FEASIBILITY OF THE USE OF CAPILLARY ELECTROPHORESIS FOR THE STUDY OF VLDL ASSEMBLY INTERMEDIATES

A Thesis

by

ELIZABETH ANNE WHITE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2005

Major Subject: Nutrition

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Approved as to style and content by:

Rosemary Walzem (Chair of Committee)	J. Martyn Gunn (Member)
Ronald Macfarlane (Member)	Nancy Turner (Chair of Nutrition Faculty)

Alan Sams
(Head of Department)

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ABSTRACT

Feasibility of the Use of Capillary Electrophoresis for the Study of VLDL Assembly Intermediates. (May 2005)

Elizabeth Anne White, B.S., University of Arizona

Chair of Advisory Committee: Dr. Rosemary Walzem

The chicken has long been a model used for the study of plasma lipoproteins due to the ability to increase VLDL production by administration of estrogen. In this study we were able to demonstrate successful isolation of VLDL assembly intermediates from the livers of hens, roosters, and estrogen treated rosters. Particle diameter of first step particles, as determined by dynamic laser light scattering, was decreased from an average diameter of 31.5 nm in untreated birds, to 16.1 nm 12 hours after estrogen treatment. Effects of estrogen waned after 24 hours and particle diameter of first step particles increased to an average of 23.9 nm. These assembly intermediates, as well as plasma VLDL and VLDLy, were successfully studied using capillary electrophoresis (CE). Effective mobilities of intact plasma VLDL and first step particles decreased after estrogen administration. Hen VLDL showed a single uniform peak whereas rooster VLDL separated into distinct "subclasses". Delipidated VLDL, VLDLy and first step assembly intermediates were also successfully separated using CE.

This thesis is dedicated to my family who always encouraged me through this process.

ACKNOWLEDGEMENTS

First of all I would like to thank God for his absolute faithfulness. He is Jehovah Jireh.

Thank you to my mom, dad, sister and the rest of my family back home who have always loved and supported me. Thank you for putting up with all my whining and encouraging me on those days when I never thought I would finish. Thanks to Naomi and YeeVoon whose friendship made the move to Texas and graduate school a lot more enjoyable. I miss you both. Thank you to my new family here in Texas; their presence and prayers are invaluable.

Thank you to my fellow graduate students, Limin, Chen and Vidya who taught me so much and helped me as I worked long distance. Thanks to Dr. Macfarlane for allowing me to use his laboratory and welcoming me as one of his own students. Thanks to the students from his lab, Christi, Richa, Leti and Sandy for giving up their time to help me. Thank you to Dr. Robert Hamilton for his assistance with the electron microscopy used in this study. Finally, I would like to thank Dr. Walzem for her guidance, patience and hospitality through this entire process.

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CHAPTER I

INTRODUCTION

Lipoproteins are essential to the transport of lipid and lipophilic molecules throughout the body. Their structure, which consists of a triglyceride rich core surrounded by a phospholipid and protein coat, facilitates movement of hydrophobic lipids in the blood. Lipoproteins are classified based on their density and on the location of their assembly. Many decades of research have provided extensive knowledge of the circulating forms of lipoproteins which include chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Each of these classes of lipoproteins have unique density characteristics and density gradient ultracentrifugation is a commonly used method to study these particles [1, 2]. Other analytical techniques such as gel electrophoresis and capillary electrophoresis have also been used to further characterize these particles [3, 4]. Chickens have a unique class of lipoproteins, termed VLDLy. Synthesis of VLDLy is induced by estrogen [5, 6]. This unique characteristic of avian species has led to the use of chickens as a model for the study of triglyceride rich lipoproteins.

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This thesis follows the style and format of the *Journal of Lipid Research.*

VLDL are triglyceride rich lipoproteins which are synthesized in the liver. Up to this point, the process leading to the assembly and secretion of VLDL has not been well studied. It has been proposed that VLDL assembly occurs in a two step process within the rough and smooth endoplasmic reticulum of hepatocytes [7, 8]. VLDL assembly intermediates have been visualized and isolated however, very little is known about the physical and chemical characteristics of these particles [8].

CHAPTER II

REVIEW OF LITERATURE

Lipoproteins

Lipoproteins are amphiphilic particles synthesized by the liver and intestine for the transport of lipids and lipophilic molecules throughout the body. The general structure of lipoproteins consists of a triglyceride (TG) rich core surrounded by a coat of protein, phospholipid and cholesterol. This coat allows the lipophilic molecules to travel in the aqueous environment of the blood. Lipoproteins are classified based on the density of the core and the location of their assembly. The classes, in birds, are: portomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL) and high density lipoproteins (HDL) [9].

Intestinal absorption of lipids in birds is accomplished via the portal circulation with subsequent uptake into the liver. Dietary triglycerides and other lipophilic molecules are assembled into portomicrons prior to direct secretion into the portal vein [10]. Triglyceride within the portomicron is hydrolyzed by lipoprotein lipase as it travels through the circulation. The portomicron remnant is then taken into hepatocytes and degraded. Upon entry into hepatocytes remnant lipids mix with triglyceride and cholesterol synthesized de novo by the liver to form what is termed the endogenous lipid pool. The endogenous lipid pool can then be assembled into VLDL.

After VLDL is assembled in the liver, it is then secreted into the circulation. As the TG in the VLDL particle is hydrolyzed by lipoprotein lipase the particle diameter

decreases and transforms first into a more dense IDL. The IDL particle can be further hydrolyzed to form LDL [9]. VLDL and its metabolites, IDL and LDL, are essential for the transport of fatty acids, cholesterol and fat-soluble vitamins throughout the body.

Apolipoproteins are those proteins found in the lipoprotein's outer coat. Because these molecules are amphipathic they are able to associate with neutral lipids and phospholipids within the aqueous environment of the bloodstream. Apolipoproteins contribute to the lipoprotein's structure and stability. Apolipoproteins also serve as ligands for receptors and co activators for enzymes as well as possessing independent catalytic and lipid transfer activity. Apolipoproteins vary in both molecular weight and physical properties such as isoelectric point, pI (Table 1). Apolipoproteins are named using the abbreviation apo in conjunction with letter and Roman numeral combinations.

Apolipoprotein B (apo B) and apolipoprotein A-I (apo A-I) are considered the primary structural apolipoproteins of triglyceride-rich (Portomicrons, VLDL, IDL and LDL) and HDL, respectively. While sequence variation exists, Apo B and Apo A-I are common to all vertebrates. Human serum lipoproteins also contain smaller apolipoproteins not found in all species. Examples of such apolipoproteins include apo A-II, apo E, apo D and the apo C proteins (-I, II and III).

Table 1
Approximate molecular weight (MW) and theoretical isoelectric points (pI) of various apolipoproteins found in avian species. Calculations of theoretical pI were done with ExPASy [11].

Apolipoprotein	Approximate MW (kDa)	Theoretical pI
Apo B	550	6.59
Apo A-I	28	5.26
Apo C-I	6.6	7.93
Apo VLDL II	9.3	9.21

The major structural protein of VLDL is Apo B. In mammals Apo B has two common forms, a full-length 550 kDa form termed Apo B_{100} and a shorter form created by RNA editing termed Apo B_{48} because it is about 48% as long as Apo B_{100} [12, 13]. Chickens do not make Apo B_{48} [14]. Because Apo B is amphipathic, it has hydrophilic regions that interact with polar surface lipids, as well as many hydrophobic regions that interact with core lipids. The amphipathic nature of Apo B is essential to its function in maintaining lipoprotein structure and stability. The sequence of apo B, specifically the lipophilic regions, is highly conserved across species [15]. Although the smaller apolipoproteins also have specific functions they are not necessary for the structure of the lipoprotein and are easily exchanged between particles. Apo B, however, is an integral structural protein for VLDL, IDL and LDL.

Apolipoprotein A-I is the defining structural protein for HDL, however, "assembly" of this particle occurs in peripheral tissues. The non-structural function of apo A-I is to activate lecithin-cholesterol acyltransferase (LCAT) [16]. LCAT is

necessary for the esterification of cholesterol in circulating HDL particles. Due to LCAT activity, HDL particles are said to mature, as synthesized cholesterol ester locates in the particle's core causing it to increase in diameter. Apolipoproteins similar to the Apo Cs have been found in avian species. Apo C-II is an activator of lipoprotein lipase and a similar protein is found on the VLDL of chickens [17].

VLDLy

Distinctive differences are found in VLDLy as compared to VLDL. These differences develop in response to elevations in plasma estrogen in the laying hen. Both physical and chemical changes occur in hepatic VLDL assembly processes in order to form VLDLy, whose metabolic fate differs from VLDL. VLDLy are resistant to hydrolysis by lipoprotein lipase and this facilitates deposition of TG into the developing egg yolk [18, 19]. The average diameter of VLDLy is decreased from the typical 60 nm of nonlaying hens, to 30 nm in a laying hen (Figure 1) [20]. The VLDLy particles are also more uniform in diameter than are generic VLDL.

The physical and chemical properties of VLDLy are essential to its function. Egg yolk must provide all of the energy requirements for the developing embryo. Most of the egg yolk is composed of TG in the form of VLDLy. Circulating VLDL must be able to support the high demand for TG during egg laying. A hen's ovary has thousands of oocytes [21]. At any one time only a few of these oocytes accumulate TG and developing into mature ova. Several layers of tissue separate the circulating VLDLy from the developing oocytes [21]. Approximately 5g of VLDLy-TG must be transferred from the circulation into the developing follicles each day [22].

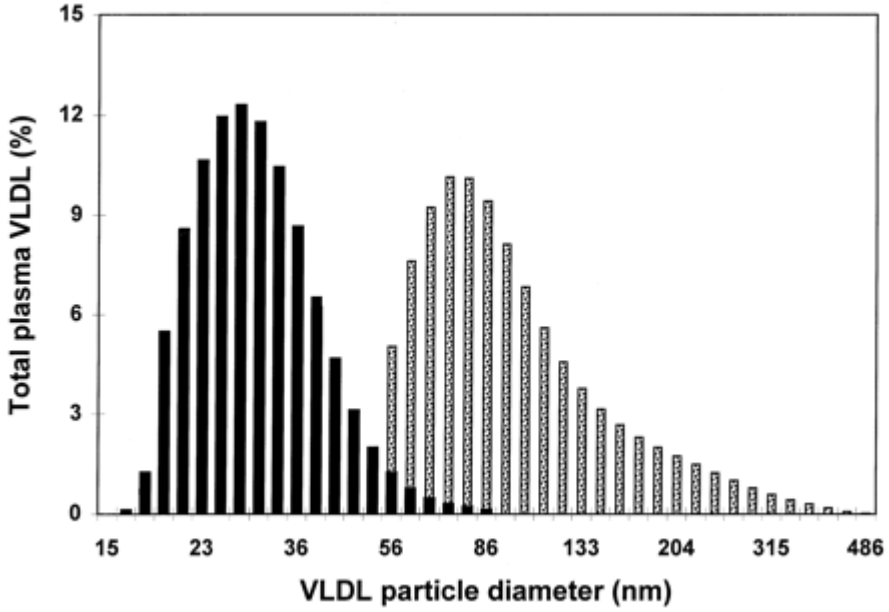

Fig. 1. Particle size distribution of plasma VLDL from adult roosters (stippled bars) and adult egg-laying hens (solid bars). Particle size determined by dynamic laser light scattering. Figure used with permission from the American Society of Nutritional Sciences: Walzem, R.L. Estrogen Induction of VLDLy assembly in egg-laying hens. *Journal of Nutrition*, **129**: 67s-472s [6].

Decreased particle diameter is essential for yolk deposition as the follicle is size selective. Using electron microscopy, Perry *et al.* concluded that only lipoproteins below 52 nm in diameter are able to pass through the various layers of connective and steroidogenic tissues that comprise the follicle wall. For this reason lipoproteins of intestinal origin, portomicrons which are generally much larger in diameter, are excluded from the yolk [22]. Although particle diameter is the primary determinant, there is also a difference in charge between the apolipoproteins and the proteins of the basal lamina which may contribute to the movement of VLDL across this membrane [22].

Lipase resistance is also necessary for the function of VLDLy. If lipoprotein lipase were to act on VLDLy it would become depleted of TG. As a result TG-deficient

yolk would form which would be unable to meet the energy requirements of the incubating embryo [19]. Apo VLDL II is responsible for the lipase resistance of the VLDLy particle [23].

Egg yolk deposition occurs by receptor-mediated endocytosis. Both apo B and apo VLDL II are integral VLDLy apolipoproteins, that is, neither are exchanged between particles. Early on it was thought that role of VLDL II was to serve as the ligand for these receptors. However, Nimpf *et al.* demonstrated that VLDL II is not the ligand [24], and it was later discovered that Apo B is the receptor ligand [25]. Although apo VLDL II is not required for endocytosis of VLDLy it is still essential for egg yolk deposition as the presence of apo VLDL II inhibits the action of lipoprotein lipase on VLDLy [23]. As apo VLDL II only appears after estrogen induction it is also possible that this protein leads to the decreased initial diameter of the VLDLy particles.

In addition to changes in the physical and functional properties of VLDL, estrogen induces an overall increase in the production apo B-containing lipoproteins in the form of VLDLy. Upregulation of VLDL synthesis can be induced in immature hens or roosters through the injection of exogenous estrogen [5]. As would be expected with such an increase in plasma VLDL there is a subsequent increase in total plasma TG [26]. This massive upregulation in VLDL synthesis provides an opportune setting to study the processes involved in the assembly and secretion of VLDL particles.

Effects of Estrogen on the Liver

The presence of estrogen directly stimulates the production of unique proteins: the vitellogenins and apo VLDL II, all of which play an important role in egg yolk

deposition. Apo VLDL II as an apolipoprotein unique to avian species. In the chicken Apo VLDL II is found as a homodimer with each subunit being 9 kDa [26]. In other avian species, such as quail, apo VLDL II is found as a monomer. Both the monomeric and dimeric forms effectively inhibit lipoprotein lipase [27]. Vitellogenins, although not physically associated with VLDL, also play a role in yolk formation and constitute a phospholipid rich very high density lipoprotein (d>1.195 g/mL) fraction in plasma [17].

Estrogen effectively increases synthesis of apo VLDL II by several mechanisms. First, estrogen directly induces transcription of apo VLDL II mRNA [28]. The presence of estrogen also increases the stability of apo VLDL II mRNA. This occurs by inducing the formation of a poly-adenosine tail which increases the half life of the mRNA [29]. The increased stability leads to an accumulation of VLDL II mRNA. For this reason VLDL II, and subsequently VLDLy, continue to be produced for an extended period of time, approximately 24 hours, after a single exposure to estrogen [5].

Assembly of VLDL

Although many individual events are required, the assembly of VLDL is proposed to occur in two broad steps. A series of physical observations led to this proposed mechanism. In 1976, Alexander *et al.* observed that within the rough endoplasmic reticulum (RER) there were regions that were richly immunopositive for apo B. However, the RER did not contain any particles matching the diameter criteria for VLDL. It was also noticed that there were regions of the smooth endoplasmic reticulum (SER) that contained large lipid filled particles of VLDL diameter that were not immunoreactive for apo B [8]. Based on these observations Alexander *et al.*

concluded that there were two separate VLDL assembly intermediates. The first of which being a small apo B containing particle, found in the RER. The second being a large lipid filled particle found in the SER which did not contain apo B.

Microsomal triglyceride transfer protein (MTP) is essential for the lipidation of apo B containing lipoproteins. MTP is a heterodimer consisting of subunits of 55kDa and 97 kDa. The smaller of these two subunits is an enzyme, protein disulphide isomerase. The larger subunit is unique to MTP. MTP is found exclusively within microsomes isolated from the liver and intestines [30]. MTP acts to shuttle lipid from the ER membrane to developing VLDL assembly intermediates. It has been shown that MTP preferentially transports hydrophobic lipid molecules namely TG and cholesterol esters, although it can transport more polar lipids as well [31].

The first step of VLDL assembly and secretion involves the synthesis of a small lipid-poor apo B-100 containing particle. In this first step lipid is added to apo B cotranslationally. As apo B is contranslationally translocated into the RER lumen, lipid is transferred by MTP and added into the core of the growing first step particle [30, 32]. Although apo B is lipidated contranslationally the bulk of the lipid is added during the second step.

The second step of VLDL assembly involves the synthesis of a large apo B-free, TG rich particle. These TG rich particles are thought to be synthesized within the smooth endoplasmic reticulum (SER) [8]. Formation of these TG rich particles is also dependent on MTP [30, 33]. These two particles, the small apo B containing first step particle and the large apo B free lipid droplet, somehow join to form the mature VLDL

[34-36]. Unlike formation of the two VLDL precursors, this final step in VLDL assembly is not MTP dependent; however the precise mediators involved in the final merging of the two VLDL precursors are unknown. Recently, Gilham *et al.*. proposed that particle fusion per se may not be involved and that TG-hydrolysis coupled to diacylglycerol acyl transferase (DGAT) activity may drive the process [37]. It is thought that the juncture of the RER and SER is where the two VLDL precursor particles join. Completion of particle remodeling to form mature VLDL occurs within the Golgi apparatus, prior to secretion into the Space of Disse.

Figure 2 provides a schematic for the proposed two step theory of VLDL assembly. The first event in step one involves the translation and concurrent tertiary folding, of apo B. As the protein folds a lipid binding cavity is created. During the second event of step one, MTP begins to deposit a small amount of triglyceride into the lipophilic cavity of apo B. Events 3 and 4 further depict the role of MTP in the transfer of lipid to the developing first step particle. After translation of apo B is complete the first step particle is released from the ribosome and the particle travels down the secretory pathway towards the SER, event 5. The first step particle then meets the large apo B free second step particle prior to formation of mature VLDL during the second step designated by event 6 [7]. Little information is available on the processes and events leading to the formation of the large apo B-free particle.

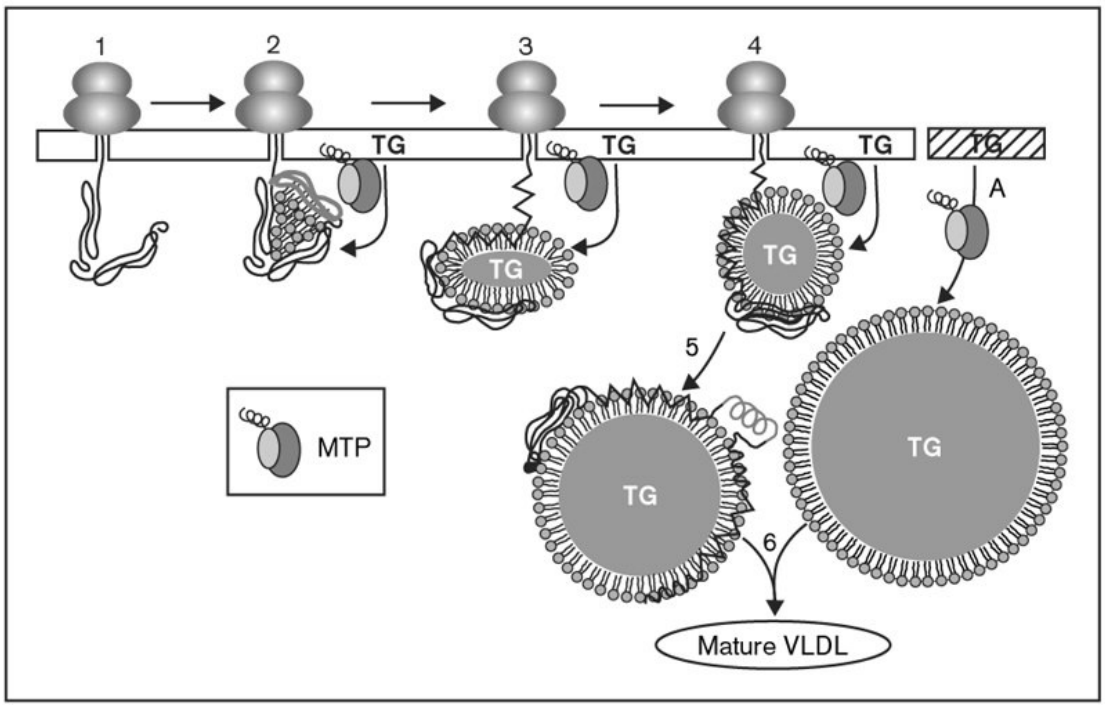

Fig. 2. Schematic of the two step model of VLDL assembly. Lipid is added to apo B cotranslationally, mediated by MTP. After being released from the ribosome the first step particle travels to the junction of the SER. It can then fuse with the large TG containing second step particle forming mature VLDL. Figure used with permission from Lippincott Williams & Wilkins: Shelness, G. Very-low-density lipoprotein assembly and secretion. *Current Opinion in Lipidology*. **12**: 151-157 [7].

Hamilton *et al.* developed a method of isolating RER that has allowed further study of VLDL assembly intermediates. This method involves calcium precipitation of the RER from whole liver homogenates [38]. Successful isolations of RER have been demonstrated in our laboratory with both hamsters and chickens using this or adaptations of this method. After RER isolation, VLDL assembly precursor particles can be released by membrane rupture with $NaCO_3$ and further isolated using density gradient ultracentrifugation.

Separation of Lipoproteins

Classes of lipoproteins can be separated by many methods. The most common method has been density gradient separation. The key to this technique is that the plasma is made to be more dense than the lipoproteins. Each lipoprotein class, VLDL, IDL, LDL and HDL has a characteristic density range. The density range for each class varies by species. The density distribution of lipoproteins shifts in avian species in response to estrogen, see Table 2. The laying hen demonstrates marked alterations in lipoprotein density when compared to estrogen naive birds. Very low density lipoproteins were found up to densities of 1.030 g/mL, overlapping with the IDL. Densities of HDL and LDL also overlap. These two lipoprotein classes were distinguishable based on apolipoprotein content with apo B being exclusive to LDL and apo AI being predominant on HDL [2, 17].

Table 2

Density profile for lipoproteins isolated from immature male chickens and laying hens which were separated by density gradient ultracentrifugation. Note the overlap of density ranges in the laying hen [2, 17].

Particle	Immature Male Density Range (g/mL)	Particle	Laying Hen Density Range (g/mL)
VLDL	<1.016	**VLDL/IDL**	<1.030
IDL	1.016 – 1.020		
LDL	1.020 – 1.046	**LDL/HDL**	1.030 – 1.158
HDL	>1.086		

Using suspending solutions of specific densities one can isolate the desired class of lipoproteins. Plasma is mixed with a suspending solution that is more dense than the desired lipoprotein class. After ultracentrifugation the desired lipoproteins will have floated to the top and can be easily collected. Fractionation can be done sequentially with suspending solutions of increasing density to isolate each lipoprotein class from a single plasma sample [39].

Density ultracentrifugation can be used as an analytical or a preparative technique. The use of density ultracentrifugation as a preparative method has allowed for further study of the chemical and physical properties of individual lipoprotein classes. This separation process has been integral to lipoprotein research for nearly 30 years. Much of the knowledge regarding lipoprotein structure and function has been made possible through the use of density ultracentrifugation.

Recent ultracentrifugation studies have focused on developing methods to observe individual variations in lipoprotein density profiles. Although the density ranges for the major classes of lipoproteins are well known there is much variability of lipoprotein subclasses between individuals. Individual variations in lipoprotein density profiles have implications for cardiovascular risk in humans. For example individuals with small dense LDL have an increased risk of developing cardiovascular disease [40].

Stability of Lipoproteins

Storage of isolated lipoproteins has long proposed a problem for researchers. Freezing is used widely for the preservation of biological samples. However, it was noted early on that the freeze-thaw process significantly alters the physical properties of

lipoproteins. Although the total cholesterol and triglyceride content of previously frozen samples is unchanged by freezing and thawing, physical changes have been observed in the lipoproteins themselves [41, 42]. In 1992, Rumsey, *et al.*, demonstrated successful cryopreservation of human LDL at -70°C with the addition of a 10% sucrose solution. Without addition of sucrose LDL particles aggregated during the freeze-thaw process. Those LDL frozen with sucrose remained in solution and did not aggregate. The LDL particles were analyzed using several methods including spectrophotometric analysis, column gel filtration and electron microscopy. Physical and chemical aspects of LDL frozen with 10% sucrose solution were similar to fresh samples [43, 44].

Capillary Electrophoresis

Capillary electrophoresis (CE) has been used to characterize and compare lipoproteins [3]. In general, CE separates ions based on charge density. The driving principle of this type of electrophoresis is that charged species will migrate at a specific rate, directly related to its charge and inversely related to its size, when exposed to an electrical potential. With CE, solutes migrate through a silica capillary tube exposed to an electrical potential gradient. The term electrophoretic migration is used to describe the movement of an electrolyte solution through a capillary that is exposed to an electric current. Capillary flow moves from the anode, the positive terminal, towards the cathode, the negative terminal. The silica-coated capillary carries a negative charge. When an electrolyte solution is introduced a bilayer is created by the formation of ionic bonds between the capillary coating and the cations of the electrolyte solution. This gives the inside of the capillary a positive charge. When a current is applied these

cations move towards the cathode pulling the electrolyte solution with them. The net movement of the electrolyte solution is determined by the strength of the current and the charged surface of the inside of the capillary. This is termed electroosmotic flow.

Since electroosmotic flow moves towards the cathode, positively charged ions will migrate more quickly, moving towards the negative terminal. Negatively charged particles will move more slowly. The velocity at which any given particle moves is the sum of the electroosmotic flow and the electrophoretic velocity. Electrophoretic velocity is proportional to the size of the ion and inversely proportional to the friction coefficient of the ion. For this reason molecules of similar size will migrate according to charge. Any charged species can be separated using this technique. The characteristics of the molecule itself are not the only determinants for the time it takes for a given ion to migrate through the capillary. Ion movement is also influenced by the length of the capillary, the ionic strength of the electrolyte solution and the applied voltage.

Capillary electrophoresis, when compared to more traditional slab electrophoresis, provides the means to achieve higher resolution separations. Increased resolution is possible because the capillary has an inner diameter of less than 100 μm and as a result radial diffusion is minimal. It also allows for high speed separations with very small sample volumes [45, 46].

There are many detection methods that can be used in conjunction with CE. The most common method of detection is absorbance of ultraviolet (UV) light. This type of detection is performed on column thereby allowing direct monitoring of individual separations. In order to monitor UV absorbance a small area of the capillary's protective

outer coating is removed to allow light of the desired wavelengths to pass through the column. UV absorbance is measured as the ions migrate past the capillary "window". This method can also be used to detect fluorescence. A more recent advance is the use of mass spectrometric detection of eluting ions. Electrospray mass spectrometry is commonly used and can be performed in conjunction with UV monitoring. This method is of particular interest because of it's applications to biochemistry and medicine [47, 48].

Graphic representations of CE elution profiles are termed electropherograms. An electropherogram is a plot of time vs. detector response. In the case of a UV detector, UV absorbance is plotted along the y-axis and time in minutes plotted along the x-axis. The effective mobility (μ_e) of individual analytes is calculated based on relative migration of the sample compared to that of a neutral marker. This marker is termed the electroosmotic flow marker (EOF). Effective mobility is calculated as $cm^2/v.s$ using the following equation:

$$\mu_e = \frac{L_c L_d}{V}\left(\frac{1}{t} - \frac{1}{t_{EOF}}\right)$$

where L_c is the total length of the capillary (cm), L_d is the length of the capillary from injector to detector (cm), V is the applied voltage (volts), t is the migration time of the sample (sec), and t_{EOF} is the migration time of the EOF marker (sec). Effective mobility of the EOF is zero. Any peak that appears before the EOF represents a cationic solute and has positive μ_e. Peaks appearing after the EOF represent anionic solutes and have negative μ_e.

Electropherograms can also be used to quantify components of a sample. This can be relative concentration comparing the areas under the peak(s) of interest. Or, if a known standard is available, peak areas can be compared to the standard solution at a given concentration.

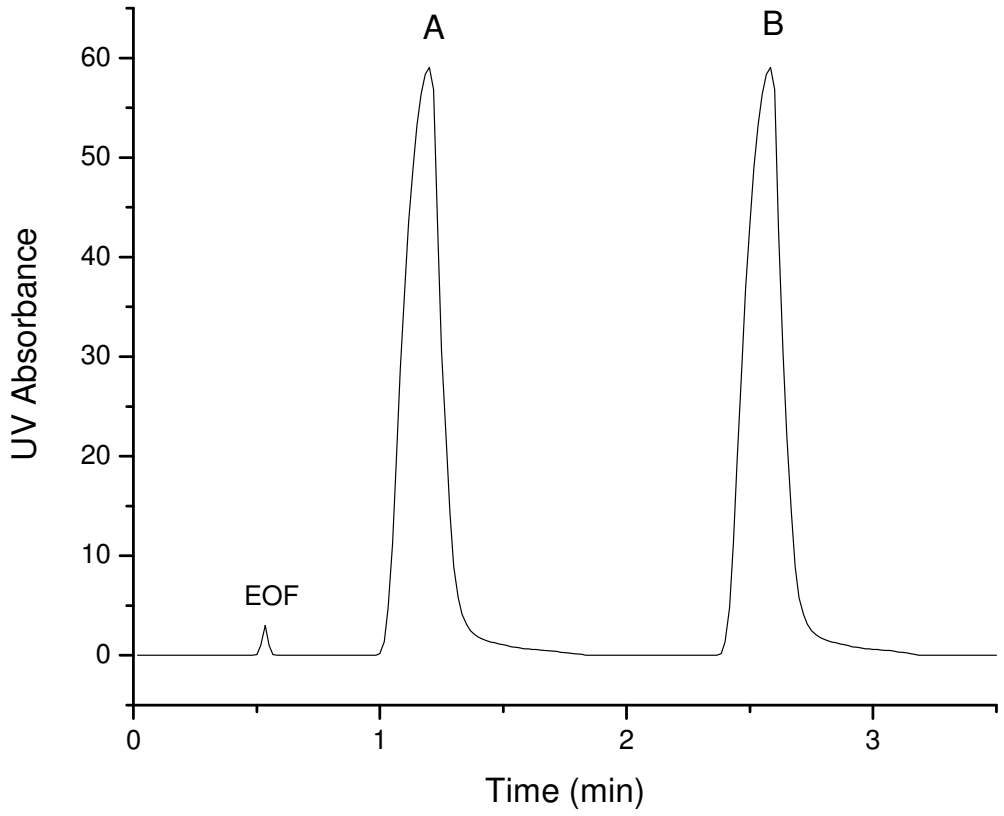

Fig. 3. Sample electropherogram of two compounds (A and B).

Figure 3 is a sample of an electropherogram. In this example both compounds appear after the EOF marker therefore they are both anionic and have negative μ_e. Compound A has a lower μ_e than compound B. Analytes with larger μ_e elute at a later

time. Large μ_e relates directly to larger charge density. In this example the electropherograms would be interpreted as compound B having a larger μ_e and therefore larger charge/mass than compound A. If the two compounds were known to have similar charge than this would indicate that compound B is smaller than compound A. If the two compounds are known to be of similar MW than this would indicate that compound B is more negatively charged than compound B. As the two peaks are of similar intensity it can be inferred that there are similar concentrations of the two compounds.

Capillary Electrophoresis of Lipoproteins

Capillary electrophoresis has been used to characterize and compare classes of lipoproteins as well as for characterization of apolipoproteins. Because lipids are neutral, and CE separates according to charge, lipoproteins migrate based on their charge density, or charge/volume ratio, which is primarily dictated by the apolipoproteins located in the phospholipid coat. Cruzado *et al.* described the movement of human HDL and LDL using this technique. Despite the larger diameter of LDL, both particles demonstrated approximately the same effective mobilities. This was thought to be related to the presence of apo B, a large, negatively charged protein, on the surface of the LDL [49].

Lipoproteins can be broken apart into their individual components using a detergent. By delipidating the lipoproteins individual apolipoproteins as well as lipids can be detected individually. Although the lipids are neutral the addition of a detergent, such as SDS, gives a negative charge to these molecules. Delipidation techniques have

been used to separate, identify and quantify apolipoproteins. Cruzado *et al.* demonstrated the use of CE in the quantification of apolipoproteins. Using SDS as a detergent they successfully separated and quantified both apo A-I and A-II from human HDL [3]. In 1998 the same laboratory successfully characterized and quantified apo B using similar techniques [50].

UV absorbance is typically measured over a range of wavelengths for any given sample. Analyzing the sample at specific wavelengths can provide information about the composition of the sample. Individual amino acids absorb at different wavelengths. The aromatic amino acids, tyrosine, tryptophan and phenylalanine, have the highest UV absorbance of all amino acids, at ~280 nm. For this reason, maximal UV absorbance of proteins occurs at this wavelength. Lipids typically absorb from 200 to 214 nm, due to the presence of carboxyl groups which absorb UV light in this range. When a lipoprotein profile is analyzed at a lower wavelength, such as 214 nm both lipid and protein components are detected in the sample. When the same sample is analyzed at 280 nm only the protein components are detected. This difference in absorption properties is useful when analyzing samples which contain high concentrations of lipids, such as VLDL. Analyzing these samples at 280 nm allow determination of peaks which represent protein components [48].

Many efforts to use CE in the analysis of lipoproteins are targeted at clinical applications, such as, creating an individual's lipoprotein profile. It has been used to separate lipoprotein classes and subclasses [51]. In vitro oxidation studies have shown that oxidation of LDL can be detected using CE[47]. This is of particular interest since

oxidation of LDL is thought to be a key event in the development of atherosclerosis [47, 52].

This study sought to determine the feasibility using CE for the study of VLDL, VLDLy and VLDL assembly intermediates. The use of CE would allow further characterization of the physical properties of these particles. Very little is known about the physical characteristics of first step particles, and isolation of these intermediates is labor intensive. The small sample volume required for CE makes it an appealing candidate method for the study of these particles. Previous studies in our collaborators laboratory have demonstrated profiling of individual human lipoprotein classes, successful delipidation of human lipoproteins as well as characterization and quantitation of apolipoproteins using CE [3, 50]. Using adaptations of these methods this study sought to increase our knowledge of VLDL, VLDLy and first step VLDL assembly intermediates. Increasing our knowledge of VLDL assembly intermediates will give us further insight into the VLDL assembly process.

CHAPTER III

OBJECTIVES

The first objective of this project was to demonstrate successful isolation of VLDL assembly intermediates from chicken liver and determine particle diameter distributions for these particles. The next objective was to establish conditions that will allow the use of CE to characterize these particles as well as plasma VLDL and VLDLy. Capillary electrophoresis allows characterization of these particles based on their overall charge density. If successful this would allow us to compare plasma VLDL to first step VLDL particles. We will also be able to determine differences in the charge densities of plasma VLDL and VLDLy. Comparisons of physical properties among lipoprotein particles at different stages in the assembly process under physiologically different settings can reveal important structural information about the products of biochemical processes.

We hypothesize that VLDLy particles will be more positively charged than VLDL particles due to the presence of a positively charged protein, VLDL II, pI 9.21. VLDLy also have decreased mass in comparison to VLDL. Overall these particles should have a lower charge:volume and therefore lower effective mobility.

Based on the two step theory of VLDL assembly Apo B is present on the first step VLDL particle. However, it is not known whether first step VLDLy particles also contain other apolipoproteins such as apo VLDL II or apo A-I. If either, or both, of these proteins are found on the first step particle it would affect the charge:volume. Apo A-I would create a more negatively charged particle. Again, Apo VLDL II, would

impart a more positive charge to the first step particle. Because overall VLDL secretion increases with estrogen induction, we would expect an increase in the area under the peak representing the VLDLy and first step VLDLy particles when compared to VLDL or first step VLDL respectively when similar volumes of similarly prepared materials are analyzed.

Our second objective was to attempt delipidation of plasma and first step VLDL and VLDLy using sodium dodecyl sulfate (SDS) and subsequent separation with CE. If successful, this approach would allow us to determine the presence of the various apolipoproteins on each these particles initially by mobility characteristics and ultimately by mass spectral analyses.

CHAPTER IV

METHODS AND MATERIALS

Animal Protocol

Thirty 8-week old single comb white leghorn roosters were obtained and housed according to an approved animal protocol (see Appendix). Ten single comb white leghorn laying hens were also obtained and housed according to the same protocol. A minimum of two mL of whole blood was collected from the brachial vein of each bird. Blood was collected via 23 gauge needle and syringe then transferred into tubes containing EDTA. (BD Vacutainer™, Franklin Lakes, NJ) Tubes were inverted 3x and held on ice until plasma separation by centrifugation at 4º C, 2500 x G for 20min.

For estrogen induction of VLDLy assembly, diethylstilbesterol (DES) dissolved in oil (10mg/mL) was injected subcutaneously in the inguinal skin-fold of 6 of these roosters at the dose of 10mg/kg-body weight. Birds were sacrificed at 12 and 24 hours after DES treatment. Three control birds and one hen were also studied; these subjects were injected with vehicle oil lacking DES.

Harvest Procedure

Birds were anesthetized by placing them in chamber filled with isoflurane and oxygen. When fully anesthetized the bird was removed from the anesthesia induction chamber and transferred to a prepared surgical table and a face mask attached to provide continued sedation via continued provision of isoflurane and oxygen. The body cavity was opened via central midline incision and blood samples obtained via heart puncture.

A minimum of 5mL of blood was collected directly into tubes containing EDTA via 20 gauge needle (BD Vacutainer™). Whole blood was held on ice until plasma separation. The liver was removed, weighed and held on ice for RER isolation. At this point the bird was exsanguinated via laceration of the heart.

Liver Perfusion

To better understand the effects of estrogen on the liver of the laying hen a method of fixation was desired that would maintain the ultrastructure of the hepatocytes as much as possible. Perfusion is a technique which uses the animal's own vasculature as a route of distributing fixative throughout the body. A method of perfusion previously described by Angermuller and Fahimi was used [53]. The fixative solution contained 1.5% glutaraldehyde (Ladd Research Industries, Williston, VT), 4% PVP, .05% $CaCl_2$, in .1M Sodium Cacodylate (Ted Pella Inc., Redding CA) buffer pH 7.4. The samples were than examined using electron microscopy.

Prior to perfusion birds were first injected with heparin in the dose of 10units/kg body weight to prevent clotting during perfusion. Approximately 45 minutes after anticoagulation the bird was anesthetized and the body cavity opened as described above. Again, a minimum of 5mL of blood was collected directly into tubes container EDTA via 20 gauge needle (BD Vacutainer) via heart puncture. The perfusion setup consisted of two flasks, one containing .1M sodium cacodylate buffer and the other containing the fixative solution. These were connected to another empty flask, which was used to maintain pressure. Y-shaped tubing was used to attach the two buffer containing flasks to a 24 gauge needle. The needle was then inserted into the heart. The

valve to the cacodylate buffer was opened and pressure was raised to and maintained at 100mm Hg using a manometer. The buffer solution was allowed to flow through the bird for 2 minutes. Following this wash the valve to the fixative solution was opened and the buffer valve closed. The fixative solution was allowed to flow through the bird for 40 minutes. After this time the valve was closed and the needle removed. A small section of the right lobe of the liver was removed and stored in a vial containing the fixative solution. These sections were sent to the laboratory of Dr. Robert Hamilton for analysis with electron microscopy.

Extraction of Rough Endoplasmic Reticulum

This method of RER extraction was adapted from the previously described method of Dr. Robert Hamilton [38]. The livers of the three birds at each time point were combined and minced finely. Approximately 20 grams of liver from each combined sample was combined with 140 mM KCl added in the proportion of 18 mL KCl/10g liver. The diluted membranes were further homogenized with a Polytron, PT 3100 for 30 seconds.

The prepared homogenate was split between two 50 mL polycarbonate centrifuge tubes (Nalgene), and the total volume brought to 40 mL with 140 mM KCl. The tubes were then centrifuged at 8K rpm, 4°C for 10 min using a Sorvall RC5C centrifuge. Floating fat was aspirated and the infranatant decanted into a fresh tube while being careful to prevent pelleted non-RER membrane transfer. This process was continued until floating fat was no longer visible or at most for 3 centrifugations.

Infranatants were combined and the volume measured. They were then titrated with 8mM $CaCl_2$. The ratio of supernatant to $CaCl_2$ was 75mL supernatant/450 mL $CaCl_2$. The titrated solution sat for at least one hour prior to centrifugation. The solution was again divided into 50 mL polycarbonate centrifuge tubes and centrifuged with a Sorvall RC5C at 8K rpm, 4°C for 20 minutes. Supernatant was discarded and the now pelleted RER membranes resuspended with 100 mM Na_2CO_3. The suspended RER membrane pellets were further diluted to a final volume of 3.63 mL Na_2CO_3 /g liver processed. This step releases the luminal contents of the RER including VLDL precursor particles. The resulting suspension was mixed vigorously every 20 minutes for 2 hours and then ultracentrifuged with a Beckman L8-70M centrifuge at 35K rpm, 4°C for 18 hours.

Following ultracentrifugation the first step particle fraction appears as a yellow cloud next to the pellet. This first step particle fraction was collected and total volume recorded. Sufficient desiccated KBr was added to the first step particle fraction to bring the solution density to 1.10 g/mL and allowed to dissolve. A volume of 2.6 mL of this solution was placed into the bottom of a 6.8 mL ultracentrifuge tube and overlaid with 1.2 g/mL NaCl/NaBr solution. The prepared tubes were then ultracentrifuged with a Beckman L8-70M centrifuge at 30K rpm, 4°C for 18 hours.

The first step particles form a band at an approximate density of 1.10 g/mL, and were collected with a pipette. Diameter of first step particles was determined by dynamic laser light scattering [18]. First step particles were then aliquotted and frozen, see cryopreservation below, prior to further analysis [43].

Separation of Plasma Lipoproteins

Plasma VLDL was separated by density gradient ultracentrifugation methods routinely used within the laboratory. Density solutions were created by adding NaBr to a stock solution of NaCl of density 1.0063g/ml containing .01% EDTA and 50ku/l each of streptomycin and penicillin. Five mL of density solution were layered over 1 mL of plasma prior to ultracentrifugation. All samples were centrifuged at 35,000 rpm for 18hrs. Final density for VLDL separation was 1.02g/ml. The top 500µl from each sample was collected using a narrow-bore pipette and particle diameter determined using dynamic laser light scattering. Samples were analyzed immediately or aliquotted and frozen at -80°. Prior to freezing stock sucrose solution was added to each sample, final concentration of sucrose was 10%.

Dynamic Laser Light Scattering

The diameter distribution of plasma VLDL and 1st step particles was determined by dynamic laser light scattering with a NanoTrac® 250 (Microtrac, Inc., Montgomeryville, PA). In this method approximately 200µl of sample was placed into a polypropylene cuvette and the probe of the detector lowered into the sample. Using system software the laser light was activated. Resultant laser scattering from the lipoprotein particles was measured for 30 sec and repeated 3 times for each sample. Averages of the three measurements were calculated by system software immediately after each run. This was done for each VLDL, VLDLy and first step particle sample.

The principle of this technique is based on the Doppler light shift that occurs when the laser light passes through the individual lipoprotein particles which are

undergoing Brownian movement. The velocity distribution of the laser scattering is a function of particle diameter, fluid temperature and fluid viscosity. Fluid viscosity and temperature are known and therefore the resultant distribution is representative of the diameter of the particles. System software is able to convert this information into a diameter distribution of the particles in a given sample. This laboratory has used this method extensively in the study of lipoproteins of both animal and human origin. Previous studies in this laboratory were able to demonstrate the decreased particle diameter of VLDLy [18, 20].

Cryopreservation

Stock solution for cryopreservation of lipoproteins was as follows; 50% sucrose, 150 mM NaCl, .24 mM EDTA, pH 7.4. A final concentration of 10% sucrose (weight/volume) was achieved by direct addition of 100 µL of the stock sucrose solution to 400 µL aliquots of VLDL or 1st step particles. Samples were stored at -80°C until further analysis [43].

Preparation of Capillary Cartridge

Capillaries were prepared using Beckman capillary cartridges (Beckman Instruments, Fullerton, CA). Approximately 60cm of untreated fused silica capillary with an inner diameter of 76µm and an outer diameter of 360µm (Polymicro Technologies, Phoenix, AZ) was measured and cut using a ceramic square. Care was taken to achieve a flat end to the cut capillary. The total length of the capillary was measured exactly to ± 1.0 mm. A place 7.6 cm from one end was measured and marked using a fine tipped pen. This point marks the location of the capillary window. To form

the window, the capillary was inserted into a small metal coil attached to a power supply. The power supply was turned on and the capillary rotated within the coil for approximately 10 seconds in order to remove the outside coating. The capillary was then removed from the coil and wiped gently with a Kimwipe™ to remove the burnt polyimide coating. The capillary was then examined under a light microscope to ensure that all coating was removed from the window. The exact length of the short, detector end of the capillary was measured to ± 1.0 mm.

Capillary assembly was based on the Beckman protocol. A place 6.5 cm from the window on the long, injector, end of the capillary was marked. This mark was placed onto the end of the spindle of the cartridge assembly; the capillary was then secured with a small piece of rubber tubing, Figure 4. The capillary was then carefully wound around the spindle three times. The other end of the capillary was secured to the opposite side of the spindle with rubber tubing. Both ends of the capillary were inserted into the cartridge assembly and the spindle placed into the cartridge. The capillary window was then aligned with the aperture of the cartridge. The capillary was then secured in place. The cartridge was then examined under a microscope to ensure proper alignment of the capillary window with the cartridge aperture. Rubber gaskets were placed around the capillary window and spindle assembly. Small rubber gaskets were also placed around the ends of each capillary to prevent leakage of the capillary coolant. The top of the cartridge assembly was then secured.

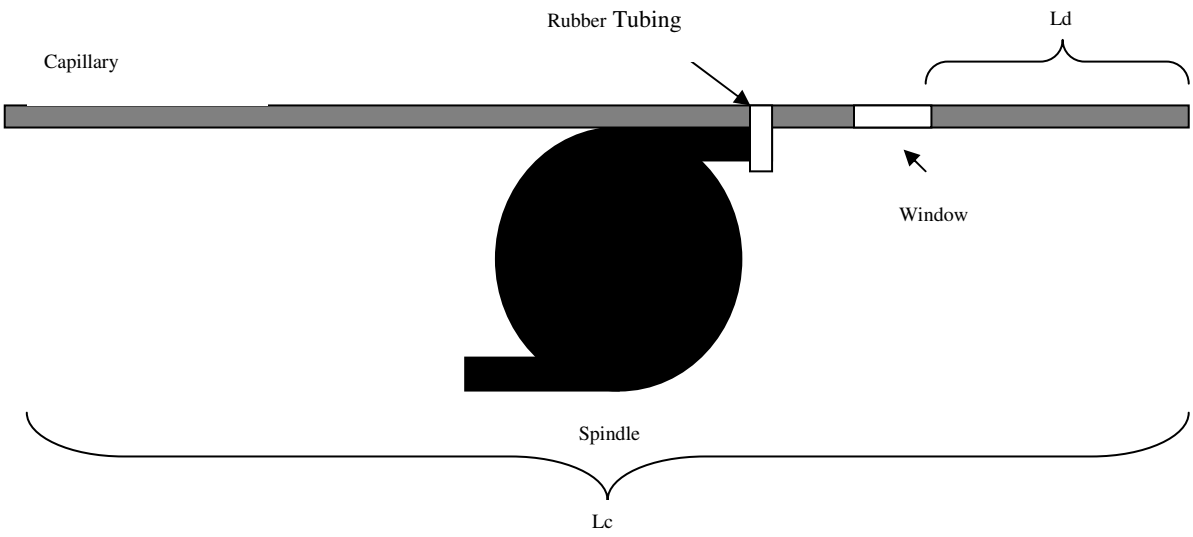

Fig. 4. Representation of capillary spindle and capillary with window (not to scale)

The ends of the capillary were measured and cut using the capillary template provided. The length of each end cut was measured to ± 1.0 mm and subtracted from the total length of the capillary. This value is referred to as the total length of the capillary, L_T. The length to detector, L_D, was calculated by subtracting the length of the windowed detector end of the capillary measured above, less the length cut off, from the total capillary length. The capillary was clearly labeled with L_T and L_D and these measurements were used to calculate effective mobilities (μ_e) using the following equation:

$$\mu_e = \frac{L_c L_d}{V}\left(\frac{1}{t} - \frac{1}{t_{EOF}}\right)$$

where L_c is the total length of the capillary (cm), L_d is the length of the capillary from injector to detector (cm), V is the applied voltage (volts), t is the migration time of the sample (sec), and t_{EOF} is the migration time of the EOF marker (sec).

Ultrafiltration

Prior to analysis by CE samples were ultrafiltrated to remove any remaining saline or sucrose solution and concentrate the lipoproteins. For analysis of intact particles samples were filtered in 2.5mM PBS. Approximately 250µL of samples, fresh or previously frozen, were mixed with 250µl of 2.5 mM PBS. Using a 100,000MW Micron filter (Millipore, Billerica, MA) samples were centrifuged for 8 min at 9000 rpm. This was repeated 3 times with addition of ~ 200µl of buffer prior to each spin. Hen VLDL samples required additional filtration and were centrifuged twice more for 8 min at 9000 rpm without addition of buffer. The filter was then inverted into a clean tube and then spun for 3 min at 6500 rpm to allow collection of all remaining sample. Prior to analysis all samples were diluted 1:1 with 2.5mM PBS.

Samples that were to be delipidated prior to CE analysis were ultrafiltrated using 100,000 MW filter and centrifuged for 8 min at 9000rpm without the addition of buffer. Prepared samples were mixed 1:2 with SDS buffer, vortexed, and allowed to incubate for 45 min at 60°C.

Capillary Electrophoresis

Capillary electrophoresis took place using Beckman P/ACE system, Model 5510, and photo-diode array detector. The background electrolyte (BGE) for intact particles was phosphate buffer (PBS). A stock solution of 38mM phosphate buffer was made which consisted of 30 mM Na_2HPO_4 and 8mM NaH_2PO_4. This solution was further diluted to 5mM for CE analysis and 2.5mM for ultrafiltration. The BGE used for delipidation of lipoproteins (SDS) contained 50mM sodium borate, 20% acetonitrile (v/v) and 3.5 mM SDS [48]. The electroosmotic flow marker (EOF) for all experiments was .001% DMSO. Prior to each use the BGE was degassed using vacuum sonication until bubbles were no longer seen.

The capillary was conditioned before being used and prior to each day's use. A series of treatments are used to properly condition the capillary. The capillary was first rinsed for 5 min with 1.0 N NaOH. This was followed by a 10 min rinse with 0.1 N NaOH and 10 min rinse with deionized H_20. Prior to injection of sample an EOF test was run to ensure adequate capillary conditioning. The EOF test was conducted as follows: the capillary was first rinsed for two minutes with the appropriate BGE followed by a 5 sec injection of EOF. Each end of the capillary was then placed in to a vial containing BGE and voltage applied across the capillary at 16kV. If the EOF marker failed to appear after 6 min, for PBS, or 8 min, for SDS, the capillary was reconditioned prior to use. Between each sample run the capillary was rinsed with 0.1 N NaOH for 1 min followed by 1 min rinse with deionized H_20.

Approximately 100µl of sample was placed into the sample vial. Prior to sample injection the capillary was rinsed with BGE for 2 min, followed by a 5 sec injection of EOF and finally a 5 sec injection of sample. Each end of the capillary is then placed into vials containing BGE and voltage, 16kV, applied across the capillary. Detection took place between 190 and 300nm. Peak areas, migration times and μ_e were determined using the Beckman P/ACE system software.

Statistics

Mean, standard deviation and coefficient of variation of particle diameters for each lipoprotein class were determined using the median particle diameter calculated by system software for each individual sample. Paired t-tests were used to determine significant differences between particle diameters of first step particles isolated from the livers of estrogen naïve and estrogen treated roosters. Observed differences in effective mobility for lipoprotein classes were also analyzed using unpaired, unequal variance, t-tests. Differences were deemed statistically significant at $p \leq .05$.

CHAPTER V

RESULTS

Diameter Distribution Studies

Particle diameter distributions were measured for plasma VLDL and VLDLy. Median particle diameter for plasma VLDL from untreated roosters was 49.2 ± 23.2 nm. Median particle diameter for plasma VLDL was 17.6 ± 1.7 nm 12 hours after estrogen treatment and 16.8 ± .8 nm 24 hours after hormone exposure. Median diameter for plasma VLDLy from hen was 19.0 ± 1.0 nm. As predicted the particle diameter distribution for untreated rooster VLDL was much more variable than the distribution from either the laying hen or estrogen treated roosters. Particle diameter for estrogen injected birds and hens were much more uniform. These results are similar to those previously established within this laboratory [6]. Table 3 provides a summary of particle diameters for plasma VLDL and VLDLy.

Table 3
Particle diameter for plasma VLDL from estrogen naïve roosters, estrogen treated roosters and laying hens. Particle diameters listed at both 12 and 24 hours after estrogen treatment for estrogen treated roosters. Particle diameter as determined by dynamic laser light scattering.

	Median Diameter (nm)	Std Dev	Coefficient of Variation	n=
Rooster	49.2	23.2	.472	18
Rooster +12	16.8	.8	.048	3
Rooster +24	17.6	1.7	.101	7
Hen	19.0	1.0	.053	11

Particle diameter distributions were also measured for first step particles. The median particle diameter of first step particles from an untreated rooster was 31.5 ± 6.8 nm. The median particle diameter of first step particles decreased dramatically, to 16.1 ± 1.6 nm, 12 hours after estrogen injection. Twenty-four hours after estrogen injection median particle diameter of first step particles increased to 23.9 ± 6.2 nm. The increase in diameter of first step particles 24 hours after estrogen exposure likely reflects the end of estrogens effect on the liver. This is consistent with previous studies which have shown that estrogen effectively induces translation of apo VLDL II for 24 hours, after which the effect wanes [5]. Table 4 provides a summary of the particle diameters of first step particles.

Table 4

Particle diameters for first step particles from estrogen naïve roosters and estrogen treated roosters (12 and 24 hours after treatment). Particle diameter as determined by dynamic laser light scattering.

	Median Diameter (nm)	Std Dev	Coefficient of Variation	n=
Rooster	31.5	6.8	.100	8
Rooster +12	16.1	1.6	.066	3
Rooster +24	23.9	6.2	.110	7

Isolation of VLDL Assembly Intermediates

Figure 5 is an electron micrograph of a thin section of a cocadylate perfused hen's liver. As hen livers are fatty, large lipid droplets are visualized in the electron micrograph. Nascent VLDLy particles, identified by open arrows, are visible within the juncture of the RER and SER. The large amount of lipid present within the hepatocyte limits resolution of VLDLy located within the secretory compartments of the Golgi (G) are not visible in this image.

Figure 6 depicts electron micrographs of plasma VLDL from immature birds and VLDLy from hens as well as VLDLy assembly intermediates isolated from hen liver. Note the dramatic reduction of particle size of serum VLDLy from the laying hen (top left), with particles ranging from 27.5-45 nm, compared to serum VLDL from estrogen naïve chicks (top right), 40-120 nm. This is similar to the reduction in particle diameter observed using the dynamic laser light scattering technique. First step particles isolated from RER of hen hepatocytes (lower right) ranged in size from 15-23 nm. This was similar to the particle diameter for first step particles isolated from RER of estrogen treated roosters which had a median diameter of 16.1 nm, as determined by dynamic laser light scattering.

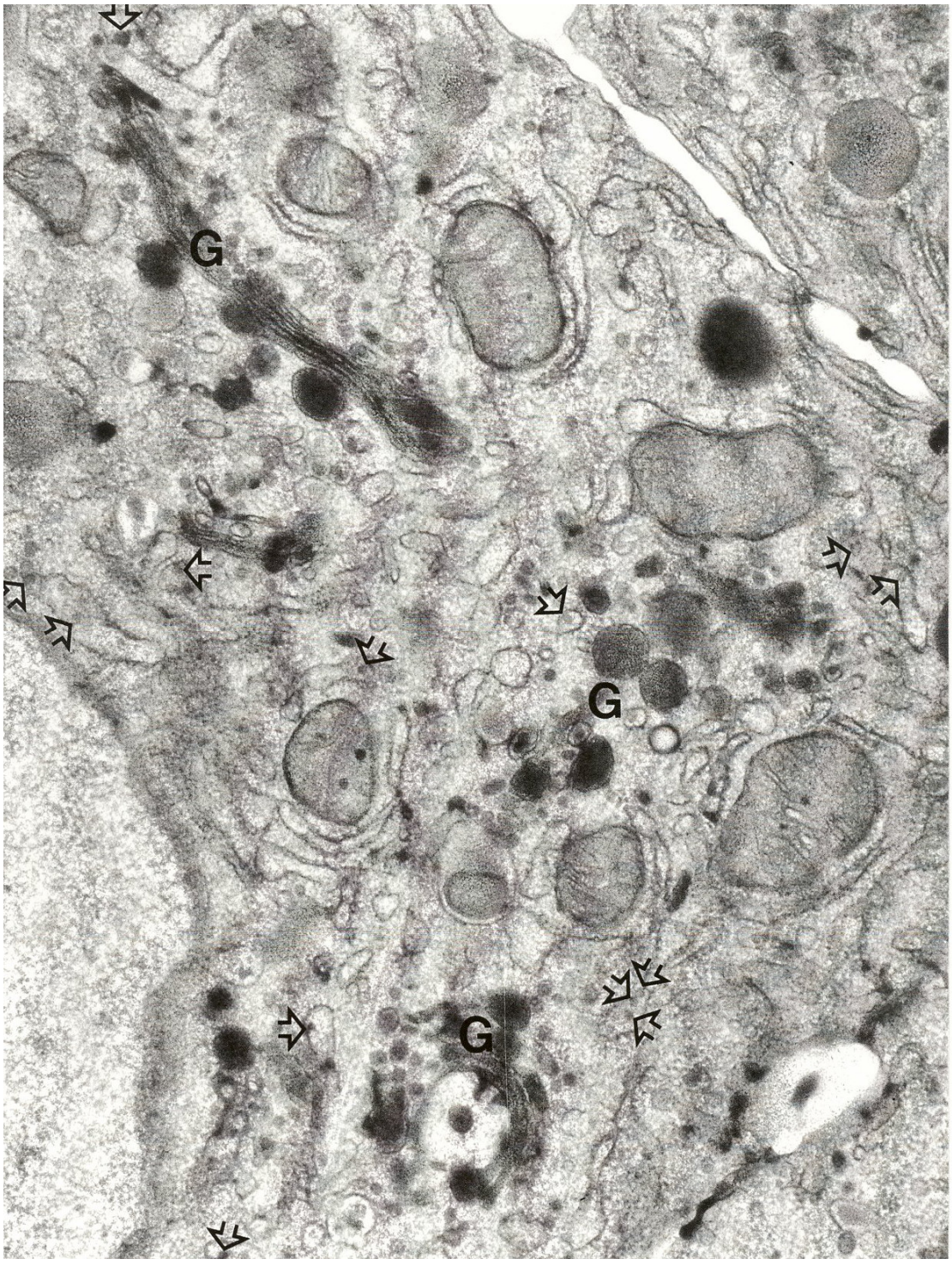

Fig. 5. Electron micrograph of laying hen hepatocyte. Open arrows point towards nascent VLDLy particles within the RER-SER membrane compartments. Particle diameter of these particles was 27.5-45nm. Due to the high levels of lipid present within hen hepatocytes there was inadequate resolution of VLDLy particles within the secretory vesicles of the Golgi (G).

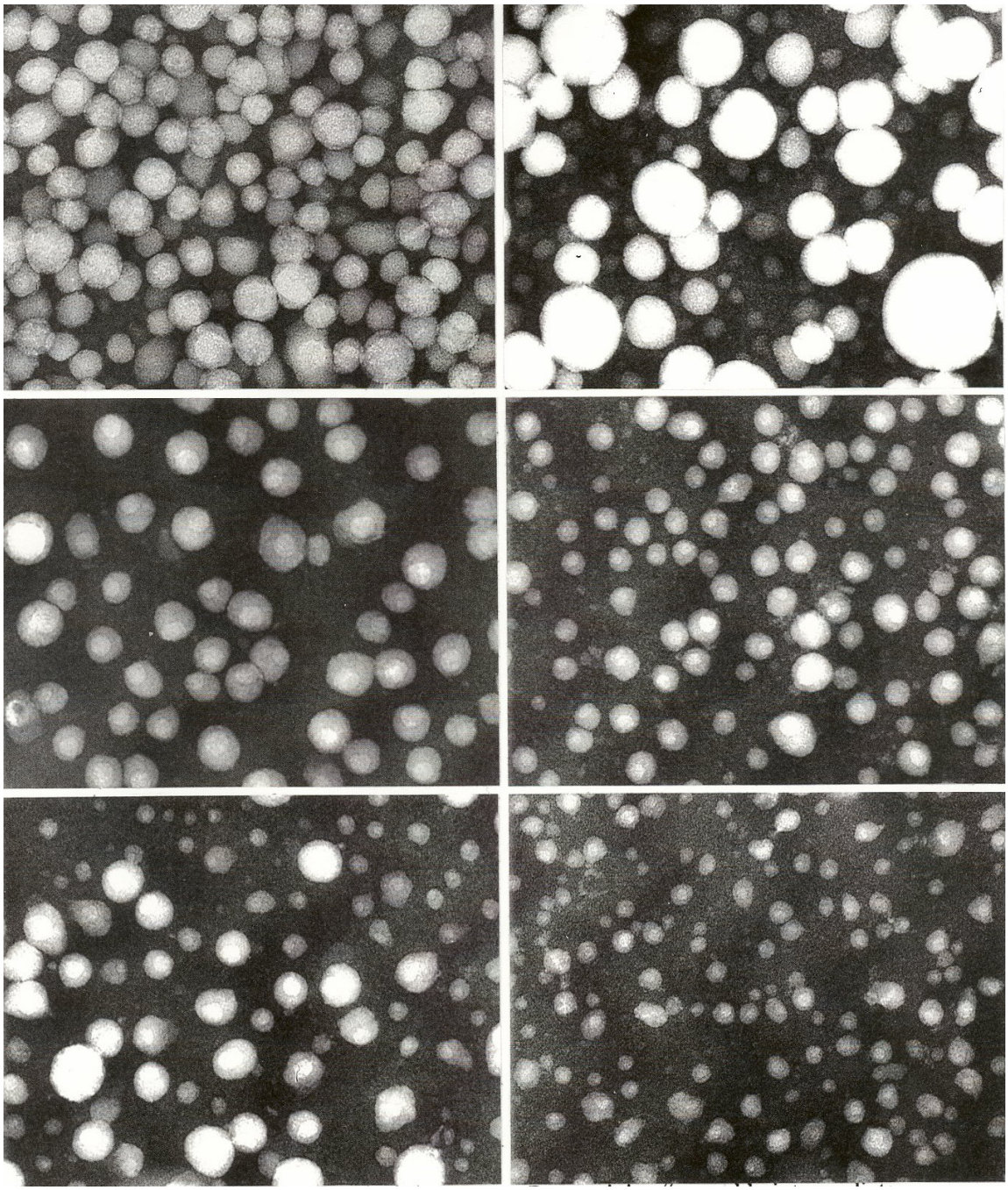

Fig. 6. Electron micrograph (x180,000) of plasma VLDL and hepatocyte VLDL assembly intermediates. UPPER LEFT: Laying hen plasma VLDLy (27.5-40nm). MIDDLE LEFT: VLDLy from Golgi of laying hen hepatocytes (27.5-40nm). BOTTOM LEFT: VLDLy from RER of laying hen hepatocytes (27.5-45nm). UPPER RIGHT: Estrogen naïve chick plasma VLDL (40-120nm). MIDDLE RIGHT: Second step particles from RER of laying hen hepatocytes (22.5-27.5nm). LOWER RIGHT: First step particles from RER of laying hen hepatocytes (15-23nm).

Capillary Electrophoresis of Intact Particles

Fresh VLDL particles were electrophoresed in untreated silica capillaries filled with 5mM PBS after being ultrafiltrated with 2.5 mM PBS using a 100,000MW Micron filter (Millipore, Billerica, MA). When intact VLDLy from hen plasma was run, a single peak was detected with an average effective mobility (μ_e) of -14.7 x 10^{-5} cm^2/V.s, Figure 7. Similar results were observed from VLDLy from estrogen treated roosters for which μ_e was -14.1 x 10^{-5} cm^2/V.s.

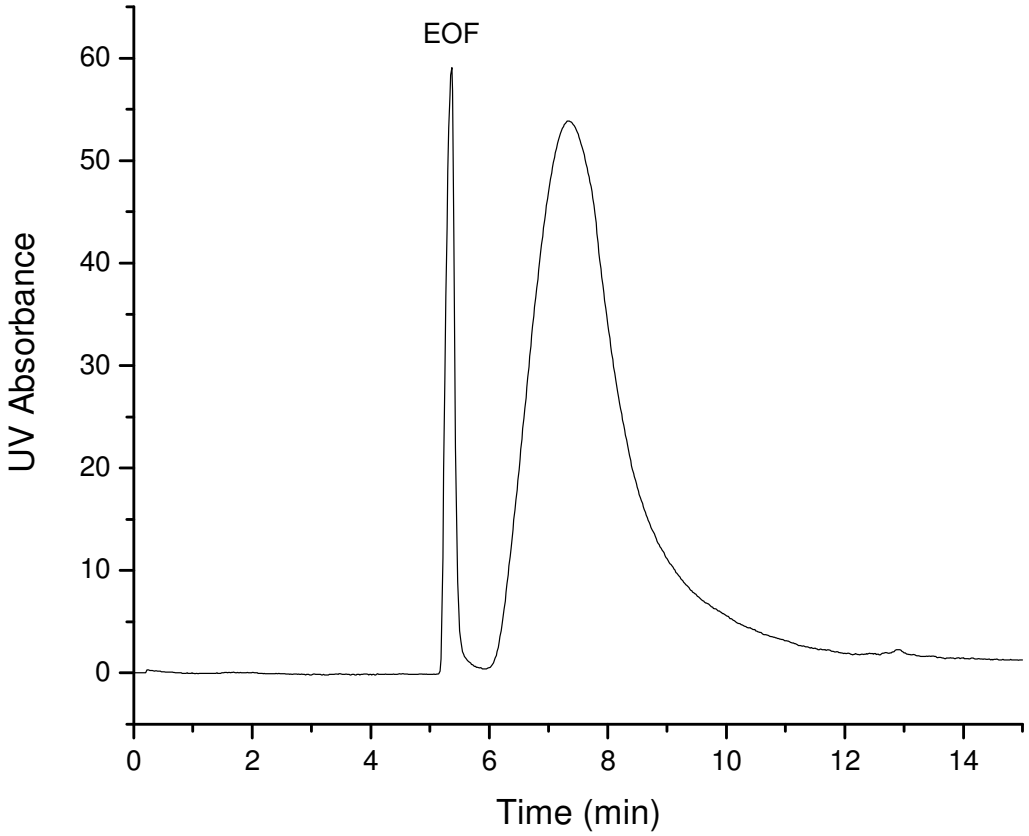

Fig. 7. Electropherogram of hen VLDL. BGE: 5mM PBS. UV absorbance at 200nm.

When intact VLDL from untreated roosters was run under the same conditions as the VLDLy, multiple peaks were observed when analyzed at 200nm, Figure 8. The number of peaks detected varied from between two and four. Effective mobilities ranged from -12.6 to -28.7 x 10^{-5} cm^2/V.s. See Table 5 for a summary of detected peaks. Assuming all particles are of similar charge due to the presence of apolipoproteins "subclasses" would represent particles of various molecular weights. These likely represent VLDL which have undergone varying degrees of hydrolysis. The particles eluting first, those with lower μ_e represent more newly produced VLDL. The peaks with larger μ_e would be particles of smaller size or partially hydrolyzed VLDL.

Table 5
Electrophoretic mobilities of plasma VLDL from 3 untreated roosters.

	Mobility (X10^{-5} cm^2/V.s)			
	Peak 1	**Peak 2**	**Peak 3**	**Peak4**
		Fresh		
Rooster 1	-14.9	-19.9	-21.7	
Rooster 2	-12.6	-18.4	-23.8	
Rooster 3	-12.8	-19.8	-24.0	-28.7

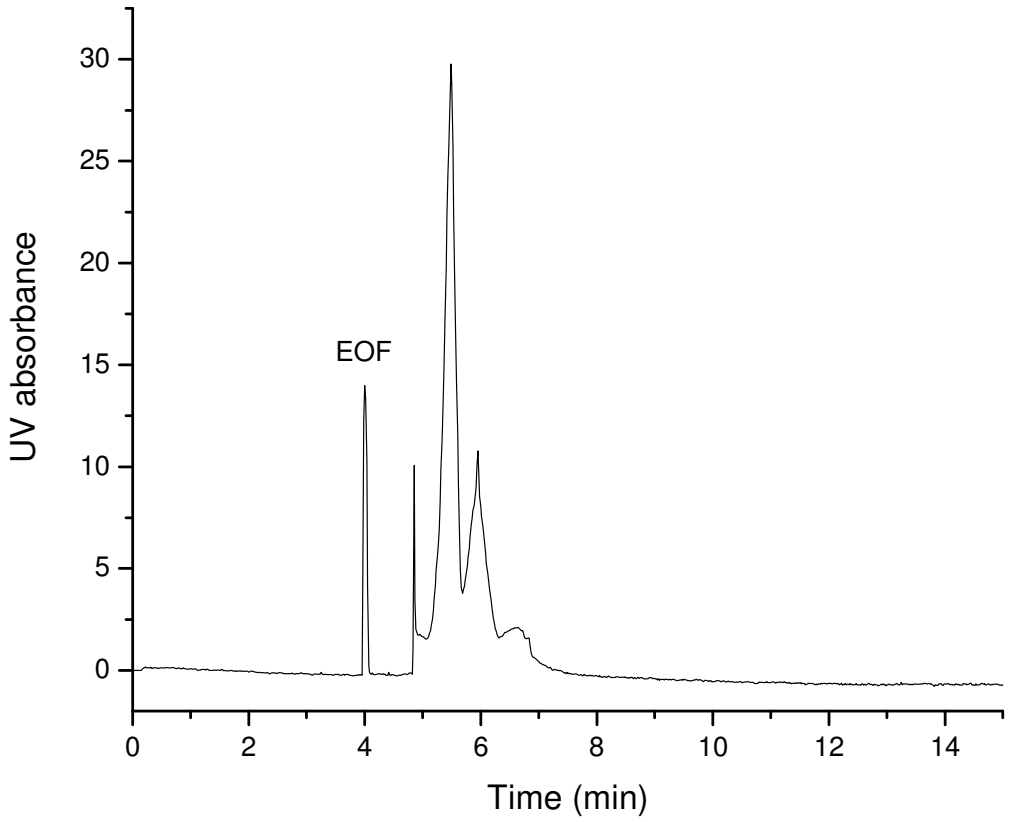

Fig. 8. Electropherogram of fresh rooster VLDL. BGE: 5mM PBS. UV absorbance at 200nm.

First step particles from estrogen naïve and estrogen treated birds, both 12 and 24 hours after treatment, were collected. First step particles were cryopreserved as described in Chapter IV. They were than ultrafiltrated and electrophoresed in the same manner as the intact VLDL and VLDLy. For all first step particles a single peak was detected when analyzed at 200nm, Figures 9 and 10. Average μ_e for first step particles from estrogen naïve rooster was -25.4 $x10^{-5}$ cm^2/V^* ± 0.38. Average mobility for first step particles from estrogen injected roosters was -23.0 ± 0.10 and -23.3 $x10^{-5}$ ± 0.17 $cm^2/V.s$ at 12 hours and 24 hours, respectively. This difference was significant at both

time points, $p<.02$. The peak areas from estrogen treated birds were significantly greater than those from the untreated roosters and the peaks much more well defined. Table 6 provides a summary of mobilities and areas for intact first step particles.

Table 6

Average area and electrophoretic mobility of first step particles from estrogen naïve roosters and estrogen treated roosters (12 and 24 hours after estrogen treatment). Standard deviation (SD) and coefficient of variation of mobilities at each time point. UV absorbance measured at 200 nm.

	Average Area	**Average Mobility** ($X10^{-5}$ $cm^2/V.s$)	**SD (Mobility)**	**Coefficient of Variation (Mobility)**
Control	173806	-25.4	0.38	.015
12 hour	482858	-23	0.10	.004
24 hour	547800	-23.3	0.17	.007

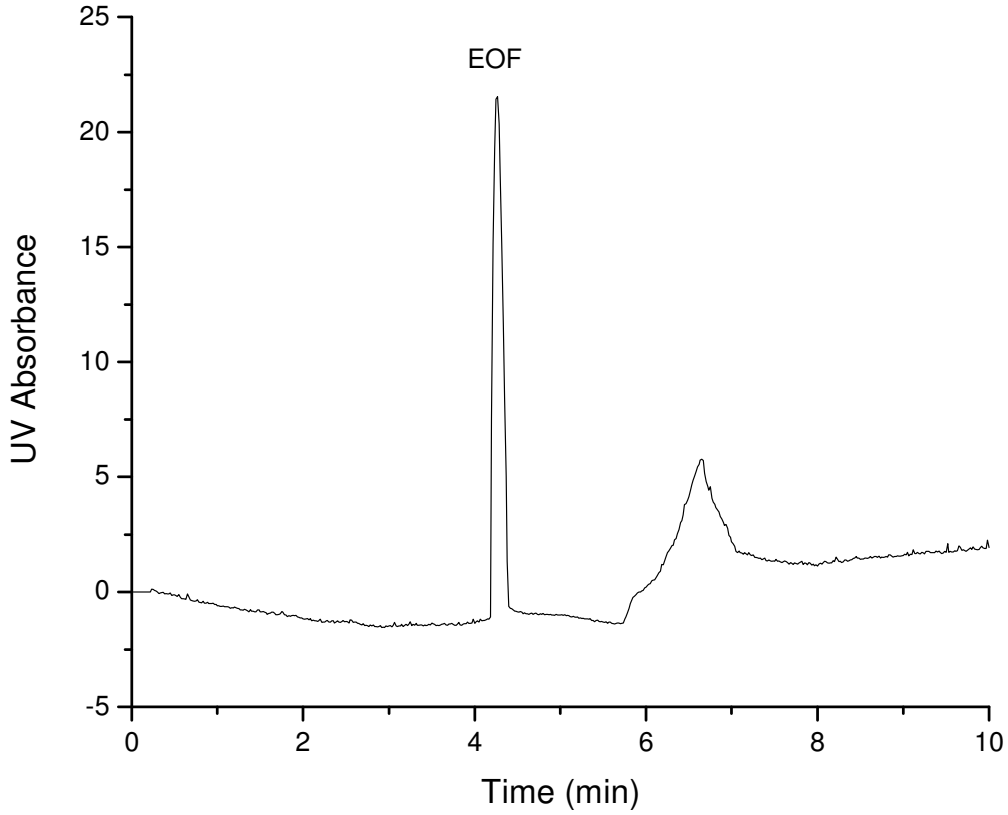

Fig. 9. Electropherogram of first step particles from an estrogen naïve rooster. BGE: 5mM PBS. UV absorbance at 200 nm.

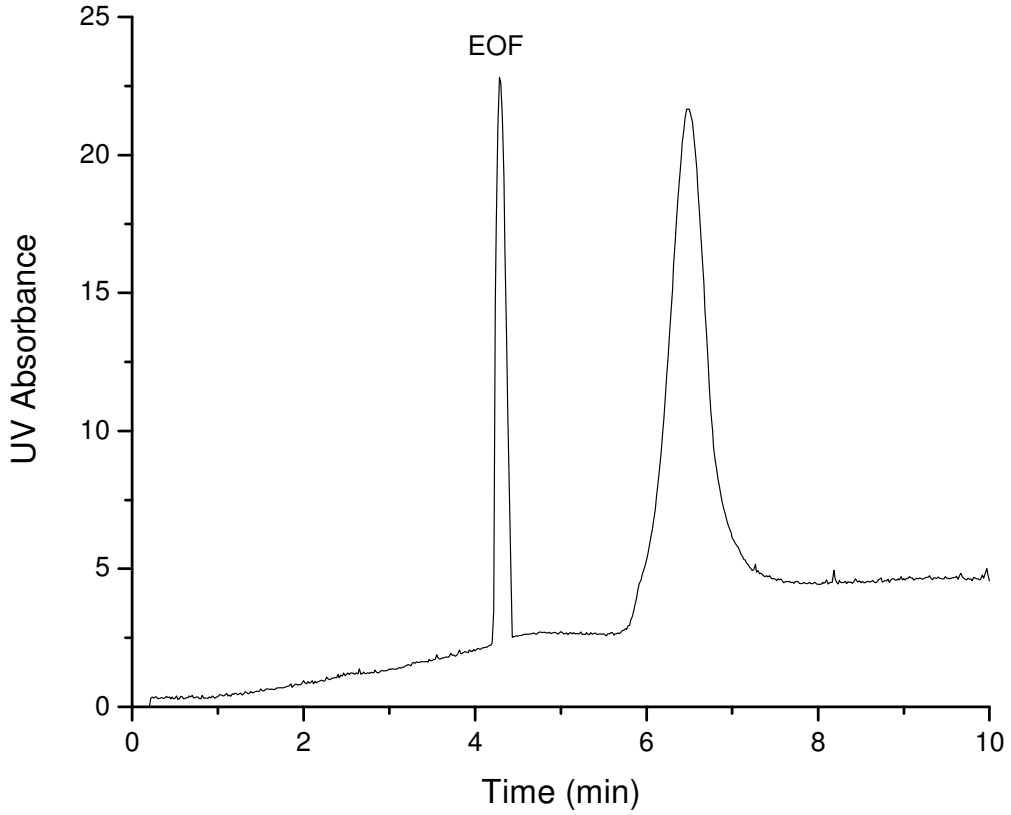

Fig. 10. Electropherogram of first step particles from an estrogen treated rooster (24 hours after estrogen treatment). BGE: 5mM PBS. UV absorbance at 200 nm.

Cryopreservation Studies

A desire for greater flexibility in experimental design prompted storage stability studies with circulating plasma lipoproteins. In these experiments the performance of freshly isolated material was compared to that of those same materials after cryopreservation as specified in Chapter IV. Intact fresh and previously frozen rooster VLDL and hen VLDLy were first ultrafiltrated and then electrophoresed in an untreated silica capillary filled with 5 mM PBS.

Freezing did not significantly affect the mobility of the intact VLDLy particles. A single peak was detected for fresh VLDLy particles with an average μ_e of -14.7 x 10^{-5} cm^2/V.s. A single peak was also detected from the frozen VLDLy particles. Average μ_e of previously frozen particles was -14.3 x 10^{-5} cm^2/V.s. The observed difference in mobility between fresh and frozen hen VLDLy of 0.4 x10^{-5} cm^2/V.s was not statistically significant, p=0.2.

The effects of freezing were significant in rooster VLDL. The distinct subclasses detected in the fresh samples were not as clearly defined in the frozen samples. Overall, mobility of the previously frozen particles increased. Average mobility for all peaks in the fresh sample was -19.3 x10^{-5} cm^2/V.s, average mobility for all peaks from the frozen samples was -25.8 x10^{-5} cm^2/V.s. This difference was deemed statistically significant with $p<.01$. See figure 11 and table 7 for comparisons of fresh and frozen rooster VLDL.

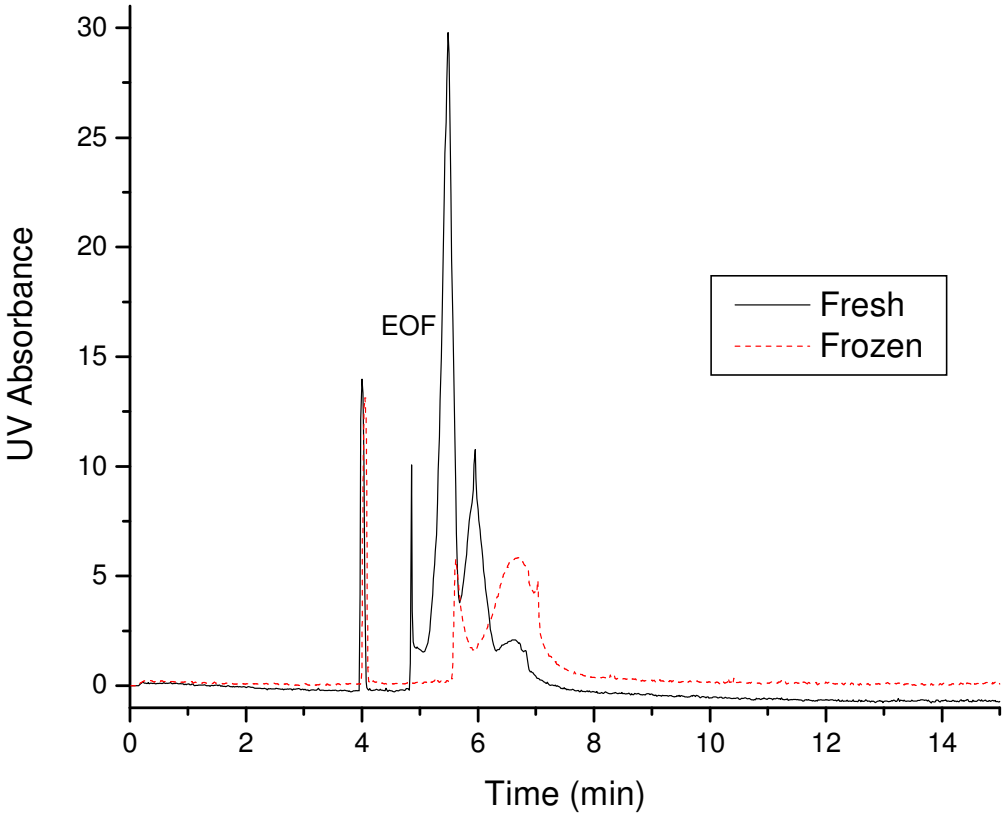

Fig. 11. Electropherograms of fresh and frozen rooster VLDL. BGE: 5mM PBS. UV absorbance at 200nm.

Table 7
Electrophoretic mobilities of fresh and frozen plasma VLDL from 3 estrogen naïve roosters.

	Mobility ($X10^{-5}$ $cm^2/V*s$)			
	Peak 1	**Peak 2**	**Peak 3**	**Peak4**
		Fresh		
Rooster 1	-14.9	-19.9	-21.7	
Rooster 2	-12.6	-18.4	-23.8	
Rooster 3	-12.8	-19.8	-24.0	-28.7
		Frozen		
Rooster 1			-25.0	-29.4
Rooster 2			-23.6	-27.7
Rooster 3			-20.1	-27.5

Capillary Electrophoresis of Delipidated Particles

Electropherograms from delipidated particles were analyzed at 214 nm. Multiple peaks were visible for all samples at this wavelength. At 214 nm both protein and lipid components will be detected. Electropherograms for delipidated samples were also analyzed at 280 nm in order to observe only the protein components of the sample. Any peaks visible at both 214 nm and 280 nm were assumed to be valid protein components of the particle.

Methods for delipidation of VLDL and first step particles were adapted from methods routinely used within the Macfarlane laboratory [48]. Human lipoprotein samples have been successfully delipidated with an incubation temperature of 37 °C. We did attempt to delipidate hen VLDLy at 37°C unsuccessfully, see Figure 12. Inadequate delipidation was achieved at that temperature. This was determined by analyzing the sample at both 214 and 280 nm. A large "lipid" peak was observed with an approximate mobility of -29 x 10^{-5} cm^2/V.s. Previous studies, using a similar CE buffer system, have established μ_e for human apo B at approximately -19 x 10^{-5} cm^2/V.s. A peak with similar μ_e, -20 x 10^{-5} cm^2/V.s, was observed in this sample. However, there was a large peak between presumed apo B peak and the "lipids" peak representing inadequately delipidated particles.

The inadequate delipidation was attributed to the facts that birds have a higher body temperature, 41°C, as compared to humans with a body temperature of 37°C. Delipidation of hen VLDLy was more successful after incubation with SDS buffer for 45 min at 60°C, Figure 13. These particles were analyzed by CE and multiple peaks were

observed when analyzed at 214 nm. One large peak was observed with a mobility of approximately -24x10^{-5} cm^2/V.s. When analyzed at 280 nm this peak was resolved into several smaller peaks. At 214 nm a large peak was also visible with a mobility of approximately -29x10^{-5} cm^2/V.s. This peak was not visible at 280 nm and was assumed to be the lipid component of the VLDL. There were several peaks with mobilities ranging from -20 to -26 x 10^{-5} cm^2/V.s. This indicated that there were likely some inadequately delipidated apo B containing particles remaining. Table 8 provides a summary of peaks observed at 280 nm from delipidated VLDLy of 4 different hens.

Table 8
Electrophoretic mobilities of separated proteins from plasma VLDLy of 4 hens.

	Mobility (X10^{-5} cm^2/V.s)						
	Peak 1	**Peak 2**	**Peak 3**	**Peak 4**	**Peak 5**	**Peak 6**	**Peak 7**
Hen 1	-1.60	-9.68	-20.11	-22.83	-23.04	-24.69	
Hen 2	-1.64	-9.47	-20.10	-22.13	23.0	-24.69	
Hen 3	-2.25	-9.61	-20.51	-21.24	-22.74	-23.55	
Hen 4	-2.27	-8.94	-19.76	-22.06	-22.82	-25.40	-26.34

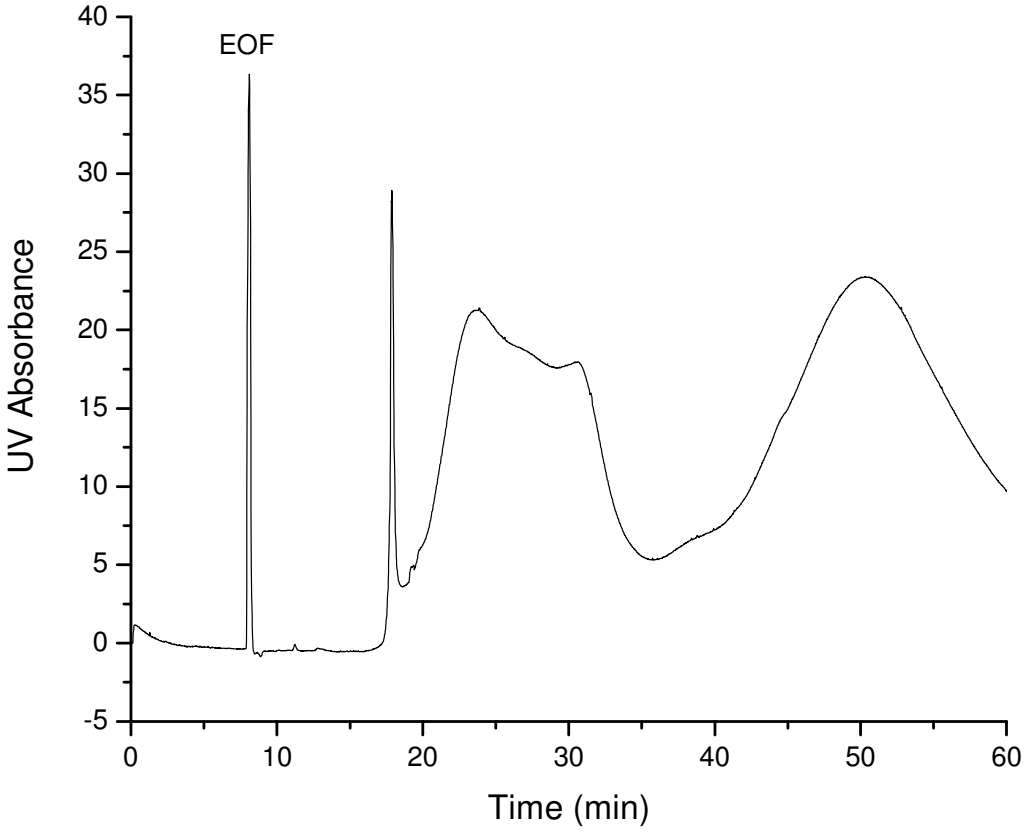

Fig. 12. Electropherogram of inadequately delipidated hen VLDLy. Sample was incubated 45 min at 37°C, 1:2 ratio (sample:SDS). BGE: 3.5 mM SDS. UV absorbance at 214nm.

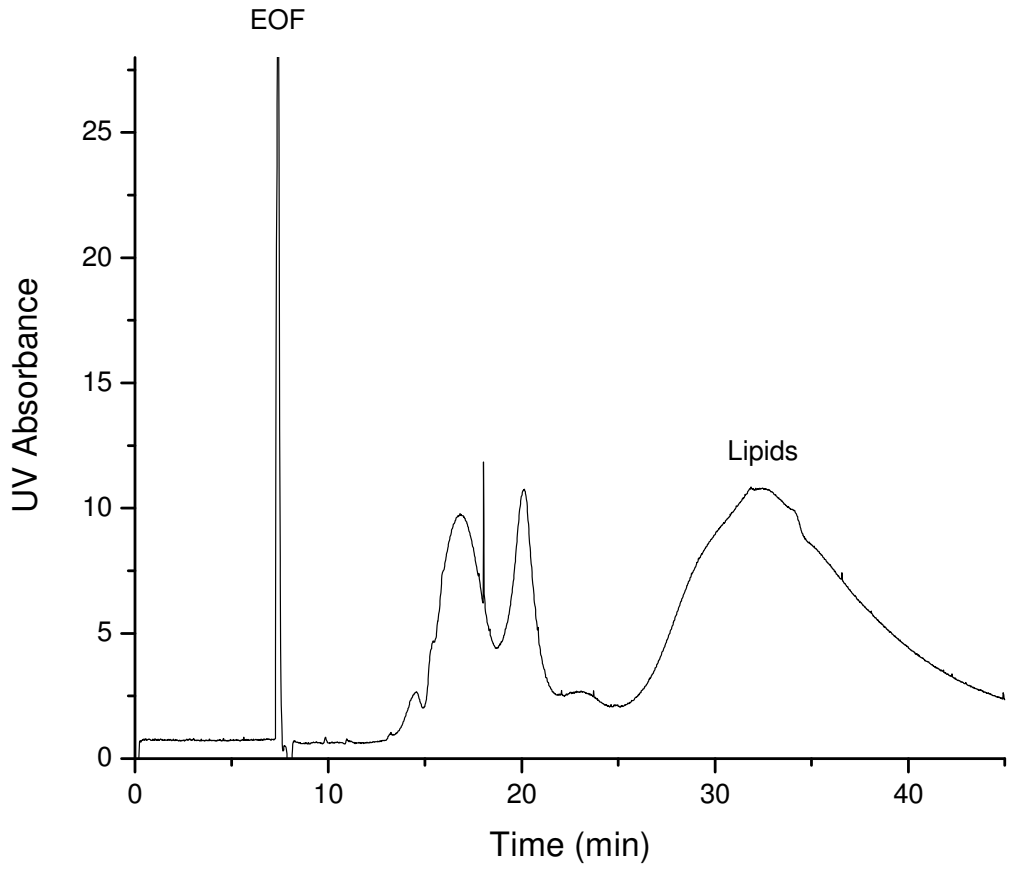

Fig. 13. Electropherogram of delipidated hen VLDLy. Sample was incubated for 45 min at 60°C, 1:2 (sample:SDS). Large "lipids" peak visible at 214 nm. BGE: 3.5mM SDS Buffer. UV absorbance at 214 nm.

VLDL from untreated roosters was delipidated in the same manner. Again, multiple peaks were visible at 214 nm, Figure 14. These peaks had similar mobilities to those observed in the hen. No peak was observed in the region of the lipids observed in then hen VLDL. Multiple peaks were observable at 280 nm as well. A summary of visible peaks at 280nm for delipidated rooster VLDL can be found in table 9. One peak was noticeably absent from the rooster VLDL in comparison to Hen VLDL. The peak

identified as Peak 2 in Table 8 with a mobility of approximately -9.6x10^{-5} cm^2/V.s, was not detected in any of the untreated rooster samples.

Table 9
Electrophoretic mobilities of separated proteins from plasma VLDL of 3 estrogen naïve roosters.

	Mobility (X10-5 cm2/V*s)						
	Peak 1	**Peak 2**	**Peak 3**	**Peak 4**	**Peak 5**	**Peak 6**	**Peak 7**
Rooster 1	-2.22	-21.86	-22.31				-26.19
Rooster 2	-1.86	-19.88	-22.96	-23.82	-24.25	-24.88	
Rooster 3	-1.97	-20.00		-24.26	-24.67	-25.02	-26.02

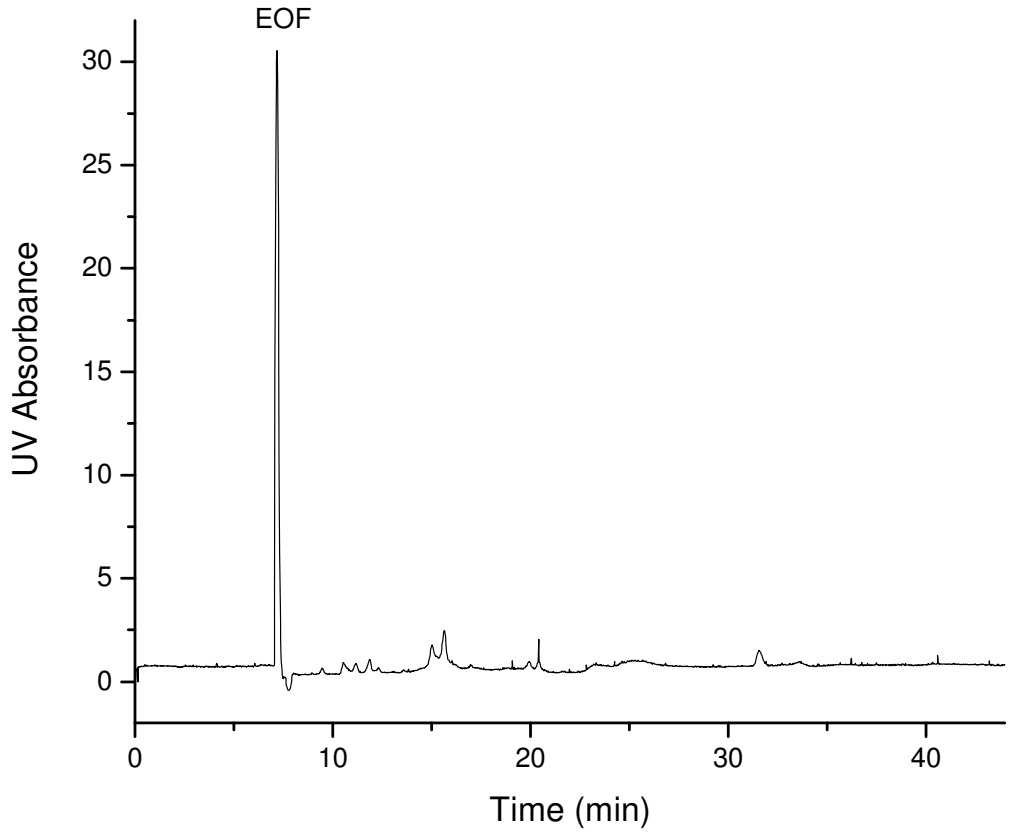

Fig. 14. Electropherogram of plasma VLDL from an estrogen naïve rooster. BGE: 3.5mM PBS. UV absorbance at 214 nm.

VLDL from estrogen treated roosters, 24 hours post estrogen injection, was also delipidated using SDS. Electropherograms for these samples were analyzed at both 214, Figure 15, and 280 nm, Figure 16. These electropherograms resembled those from the hen. A "lipid" peak was detectable at 214 nm, which was not observed when analyzed at 280 nm. An additional peak was observed with a mobility of approximately -10.5x10^{-5} cm^2/V.s, which was not observed in the untreated rooster samples. Table 10 provides a summary of peaks detected at 280 nm in VLDL from estrogen treated roosters.

Table 10
Electrophoretic mobilities for separated proteins from plasma VLDLy of 3 estrogen treated roosters (24 hours after estrogen treatment).

	Mobility ($X10^{-5}$ cm^2/V*s)						
	Peak 1	**Peak 2**	**Peak 3**	**Peak 4**	**Peak 5**	**Peak 6**	**Peak 7**
Rooster 1	-1.54	-10.58	-19.59	-20.39	-22.10	-23.64	-25.61
Rooster 2	-1.63	-10.49	-19.11	-20.11	-21.79	-23.72	-25.62
Rooster 3	-1.77	-10.45	-19.42	-20.70	-21.70	-21.70	-25.99

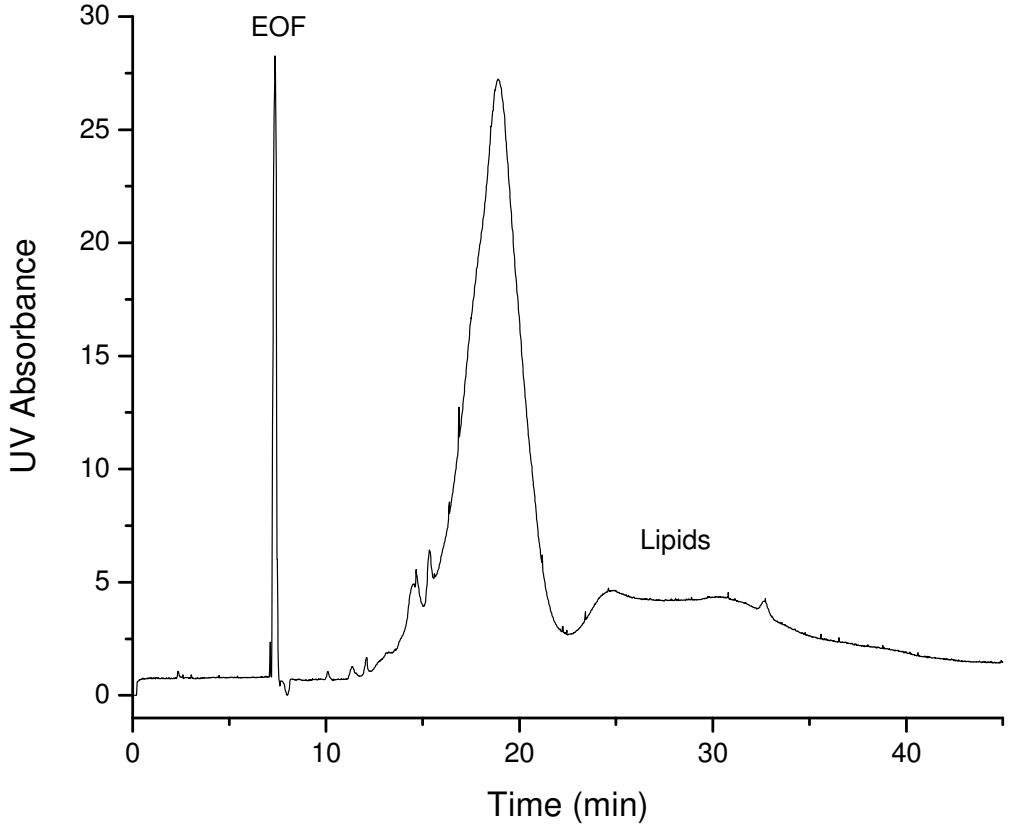

Fig. 15. Electropherogram of plasma VLDLy from an estrogen treated rooster, UV absorbance at 214 nm (24 hours after estrogen treatment). BGE: 3.5mM SDS. UV absorbance at 214 nm.

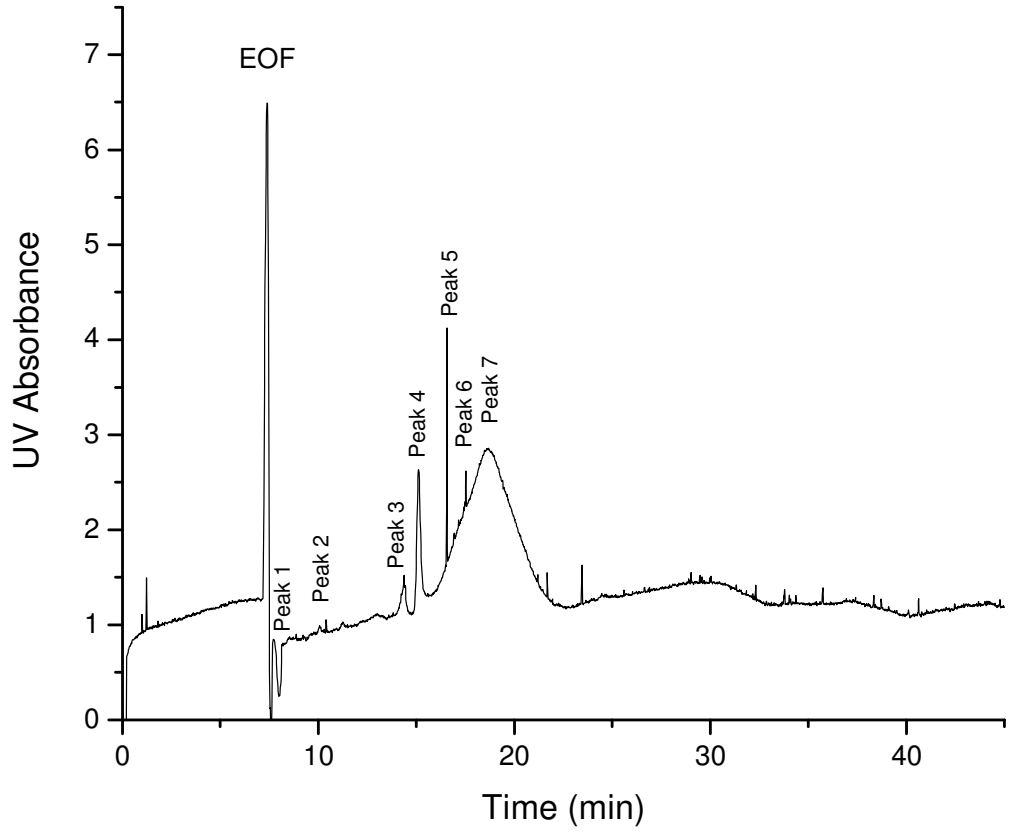

Fig. 16. Electropherogram of plasma VLDLy from an estrogen treated rooster, UV absorbance at 280 nm (24 hours after estrogen treatment). BGE: 3.5mM SDS.

First step particles from both control and estrogen treated rooster were delipidated by incubation at 60°C for 45 min in 3.5mM SDS. After incubation with SDS particles were separated with CE and analyzed at both 214 and 280 nm. Multiple peaks were observable at 214 nm for both control, Figure 17, and estrogen treated, Figure 18, first step particles. A small "lipid" peak was visible at 214 nm.

When analyzed at 280 nm only two peaks were visible for first step particles from control birds. Delipidated first step particles from estrogen treated birds had multiple observable peaks when analyzed at 280 nm. A summary of peaks observed at 280 nm for first step particles from control and estrogen treated birds are found in Tables 11 and 12.

Table 11
Electrophoretic mobilities for separated proteins from delipidated first step particles from three estrogen naïve roosters.

	Mobility ($X10^{-5}$ $cm^2/V*s$)	
	Peak 1	**Peak 2**
Rooster 1	-19.83	-21.06
Rooster 2	-19.94	-20.91
Rooster 3		-21.20

Table 12
Electrophoretic mobilities for separated proteins from delipidated first step particles from four estrogen treated roosters (24 hours after estrogen treatment).

	Mobility ($X10^{-5}$ $cm^2/V*s$)					
	Peak 1	**Peak 2**	**Peak 3**	**Peak 4**	**Peak 5**	**Peak 6**
Rooster+ 1	-4.85	-10.56	-21.12	-24.63	-25.56	-26.41
Rooster+ 2	-5.61	-9.84	-20.11		-26.93	
Rooster+ 3	-5.93	-10.90	-22.02	-23.25	-25.28	-26.52
Rooster+ 4	-5.60		-21.85	-23.72	-24.63	-27.96

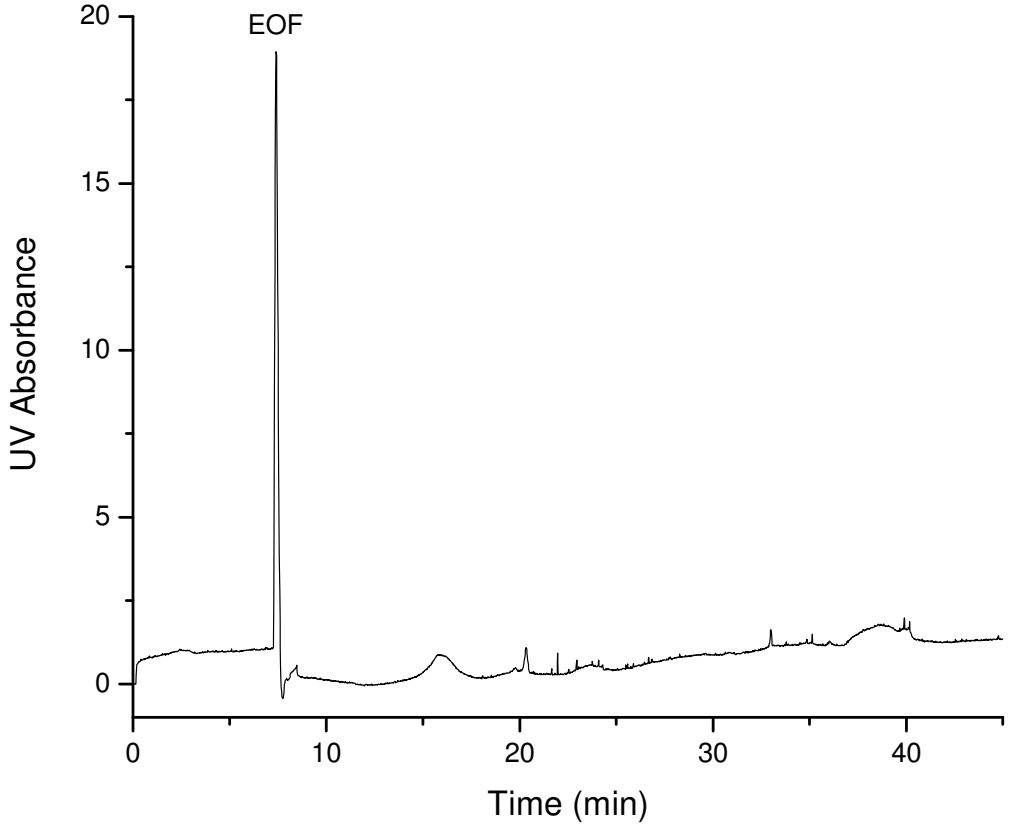

Fig. 17. Electropherogram of first step particles from an estrogen naïve rooster. BGE: 3.5mM SDS. UV absorbance at 214 nm.

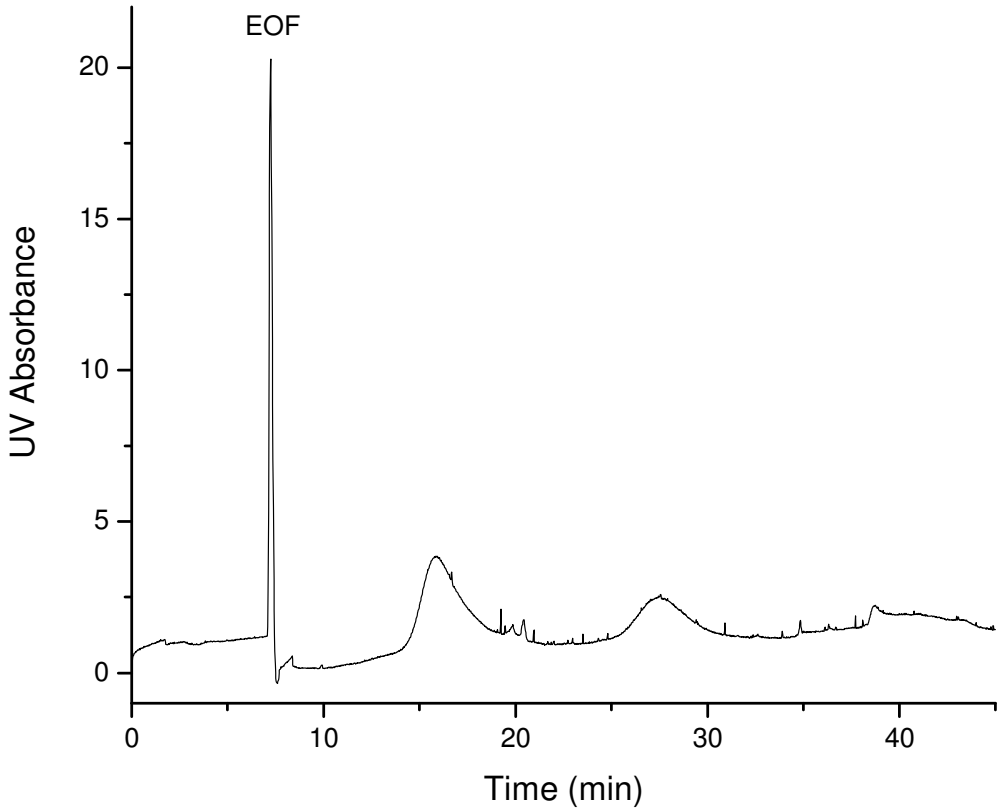

Fig. 18. Electropherogram of first step particles from an estrogen treated rooster (24 hours after estrogen treatment). BGE: 3.5mM SDS. UV absorbance at 214 nm.

CHAPTER VI

DISCUSSION

The chicken is an excellent model for studying triglyceride-rich lipoprotein assembly in liver due to the ability of injected estrogen to rapidly increase VLDL production. The affects of estrogen on plasma lipoproteins has been well studied, but relatively little is known about the intermediate forms of these particles. This study has demonstrated successful isolation of VLDL assembly intermediates from chicken liver. The concentration of first step VLDL particles found in the RER of rooster livers increased dramatically in response to exogenous estrogen injection. This was demonstrated by comparing the area under the curve values obtained from electropherograms of first step particles from untreated and estrogen treated roosters.

Estrogen not only increased the number of first step particles but it also altered the diameter of the first step particles. This study demonstrated that first step particles from estrogen treated birds have significantly smaller diameters than those isolated from the livers of estrogen naïve roosters. The diameter of first step particles increases 24 hours after estrogen treatment. This is consistent with previous studies which have shown that estrogen's effects on the liver decline 24 hours after exposure [5].

The present studies suggest that CE is an effective method for the study of intact VLDL and first step VLDL particles. Determination of charge:volume is a unique physical characteristic which has not been well studied in these particles. This study demonstrated that effective mobilities for plasma VLDLy from hens and estrogen treated

roosters, $\mu_e \approx -14 \times 10^{-5}$ cm²/V.s, was lower than the average effective mobility for plasma VLDL from estrogen naïve roosters in which average μ_e for all subclasses was $\approx -25.4 \times 10^{-5}$ cm²/V.s. This difference in mobility indicates that rooster VLDL has a larger charge:volume than VLDLy. This is consistent with our hypothesis as the presence of a relatively positively charged apolipoprotein, apo VLDL II, would be expected to decrease μ_e.

This study also demonstrated the presence of possible "subclasses" within rooster plasma VLDL. A wide distribution of particle diameters would be expected in rooster VLDL as it is susceptible to lipoprotein lipase. Hydrolysis of the VLDL would lead to lipoproteins of various diameters. Charge would remain relatively constant due to the presence of apo B and other apolipoproteins. The neutral lipids lost due to hydrolysis would lead to decreased mass of the particle but would not affect the net charge. Therefore, the charge:volume of the VLDL particle would increase as a result of hydrolysis. If indeed these "subclasses" represent VLDL at varying degrees of hydrolysis than the peaks furthest to the left, those with the lowest μ_e, would represent newly produced VLDL particles. Those peaks with higher μ_e represent older, more hydrolyzed VLDL. In previous studies of lipoprotein particle diameters our laboratory has shown that particle diameter of VLDL from untreated roosters is widely variable. However, we have never observed such distinct "subclasses" as were observed when these particles were separated using CE. VLDLy is not susceptible to hydrolysis by lipoprotein lipase and "subclasses" were not observed in plasma VLDLy from hens or estrogen treated roosters.

Intact first step VLDL assembly intermediates isolated from rooster liver were also analyzed using CE. First step particles from estrogen treated roosters had lower μ_e than those from estrogen naïve roosters. This difference was significant at both 12 and 24 hours after estrogen treatment. This decrease in μ_e represents decreased charge:volume. Based on the particle diameter studies we know that first step particles from estrogen treated roosters have smaller particle diameters than those from estrogen naïve birds. If decreased diameter were the only change to occur in response to estrogen than we would expect an increase in charge:volume and therefore higher μ_e. The opposite effect was observed in these studies, suggesting that some other change, other than decreased particle diameter, occurs in these particles. The presence of a positively charged protein, such as apo VLDL II, in conjunction with decreased particle diameter could explain this decrease in μ_e. The calculated area under the curve for first step particles increased significantly after estrogen exposure. This is consistent with the known increase in VLDL production that occurs in response to estrogen.

It is important to note that these studies were done with previously frozen first step particles. If cryopreservation affects charge:volume equally for first step particles from both estrogen naïve and estrogen treated roosters than these differences would still be apparent. However, the effects of cryopreservation with sucrose are unknown for first step particles. As cryopreservation did affect the electrophoretic mobility of some of the particles examined in these studies, the affects of the freeze/thaw process on first particles will need to be further evaluated.

The effects of freezing are only problematic when looking at intact particles. The presence of SDS used to delipidate the lipoproteins would eliminate any aggregation that had occurred during the freeze/thaw process. In this study charge:volume of intact rooster VLDL was altered by cryopreservation even with the addition of sucrose. Average effective mobility of previously frozen, intact rooster VLDL was greater than that of fresh samples. This effect was not significant in hen VLDLy. It is possible that some physical chemical property of VLDLy make it less susceptible to the effects of freezing. It may be that the smaller diameter of VLDLy is cryoprotective. The hen VLDLy in this study had an average diameter of ~20nm, similar to that of the human LDL used in Rumsey's cryopreservation study. Their study demonstrated successful cryopreservation of human LDL by the addition of sucrose [43]. This method of cryopreservation has not previously been tested on VLDL particles. Our study suggests that the addition of sucrose may not be protective for all types of lipoproteins during the freeze thaw process. The level of protection provided may be dictated by the diameter of the lipoprotein particles. The effects of cryopreservation with sucrose were not evaluated for first step particles. If indeed size is a factor than the smaller first step particles may be protected by the addition of sucrose.

This study also demonstrates the potential use for CE in the further elucidation of the proteins involved in the VLDL assembly process. The presence of apo B on the first step particle is key to the process of VLDL assembly. It is unknown whether or not first step particles also contain other apolipoproteins. Previous studies within our collaborators' laboratory demonstrated that human apo B-100 has an effective mobility

of approximately -19 x 10^{-5} cm^2/V.s when a similar SDS-containing CE buffer was used [48]. As the amino acid sequence of apo B is highly conserved across species [15] it seemed likely that chicken apo B would demonstrate similar mobility. Delipidated plasma VLDL and VLDLy showed a peak with approximate mobility of -20 x10^{-5} cm^2/V.s. As this peak appeared consistently throughout all plasma VLDL, VLDLy and first step particles and has a similar mobility to those previously observed, it may represent apo B.

In most samples multiple peaks with larger effective mobilities, ranging from -21 to -26 x10^{-5} cm^2/V.s, were observed. These likely represent particles that contain apo B but have retained some of the lipid components. Such an outcome would be consistent with previous studies in which VLDL from hyperlipidemic humans demonstrated a similar profile when separated using the same buffer system [48].

In the presence of SDS all proteins have the same charge:volume ratio as SDS binds to proteins in a specific ratio. This means that separation of proteins is entirely based on molecular weight. A small peak with an effective mobility of approximately -2 x 10^{-5} cm^2/V.s was detected in all of the plasma VLDL and VLDLy sample. It is likely that this peak represents one of the smaller apolipoproteins such as apo C-I with an approximate MW of 6.6 kDa.

A unique peak was present exclusively in delipidated plasma VLDLy from hens and estrogen treated roosters. This peak had a mobility of approximately -10 x 10^{-5} cm^2/V.s. This peak may represent apo VLDL II as it is seen only in those birds exposed

to estrogen. This peak had a lower effective mobility than apo B and therefore represents a protein of lower molecular weight.

A peak with similar mobility, to that of the presumed apo VLDL II peak, was also observed in the delipidated first step particles from estrogen treated roosters. This, along with the shift in mobility of intact first step particles discussed above, would further support the presence of apo VLDL II on the first step particles of estrogen treated roosters. A second peak with an effective mobility of approximately -5 x 10^{-5} $cm^2/V.s$ was detected for first step particles from estrogen treated birds. This peak was not detected on any of the other samples. This peak has a mobility half that of the presumed apo VLDL II peak. In chickens apo VLDL II functions as a homodimer. It may be that this peak represents the monomeric form of apo VLDL II. Apo VLDL II is responsible for the decreased particle diameter of VLDLy, its presence on the first step particle would suggest that the diameter of the mature VLDLy may be determined during the first step of VLDL assembly.

In order to positively identify these protein components, application of additional analytical techniques would be required. One possibility would be to obtain known protein standards and determine effective mobilities of those standards using this CE buffer system. Another option would be to combine the use of CE with mass spectrometry, allowing more accurate identification of each of the protein components.

CHAPTER VII

SUMMARY AND CONCLUSIONS

This project was able to demonstrate successful isolation of first step VLDL assembly intermediates from hen and rooster liver. CE was shown to be a useful method for the study of intact VLDL, VLDLy and first step VLDL particles. Cryopreservation with sucrose significantly altered the charge:volume of intact rooster VLDL. This study suggests that CE may be useful in the study of VLDL subclasses. CE can also be used to separate individual protein components from delipidated VLDL, VLDLy and first step VLDL particles. Use of this method, in conjunction with mass spectrometry, may lead to the identification of those proteins found on the first step particle. Increasing our knowledge of VLDL assembly intermediates gives us further insight into the VLDL assembly process.

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APPENDIX

TEXAS A&M UNIVERSITY
University Laboratory Animal Care Committee
1112 TAMU, College Station, TX 77843-1112
(979) 845-1828 FAX (979) 862-3176
318 Jack K. Williams Administration Building

Dr. Dianne C. Kraemer, Chair
Dr. Elizabeth Browder, Attending Veterinarian
Dr. Michael W. Buckley, Director, Research Compliance
Olivia Ash, Program Coordinator

September 1, 2004

MEMORANDUM

TO: Dr. Rosemary Walzem
Poultry Science
MS 2472

FROM: Olivia Ash, Program Coordinator OA

SUBJECT: Annual Review
AUP # 2002-317
Title: "Apolipoprotein B-100 VLDLy Assembly by Bird Hepatocytes"
Original Approval Date: 10/28/2002
Expiration Date: 10/28/2005

Your Animal Use Protocol (AUP) Annual (Continuing) Review was approved based upon the information provided. The committee thanks you for keeping your protocol current.

The A&M ULAC Committee must approve documentation from you during the term of your experimental study as established by federal regulations. The ULAC Committee bases the review date on the approval date of the original AUP. Documentation required will include but not be limited to the following:

- This AUP must continue to be reviewed annually. The ULAC Committee will not consider requests for amendments to the AUP unless your annual review documentation is current and approved. You will provide documentation for the annual review on a form available from the ULACC office.
- A new AUP will be required to continue this research beyond the approved three years.

If you have any questions, please contact me at 845-1828.

JOA/brg

Enclosure: form

Pc: Routing Agency: Alecia Arnold, TAES, MS 2147
Housing Facility: Aleta Susnik, Poultry Farm, MS 2471
Office of Campus Veterinarian: Dr. Elizabeth Browder, LARR, MS 4473
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EDUCATION

Master of Science, Nutrition, Texas A&M University, College Station, 5/05

Bachelor of Science, Nutrition, University of Arizona, Tucson, 5/01 Magna Cum Laude

PROFESSIONAL

Nutrition Support Dietitian, Scott and White Memorial Hospital Temple, TX, 6/04 – Present

Clinical Dietitian, Scott and White Memorial Hospital Temple, TX, 8/03 – 6/04

Dietetic Intern, Texas A&M University, 1/03 – 7/03

Research Assistant, Texas A&M University, 1/02 – 12/02

Teaching Assistant, Texas A&M University, 8/01 – 12/01