63.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193,* 265.

Mahley, R. W. (1978) in *The Physiology of Lipids and Lipoproteins in Health and in Disease* (Dietschy, J., Ed.) American Physiology Society, Bethesda, Md. (in press).

Mahley, R. W., & Innerarity, T. L. (1977) *J. Biol. Chem. 252,* 3980.

Mahley, R. W., & Innerarity, T. L. (1978) in *Sixth International Symposium on Drugs Affecting Lipid Metabolism* (Kritchevsky, D., Paoletti, R., & Holmes, W. L., Eds.) Plenum Press, New York, N.Y. (in press).

Mahley, R. W., & Weisgraber, K. H. (1974) *Biochemistry 13,* 1964.

Mahley, R. W., Weisgraber, K. H., & Innerarity, T. (1974) *Circ. Res. 35,* 722.

Mahley, R. W., Weisgraber, K. H., Innerarity, T., Brewer, H. B., Jr., & Assmann, G. (1975) *Biochemistry 14,* 2817.

Mahley, R. W., Weisgraber, K. H., & Innerarity, T. (1976) *Biochemistry 15,* 2979.

Mahley, R. W., Innerarity, T. L., Weisgraber, K. H., & Fry, D. L. (1977a) *Am. J. Pathol. 87,* 205.

Mahley, R. W., Innerarity, T. L., Pitas, R. E., Weisgraber, K. H., Brown, J. H., & Gross, E. (1977b) *J. Biol. Chem. 252,* 7279.

Marchalonis, J. J. (1969) *Biochem. J. 113,* 299.

Margolis, S. (1967) *J. Lipid Res. 8,* 501.

Miller, N. E., Weinstein, D. B., & Steinberg, D. (1977a) *J. Lipid Res. 18,* 438.

Miller, N. E., Weinstein, D. B., Carew, T. E., Koschinsky, T., & Steinberg, D. (1977b) *J. Clin. Invest. 60,* 78.

Nelson, G. J. (1975) in *Analysis of Lipids and Lipoproteins* (Perkins, E. C., Ed.) p 70, American Oil Chemists Society, Champaign, Ill.

Rudel, L. L., Pitts, L. L., & Nelson, C. A. (1977) *J. Lipid Res. 18,* 1977.

Van Harken, D. R., Dixon, C. W., & Heimberg, M. (1969) *J. Biol. Chem. 244,* 2278.