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DETERMINATION OF AGENT RESPONSIBLE FOR HYDROLYSIS OF CHOLESTERYL ESTERS IN LOW DENSITY LIPOPROTEINS

BY

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ABSTRACT

A recent investigation revealed an apparent cholesteryl ester hydrolysis in low density lipoproteins (LDL) upon incubation with reconstituted high density lipoproteins (r-HDL). Since r-HDL contains apolipoprotein A-I (apoA-I) and egg phosphatidylcholine (egg-PC), this work attempts to reveal if the apoA-I, the egg-PC in vesicle form, or the r-HDL is the agent responsible for this hydrolysis. The results of this study show that there is no increase in free cholesterol in LDL upon incubation with either apoA-I or egg-PC vesicles. Thus, it is concluded that the intact r-HDL upon incubation with LDL causes the change in LDL composition.

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LIST OF ABBREVIATIONS

LDL	Low density lipoproteins
HDL	High density lipoproteins
VLDL	Very low density lipoproteins
ApoB	Apolipoprotein B
ACAT	Acyl-CoA:cholesterol acyltransferase
LCAT	Lecithin cholesterol acyltransferase
LCP	Lipoprotein complexing proteoglycan
CETP	Cholesteryl ester transfer protein
PLTP	Phospholipid transfer protein
DPPC	Dipalmitoyl phosphatidylcholine
Egg-PC	Egg Phosphatidylcholine
CEME	Cholesterol ester-phospholipid microemulsions
DMPC	Dimyristoyl phosphatidylcholine
r-HDL	Reconstituted high density lipoproteins
ApoA-I	Apolipoprotein A-I

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

INTRODUCTION

The plasma lipoproteins are the major source for transport of insoluble lipids in an aqueous environment. They consist of a neutral lipid core containing primarily triglycerides and cholesteryl esters and an outer shell of phospholipid, apolipoproteins, and some cholesterol. The various lipoproteins can be distinguished based on the differing proportions of these constituents. These complexes can be divided into four basic categories. Chylomicrons transport dietary triglycerides and cholesterol while very low density lipoproteins (VLDL) transport triglycerides which have been synthesized in the liver. Low density lipoproteins (LDL) and high density lipoproteins (HDL) are the major carriers of endogenous cholesterol. These lipoproteins are readily isolated by ultracentrifugation at the appropriate density.

Low density lipoproteins are a major topic of research since a positive correlation between them and atherosclerosis has been determined. These lipoproteins of density 1.019-1.063 g/ml contain approximately 29% protein, 21% phospholipid, 9% free cholesterol, 4% triglyceride, and 40% cholesteryl esters (1) in a quasi-spherical particle with a diameter of 200-250Å and a molecular weight of 2-3.5 x 10^6 million. However, Shen et al. (1) has determined that low density lipoproteins are heterogeneous even within the individual. They have separated LDL into at least three subgroups which differ in particle size, hydrated density, and chemical composition. These subspecies were shown to correlate differently between men and women.

Like other lipoproteins, LDL contains the triglycerides and cholesteryl esters in the core of the molecule. The cholesteryl ester/triglyceride ratio determines whether the core is in a smectic-like liquid crystalline or liquid state. The phospholipid and protein form a coat around the core with the protein, apolipoprotein B (apoB) extended 5-10Å from the polar heads of the phospholipid. Twenty percent of the phospholipid appears to be in a restricted conformation due to its interaction with apoB. Lund-Katz and Phillips (2) have shown that 65% of the free cholesterol content of LDL is located with the phospholipid in the outer layer while the rest is associated with the core. These two cholesterol locations are in continual exchange and are described as one physiological pool. Lund-Katz and Phillips (2) have also shown that the intramolecular exchange as well as the intermolecular exchange of cholesterol in LDL is much slower than that in HDL. They attribute this to a greater acyl chain saturation of phosphatidylcholine, and higher sphingomyelin/phosphatidylcholine and free cholesterol/phospholipid ratios in LDL. In general, they feel that the phospholipid and cholesterol are more tightly packed in the LDL.

The protein in LDL is 95-98% apoB. This represents approximately 17-32 mg/100 mg of LDL. ApoB is a huge, insoluble protein which is susceptible to degradation and oxidative cleavage, with a molecular weight of 512,000 daltons. Glutamic acid is the amino terminal residue and a blocked serine is the carboxyterminal residue. ApoB is also a glycoprotein with carbohydrates representing 5% of its total mass. The main carbohydrates present include glucose, galactose, mannose, fucose, glucosamine, and sialic acids which are all N-linked to glucosamine through asparagine residues (3).

There are six pairs of intramolecular disulfide bonds and two free half-cystine residues in 250,000 g of protein. Pertaining to these cystine residues is a recent discovery by Huang, Lee, and Singh (4) who investigated the presence of thiol ester linked palmitate and stearate in the apoB structure which are labile to mild alkaline conditions.

ApoB is the feature that the LDL receptor recognizes as postulated by Brown and Goldstein (5,6), thus revealing the importance of studying its primary structure. ApoB-48 of intestinal origin and apoB-100 of hepatic origin are the major forms of apoB with B-48 found mainly in chylomicrons and B-100 found in VLDL, IDL, and LDL. There exists a proteolytic cleavage within B-100, the major apoprotein in LDL, in plasma which reveals two fragments, B-74 and B-26. Cardin et al. (7) incubated LDL with kallikrein which degraded B-100 into four fragments in the same order as that of natural proteolytic cleavage. This involved the B-100 separating into two fragments, B-26 and B-74 and subsequent cleavage of B-74 into B-44 and B-30. B-26 was found at the amino terminus while B-74 was located at the carboxy terminus with B-44 contained within the sequence and B-30 found at the very end. All of these fragments have been found to some degree in LDL but have been identified merely as artifacts.

Also, the sequence of B-48 was located along with B-26 at the amino terminus of B-100. A recent investigation revealed that the mRNA encoding B-48 contains an in-frame UAA stop codon produced from a C to U change in the CAA codon in B-100 mRNA (8). The termination codon ends the protein with an isoleucine residue. However, a methionine residue is the actual carboxy

terminus which is possibly due to the removal of the carboxy terminal residue by carboxypeptidase A, an enzyme which removes nonpolar residues from the carboxy terminus of proteins. This result is important since it essentially states that B-100 and B-48 are the products of a single gene even though the mechanism involved is unknown.

The complete cDNA and amino acid sequence of human B-100 has been determined by two methods including sequencing tryptic peptides and partial cDNA clones (9,10). These studies revealed a 4563-amino acid protein comprised of a 27-amino acid signal peptide and a mature protein of 4536 residues. Other characteristics of B-100 revealed included a high hydrophobicity of 0.916 kcal/residue with a large number of these residues located at the carboxy end and the proportions of secondary structure including 42% α-helix, 21% β-sheet, 20% random structure, and 16% β turns. Dot matrix analysis revealed many long (> 70 residues) internal repeats and many more shorter repeats. Yang et al. (10) suggests from these results that the sequence of B-100 is derived from internal duplications. Further experimentation with trypsin revealed five hypothetical domains since trypsin-accessible and -inaccessible peptides were found to be nonrandom on the LDL. Finally, 20 possible N-glycosylation sites with 13 being glycosylated and 4 unglycosylated were located along with a potential LDL receptor binding domain.

Formation of LDL is through a process involving VLDL, a triglyceride-rich lipoprotein containing apoB as well as other apoproteins. This particle is released into the plasma from the liver and is transported to adipose

tissue. An enzyme, lipoprotein lipase, removes triglycerides from the VLDL causing all proteins other than apoB to leave also. The cholesteryl ester content of this particle increases mainly due to the effect of lecithin cholesterol acyl transferase (LCAT), an enzyme which removes a fatty acid from the sn-2 position of lecithin and transfers it to cholesterol. HDL is the main source of the cholesteryl esters since it appears to be the substrate for LCAT, and a lipid transfer protein will exchange cholesteryl esters and triglycerides between LDL and HDL. Once these processes take place to a sufficient level the VLDL becomes LDL (5). Although this is the accepted pathway, Rudel, Parks, Johnson, and Babiak (11) have discovered that a certain amount of LDL may be directly secreted into the circulation. From 25% to 75% of LDL is from VLDL conversion while the rest is due to this secretion depending on the particular laboratory animal.

LDL catabolism takes place through receptor-mediated endocytosis. LDL binds to a receptor which is located in a coated pit containing the protein clathrin. The LDL and receptor are internalized and form a coated vesicle. Eventually the coated vesicle is delivered to a lysosome as the receptor is recycled to the cell surface. In the lysosome, the coat breaks down and the fatty acids are cut from the cholesteryl esters. The free cholesterol is used in synthesizing membranes and hormones. If the cholesterol concentration increases three control mechanisms take over. First, HMG CoA reductase, an enzyme in cholesterol biosynthesis is inhibited. Second, acyl-CoA cholesterol acyltransferase (ACAT) esterifies the cholesterol for storage. Finally, the synthesis of LDL receptors is stopped. A small number or the absence of LDL

receptors and a defect in the receptor mechanism contribute to an increase in LDL in the circulation and an increased risk of atherosclerosis.

Certain characteristics of the LDL currently being studied may have a direct effect on the interaction with receptors. Among the properties which affect the binding of LDL to its receptor or proteoglycans, constituents of the arterial wall, include the molecular weight of LDL, its surface charge, the state of the core cholesteryl esters, the conformation of apoB, and the presence of other apoproteins (11). Camejo et al. (12) magnified some of these effects by studying complexes formed between LDL and a lipoprotein complexing proteoglycan (LCP) which is extracted from the human arterial intimal media. This LDL/LCP complex may be important in the formation of atherosclerotic plaque. LDL which contains more cholesterol, less sialic acid, and has a higher isoelectric point seems to promote the formation of the LDL/LCP complex. Also, a very important result by Camejo et al. (12) showed that HDL inhibits this complex formation. This apparently is associated with the apoprotein present in HDL and presents a possible way in which HDL may deter atherogenesis.

Many modifications of LDL are used to analyze its physiological properties. Specifically these modifications are used to measure the effects of cholesteryl ester exchange and transfer proteins (CETP), LCAT, and phospholipid transfer proteins (PLTP). The main effect of CETP and LCAT on LDL is the incorporation of cholesteryl esters into its neutral lipid core. LCAT acts on HDL to esterify the cholesterol while the CETP is responsible for the bidirectional exchange of cholesteryl esters and triglycerides between LDL

and HDL. Zechner et al. (13) recently investigated plasma incubations with both LCAT and CETP or just CETP alone. The CETP incubation alone resulted in an increase in triglycerides and a decrease in cholesteryl esters in the LDL core with no structural changes in the LDL itself while the incubation with LCAT decreased the polar lipids, phospholipids and cholesterol in LDL and reduced the LDL particle size without changing its properties. They feel this reduction in particle size due to LCAT may have a negative effect on LDL binding to its receptor.

Various studies have been done to develop a technique to study cholesteryl ester exchange. Krieger et al. (14) extracted the cholesteryl ester core of LDL and incorporated two fluorescent probes PMCA oleate and dioleyl fluorescein. These probes were used to monitor LDL uptake by its receptor and to identify receptor-deficient cells. Incubation of plasma or LDL plus lipoprotein-free plasma with cholesteryl ester-rich microemulsions leads to a transfer of cholesteryl esters into the LDL core as monitored by fluorescent probes (15). This transfer is mediated by a cholesteryl ester transfer protein in the plasma and has no deleterious effects on the LDL structure or properties. A similar process to create a cholesteryl ester probe involved incubation of plasma with density > 1.006 at 37°C with [^{14}C]cholesteryl ester containing liposomes (16). Again the radiolabeled cholesteryl esters were found in the core of the LDL with no structural or functional changes in LDL occurring. LDL with radiolabeled cholesteryl esters from this procedure was incubated with HDL to monitor the kinetics of transfer. The result agreed with a previous equilibrium transfer model.

The ability to monitor the phospholipid exchange protein activity with LDL is possible with the use of phospholipid vesicles. Jackson, Cardin, Barnhart, and Johnson (17) isolated bovine liver phosphatidylcholine exchange protein and prepared an incubation of this protein, LDL, and di[^{14}C]palmitoyl phosphatidylcholine (DPPC) vesicles. The result included a transfer of the radiolabeled DPPC into the LDL depending on the temperature. At 42°C there was complete exchange of the phospholipid. An increase in the total phospholipid of LDL along with an increased lipid rigidity was discovered with fluorescence polarization studies. Another experiment showed conversion of LDL particle size with incubation of plasma ($d > 1.2$ g/ml) and phosphatidylcholine (PC) vesicles (18). Upon ultracentrifugation to isolate the LDL, the size change was no longer observed when incubated with PC vesicles. This proves that a protein factor in the plasma must mediate this process.

Similar incubations of isolated LDL and PC vesicles or cholesterol ester-phospholipid microemulsions (CEME) have produced aggregation of the LDL with the particles (19,20). Hunter, Shahrokh, Forte, and Nichols (19) noticed turbidity in the LDL/PC vesicle incubation along with an extreme increase in the phospholipid/protein ratio. They also discovered that upon tryptic digest, the LDL aggregation did not occur showing a connection with apoB. The incubations of LDL and CEME produced particles with intermediate density and electrophoretic mobility to those of LDL or CEME alone; hence, leading to the conclusion of LDL/CEME aggregation. Also, this experiment revealed that the

addition of apoA-I, the major protein in HDL, inhibited the fusion of CEME and LDL. A possible conclusion to these two experiments as outlined by Parks, Martin, Johnson, and Rudel (20) states that apoB and apoA-I interactions may be responsible for LDL particle integrity.

Modifications of apoB are also important in determining structural and functional properties of LDL in addition to those of the protein. When carboxamidomethylated, tryptic digests of apoB were incubated with dimyristoyl phosphatidylcholine (DMPC) liposomes, lipid-peptide complexes were formed (21). After isolation of these complexes by ultracentrifugation, they were shown to be thermally stable and more rigid in structure than native LDL. The α-helices and β-pleated sheets underwent extreme changes with an increase in the α-helices and a decrease in the β-pleated sheets. Also, this lipid-peptide complex did not bind to the LDL receptor.

Finally, chemical modifications on lipoprotein B and apolipoprotein B were used to analyze the structure of this protein. Singh and Lee (22) revealed several interesting features about apolipoprotein B. First of all, they found that upon storage of lipoprotein B the secondary structure of the protein changed even though preventive measures for peroxidation and degradation were taken. Second, apoB in the presence of 6M guanidine·HCl caused the disappearance of the α-helical structure and an increase in the number of β-sheets with 6M urea restoring some of the helical structure and aqueous buffer restoring the structure to its normal state. Third, carboxymethylated and reduced-carboxymethylated apoB showed no changes in secondary structure. The final observation showed no change in the structure

of apoB with a pH variance from 2.7 to 9.0. Their major conclusions from these results state that apoB is less stable to pH changes in the presence of lipids, that disulfide bonds are insignificant on the conformation of apoB and that the α-helical changes due to delipidation and denaturation on lipoprotein B are reversible.

A more recent investigation of lipid transfer activity involving lipoproteins contains the background information for the work in this paper. The investigation examined the incubation of reconstituted high density lipoprotein discs (r-HDL) containing egg-PC or DPPC with apoA-I and cholesterol with low density lipoproteins (LDL) (23). These incubations took place in the presence and absence of LCAT and phospholipid transfer protein (PLTP). The compositional and particle sizes of the r-HDL were determined. However, with more relevance to this work, the compositional changes of LDL were determined. A spontaneous transfer of phospholipids from r-HDL into LDL was noted with a half-time from 5.8 to 6.9 hours. This reaction was further facilitated with the presence of PLTP increasing the rate of transfer four-fold. When LCAT was added to this incubation the composition of LDL returned to normal with a decrease in the phospholipid and free cholesterol and an increase in the cholesteryl ester content.

During the above investigation a few slow reactions surfaced. One of these reactions seemed to be a deesterification of the cholesteryl esters within the LDL. Since this compositional change was not observed in the LDL control, the r-HDL must be relevant to the process. This work attempts to

determine the agent responsible for this reaction--r-HDL, phospholipid, or apoA-I. LDL was incubated with egg-PC vesicles and apoA-I, separately. Analyzing the composition of LDL after these incubations would determine if the phospholipid or apoA-I contained within the r-HDL is the plausible agent or whether the reconstituted particle itself is.

EXPERIMENTAL

A. Materials

Plasma used for the LDL and apoA-I preparations was generously given by the Champaign County Blood Bank. ApoA-I was isolated by the method of Edelstein et al. (24). The dialysis tubing used in the LDL preparation was Spectrapor 4 with a molecular weight cut-off of 12,000-14,000 obtained from American Scientific Products, Division of American Hospital Supply Corporation, McGraw Park, IL. The reagent used in the Lowry protein assay, Phenol Reagent Solution, 2N was obtained from Fisher Scientific Company, Orangeburg, NY. Bovine serum albumin, cholesterol oxidase, horseradish peroxidase, cholesterol, cholesterol oleate, and egg phosphatidylcholine (egg-PC) were obtained from Sigma Chemical Company, St. Louis, MO. P-hydroxyphenylacetic acid was obtained from Aldrich Chemical Company, Incorporated, Milwaukee, WI, while the cholesterol esterase used in the enzymatic total cholesterol assay came from Calbiochem, Behring Diagnostics, LaJolla, CA. A High Performance Total Cholesterol Assay was obtained from Boehringer Mannheim, Indianapolis, IN.

B. Instruments

A Beckman Ultracentrifuge L8-70 with a Ti 55.2 rotor was used for LDL preparation. Absorbance values were read on a Beckman Model 35 Spectrophotometer. For the free and total enzymatic cholesterol assays a Perkin-Elmer MPF-66 Fluorescence Spectrophotometer was used. Preparation of

phospholipid vesicles by sonication was performed with a Heat Systems Ultrasonic Processor.

C. Methods

1. LDL Preparation

LDL from human plasma was isolated by ultracentrifugation by flotation at the appropriate densities (25). Plasma was adjusted with NaCl to a density of 1.019 g/ml and spun for 24 hours at 10°C and 50,000 rpm. After the spin, the top portion containing mainly VLDL, LDL, and chylomicrons was discarded. The bottom portion was adjusted to a density of 1.063 g/ml with NaCl and was spun again under the same conditions. The LDL was then isolated from the top portion of the tube. A final spin at 1.063 g/ml was then used to wash the LDL. Following these steps, the LDL was dialyzed against buffer composed of 10 mM Tris HCl, 150 mM NaCl, 1 mM NaN_3 and 0.01% EDTA at pH 8.0, and stored under N_2 at 4°C.

2. Egg-PC Vesicle Preparation

A measured amount of egg-PC, equal to 0.400 ml, was dried under N_2. Then 5 ml of buffer previously described was added to make a concentration of 10 mg/ml. This concentration of egg-PC was then sonicated five times for three minutes with a one to two minute interval between the sonications.

3. ApoA-I Preparation

ApoA-I was prepared by the Edelstein et al. (24) method. The first step involved isolating HDL from plasma. An appropriate amount of NaCl was added to the plasma to give a density of 1.063 g/ml and was centrifuged using

a Ti 55.2 rotor at 15°C and 50,000 rpm for 24 hours for separation. The top layer was removed and discarded. Another spin with added NaBr took place at a density of 1.21 g/ml to finally isolate the HDL. The HDL was then dialyzed in Spectrapor 4 tubing against 10 mM Tris, EDTA/Azide (No salt) in a four liter flask with five buffer volumes for nine hours. The next step involved delipidation. HDL was slowly added to polypropylene bottles containing cold ethanol:diethylether 1:3, v/v and was stored overnight at 0°C. It was then spun in a centrifuge at 8-10,000 rpm at 5°C for 30 min. Then three ether washes were spun under the same conditions. Next the protein sample was dissolved in fresh 3 M Guanidine Tris buffer pH 8.2 and applied to a Sephacryl S-200 column. Finally, the pooled fractions containing apoA-I were dialyzed against five volumes of 0.01% EDTA for 6-8 hours.

4. Incubations of LDL with ApoA-I or Egg-PC Vesicles

The LDL was heat inactivated at 55-60°C for one hour prior to the incubations. These incubations were carried out under N_2 for differing time intervals in a shaking water bath at 37°C. The incubations with apoA-I contained 0.5 mg of apoA-I protein to 0.5 mg of LDL phospholipid in a total volume of 0.640 ml and were incubated for 0, 2, 6, and 18 hours. The egg-PC vesicle incubations were carried out for 0 and 16 hours with different ratios of LDL/egg-PC. These included 2.0 mg of LDL protein to 0.5, 1.0, 2.0, and 4.0 mg of egg-PC in a total volume of 1.122 ml. Both of the above experiments contained controls of LDL incubated at the same time intervals.

5. Protein Determination

LDL protein concentrations were determined by the Lowry et al. (26) method as modified by Markwell et al. (27). Bovine serum albumin was used as the standard for the procedure.

6. Phosphate Determination

The phosphate concentration in the incubations was determined using the Chen et al. (28) method. A standard curve using an inorganic phosphate solution was constructed allowing the phosphate values of the incubations to be calculated.

7. Free and Total Cholesterol Determination

Initially, an enzymatic assay for free and total cholesterol developed by Heider and Boyett (29) and modified by Gamble, Vaughan, Kerth, and Avigan (30) was used. This involved preparation of a reagent containing phosphate buffer, cholesterol oxidase, horseradish peroxidase, and P-hydroxyphenylacetic acid with an addition of cholesterol esterase for the total cholesterol assay only. The standards used were free cholesterol and cholesterol oleate for the free and total cholesterol assays, respectively. The samples and standards plus the added reagent were inculated at 37°C with the incubation time for the free cholesterol assay being 30 minutes and that for the total cholesterol assay 60 minutes. Fluorescence measurements were then made within two hours of incubation with an excitation wavelength of 325 nm and an emission wavelength of 415 nm. A total cholesterol assay from Boehringer Mannheim was used to compare the results with the fluorometric assay above.

RESULTS AND DISCUSSION

A. Evaluation of Total Cholesterol Assay

The total cholesterol assay used in evaluating the incubation mixtures was an enzymatic assay developed by Heider and Boyett (29) and modified by Gamble, Vaughan, Kerth, and Avigan (30). As previously described this assay involved preparing a reagent containing phosphate buffer, cholesterol oxidase, horseradish peroxidase, cholesterol esterase, and p-hydroxyphenylacetic acid. The sequence of events in this reaction mixture involved, first of all, the cholesterol esterase hydrolyzing the cholesteryl ester bond. The free cholesterol would then release peroxide (H_2O_2) and choles-4-en-3-onl upon reaction with the enzyme cholesterol oxidase. The final step involved the production of a fluorescent product due to the effect of horseradish peroxidase on peroxide and p-hydroxyphenylacetic acid. The fluorescence was then easily measured using an excitation wavelength of 325 nm and an emission wavelength of 415 nm.

When evaluating the percent composition of LDL through chemical assays, the values for the cholesteryl esters were found to differ significantly from the accepted values. Table 1 lists the percent composition for LDL protein, phospholipid, cholesterol, and cholesteryl esters including the accepted values by Shen et al. (1) and my own. All of the values vary to a certain degree. There are possibly two main reasons for this. First, the difference in values could be due to experimental error in my technique. Second, LDL is known to be heterogeneous in composition in the population as well as the

individual. Since the LDL was usually isolated from plasma belonging to one individual, it is not a representative pool. The accepted percent composition of cholesteryl esters in LDL is approximately 40% by weight. The results of my assays ranged from 20.4-24.1%. This is very significant difference since it has been shown that cholesteryl esters are the major lipid in LDL.

Along with the total cholesterol assay, an assay measuring free cholesterol using the same reagent minus the cholesterol esterase was used. The results from the assay seemed accurate. This revealed that a possible cause for the difference in cholesteryl ester composition was the cholesterol esterase. However, in the analysis the standard curve using cholesterol oleate as the standard was a linear, reproducible curve. This may suggest that the enzyme itself was not the problem but perhaps it was the accessibility of the enzyme to the cholesteryl esters located in the core of the LDL. The assay did call for a solution of sodium cholate:Triton X-100: absolute alcohol (1:1:2), but this may not be effective in breaking up the LDL particle and revealing its core constituents.

A total cholesterol kit from Boehringer Mannheim which is a colorimetric assay was used to compare its values with those of the fluorometric assay. Table 2 shows that there is a significant increase in the total cholesterol concentration using the kit proving that the fluorometric assay is not accurate. However, time did not permit the experiments to be repeated using the kit to analyze the cholesteryl ester content, so the cholesterol compositional analysis is based on the fluorometric assay for free cholesterol only since it is reproducible and reliable.

B. Compositional Analysis of LDL Incubated with ApoA-I

An incubation of LDL containing 0.5 mg of phospholipid and 0.5 mg of apoA-I was carried out at 37°C for different time periods including 0, 2, 6, and 18 hours. LDL alone was also incubated at 37°C for the same time periods as a control. The reaction mixtures were analyzed for protein, phospholipid, and cholesterol concentrations to see if there was any change in the composition of the LDL in this incubation. The Lowry protein determination as shown in Table 3 revealed that the protein concentration within the LDL control and within the LDL/apoA-I incubation remained constant at approximately 0.962 mg/ml and 1.87 mg/ml, respectively. These values are different since the apoA-I adds more protein to the incubation. The phospholipid concentration remained the same for all eight incubations at an approximate value of 0.773 mg/ml. Most importantly the free cholesterol values remained constant in the LDL control with increasing incubation times as well as in the LDL/apoA-I incubations. In fact, the values for the separate incubations did not vary significantly with an average concentration being 0.534 mg/ml which is shown graphically in Figure 1. Therefore, the composition of LDL is not changing upon incubation with the apoA-I.

C. Compositional Analysis of LDL Incubated with Egg-PC Vesicles

A similar incubation containing LDL and egg-PC vesicles was also done. However, for these incubations varying amounts of the egg-PC vesicles were incubated with LDL for the same time period (16 hr) to determine whether a compositional change would occur in LDL in this reaction and to find the

proper ratio of LDL/egg-PC vesicles. In the incubations 2 mg of LDL protein to 0.5, 1.0, 2.0, and 4.0 mg of egg-PC vesicles were incubated for 0 and 16 hours at 37°C. LDL incubated alone for 0 and 16 hours was also included as a control. The results shown in Table 4 included a slight increase in the protein content since PC will contribute slightly to the protein determination. The cholesterol concentration remained the same at approximately 0.985 mg/ml within the LDL control and the incubations of LDL and egg-PC vesicles together. Figure 2 shows a much easier presentation of the unchanging cholesterol content. This indicates that the incubations between LDL and egg-PC vesicles also do not cause compositional changes in the LDL particle.

D. Concluding Remarks

Clearly the results from the above incubations show that neither apoA-I nor the addition of egg-PC vesicles to LDL caused a change in the LDL composition. However, this conclusion is based solely on the free cholesterol concentration and not on the cholesteryl ester concentration due to the unreliability of the total cholesterol assay. This leads to an accurate but rather incomplete compositional analysis. The ideal situation would be to repeat the incubations and use the Boehringer Mannheim total cholesterol kit to analyze the cholesteryl ester concentration.

Since neither the apoA-I nor the egg-PC vesicles caused the LDL composition to change, it must be that LDL only changes in composition upon incubation with the intact r-HDL particle. The apparent cholesteryl ester

hydrolysis seen by Jonas et al. (23) in the LDL incubated with r-HDL must depend on the interaction between LDL and the intact r-HDL.

A possible mechanism for this cholesteryl ester hydrolysis may lie within the apoB in the LDL particle itself with the r-HDL possibly acting as a catalyst. As was described previously apoB is a huge, insoluble protein of which little is known except that it contains the recognition site for the LDL receptor. ApoB was shown to have a protease activity that produced at least four fragments that have been found within the LDL. Perhaps one of these cleaved fragments contains an apparent cholesterol esterase activity. The active site for esterases has been shown to exhibit the amino acid sequence of glycine-variable amino acid-active serine-variable amino acid-glycine (Gly-X-Ser-X-Gly) as was located in LCAT (31). The complete cDNA and amino acid sequence of apoB-100 deduced by Chen et al. (9) however does not show this sequence. This still does not rule out a possible esterase activity.

Also found within the apoB structure by Huang, Lee, and Singh (4) is the presence of labile thiol ester linked palmitate and stearate. This could be important to this work since Juahiainen and Dolphin (32) have proposed a mechanism for forming cholesteryl ester from cholesterol and lecithin by LCAT that involves a thiol ester bond as an intermediate. Their mechanism involves first the transfer of the sn-2 fatty acid of lecithin to the serine in the active site of LCAT forming lysolecithin. Next, the fatty acid is transferred to a cysteine from the serine forming a thiol ester bond. Finally, the fatty

acid in the thiol ester bond is transferred to cholesterol forming a cholesteryl ester. Perhaps a thiol ester bond in apoB is an intermediate in the hydrolysis of cholesteryl esters.

This cholesteryl ester hydrolysis in LDL is clearly important since LDL with different compositions have been isolated and correlated positively and negatively with atherosclerosis. Therefore, future experiments are necessary to reveal the mechanism of this spontaneous hydrolysis. Two possible experiments include tryptic digest and chemical modifications of apoB on the LDL and subsequent incubation with r-HDL.

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TABLE 1. Percent Composition Of LDL

	Protein	Phospholipid	Cholesterol	Cholesteryl Ester
Accepted (1)	29%	21%	9%	40%
LDL*	34.0%	27.8%	18.5%	20.4%
LDL*	29.3%	29.6%	16.9%	24.1%

The sum of the mass of the above components was taken as 100% in each case to calculate the percent composition.

*This LDL represents the compositional analysis that was done in this work using the fluorometric assay for total cholesterol.

TABLE 2. Comparison Of Total Cholesterol Assays

	Fluorometric Assay	Boehringer Mannheim Kit
Total Cholesterol (mg/ml)	2.05*	4.33**
	1.99*	4.22**

*LDL control used in egg-PC experiment which was incubated at 37°C for 0 and 16 hours, respectively.

**The same LDL as above stored at -16.8°C.

TABLE 3. Composition Of LDL After Incubation With ApoA-I At 37°C

Incubation Components	Incubation Time (hrs)	Protein (mg/ml)	Cholesterol (mg/ml)
LDL	0	0.954	0.548
LDL	2	0.950	0.526
LDL	6	0.966	0.503
LDL	18	0.980	0.516
LDL + ApoA-I	0	1.92	0.493
LDL + ApoA-I	2	1.84	0.569
LDL + ApoA-I	6	1.87	0.549
LDL + ApoA-I	18	1.86	0.566

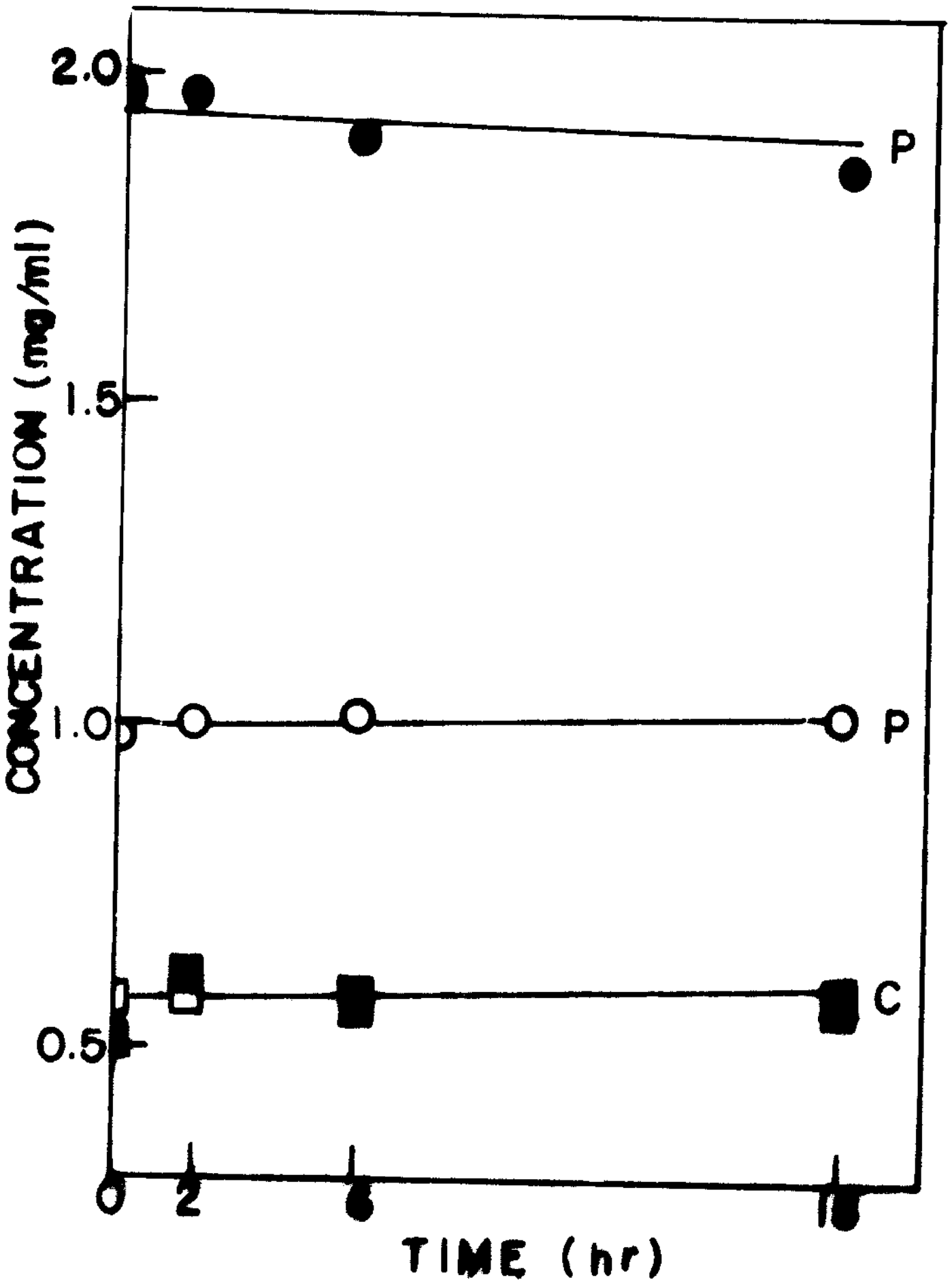
TABLE 4. Composition Of LDL After Incubation With Egg-PC Vesicles At 37°C

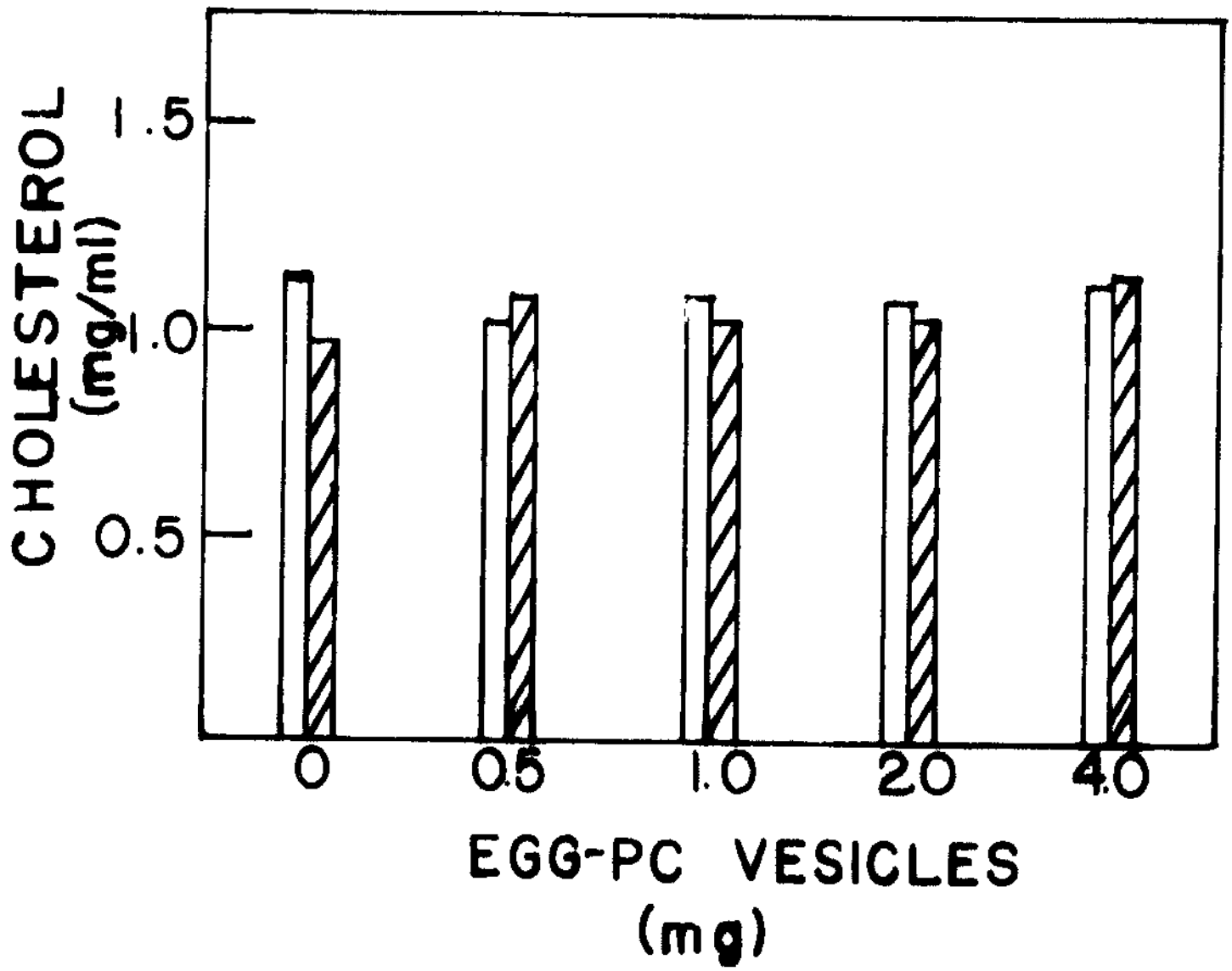
Incubation Components	Incubation Time (hrs)	Protein (mg/ml)	Cholesterol (mg/ml)
LDL	0	1.14	1.01
LDL	16	1.17	0.807
LDL + 0.5 mg Egg-PC	0	1.15	0.873
LDL + 0.5 mg Egg-PC	16	1.18	0.970
LDL + 1.0 mg Egg-PC	0	1.20	0.981
LDL + 1.0 mg Egg-PC	16	1.22	0.884
LDL + 2.0 mg Egg-PC	0	1.24	1.00
LDL + 2.0 mg Egg-PC	16	1.29	0.966
LDL + 4.0 mg Egg-PC	0	1.36	1.16
LDL + 4.0 mg Egg-PC	16	1.35	1.20

FIGURE LEGENDS

Figure 1 Composition changes, as a function of time, of LDL incubated with (filled symbols) and without (open symbols) apoA-I. The cholesterol (□,■) and protein (○,●) values are normalized to 0.807 mg/ml of phospholipid since the phospholipid concentration remained constant throughout the incubations. These incubations took place at 37°C with 0.5 mg of LDL phospholipid and 0.5 mg of apoA-I protein.

Figure 2 Changes in cholesterol concentration upon incubation with various amounts of egg-PC vesicles. Incubations containing 2.0 mg of LDL protein and either 0, 0.5, 1.0, 2.0, or 4.0 mg of egg-PC took place at 37°C for 0 hours (plain bars) or 16 hours (slashed bars). These cholesterol values are normalized to 1.36 mg/ml of protein.


2.0
1.5
1.0
0.5
P
P
C
0
2
6
18
CONCENTRATION (mg/ml)
TIME (hr)


CHOLESTEROL (mg/ml)
1.5
1.0
0.5
0
0.5
1.0
2.0
4.0
EGG-PC VESICLES (mg)