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Chen, Shwu-Pyng T.

LIPOPROTEINS IN HUMAN MILK

The Ohio State University

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LIPOPROTEINS IN HUMAN MILK

DISSERTATION

Presented in Partial Fulfillment of the Requirements
for the Degree Doctor of Philosophy in the
Graduate School of The Ohio State University

By

Shwu-Pyng T. Chen, B.S., M.S.
The Ohio State University
1986

Dissertation Committee:
P. M. T. Hansen
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Adviser

Department of Food Science and Nutrition
To my parents and my beloved husband, Loren
ACKNOWLEDGMENTS

I express my sincere gratitude to my advisor, Dr. P. M. T. Hansen, for his guidance and continued encouragement throughout the study.

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Special gratitude is expressed to my sister's family in Connecticut and my Chinese friends in Columbus, especially Ling, Shih, Debbie, Lam and Yang's family, for their spiritual concern and intellectual support.

I greatly appreciate the financial support received from the Department of Food Science and Nutrition. Without such support, my study could not have been completed.
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          2. Providing Services of analyzing milk compositions for
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           2. Food structure and interaction. Winter / 1984, 85, 86
           3. Food system II. Concentrated and frozen foods. Spring / 1984
           4. Seminar - analysis of technical problems in food science.
              Fall / 1984

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INTRODUCTION

Lipoproteins, especially human blood serum lipoproteins, have received a surging interest in recent years. The attention has resulted mainly from the roles they are playing in transporting plasma cholesterol, an important risk factor in cardiovascular disease. Because of this important function, the physical, chemical properties as well as the methods for the isolation and characterization of those lipoproteins have been well studied and documented.

In milk systems, "spontaneous lipolysis" causes an elevation of free fatty acids. This increase results in flavor impairment in milks and dairy products. It is also linked to breast milk jaundice where breast-fed infants are concerned. The cause of such lipolysis is found to relate to the presence of components of blood serum lipoproteins.

It has been indicated that serum-related lipoproteins are present in human milks. However, these milk lipoproteins have received very little study. Whether they are identical to those present in blood is still unknown. It is the attempt of this investigation to adapt the methods established for studying blood serum lipoproteins to closely examine the serological and physical properties of the serum-related lipoproteins in human milk system.
LITERATURE REVIEW

Lipoproteins are macromolecules which are formed by association of specific proteins with certain lipids through non-covalent interactions (Owen and McIntyre, 1982). Based on their function in living systems, they can be classified into two major types -- namely transport lipoproteins and membrane lipoproteins. Each type of the lipoproteins has its distinct characteristics.

I. Transport Lipoproteins in human blood serum

Human serum lipoproteins provide a system capable of transporting hydrophobic lipids in an aqueous medium (Pownall et al, 1982). Through this system, the lipids can be transported from one tissue to another for metabolism.

A. Classifications and Nomenclatures

The major constituents of serum lipoproteins are proteins and lipids. Because of the inclusion of lipids, serum lipoproteins are characterized by relatively low hydrated densities (Lewis and Oppl, 1980). This distinct property has been utilized as a popular classification criterion for serum lipoproteins. Based on buoyant density, serum lipoproteins can be separated into four major density classes designated as chylomicrons (CM), very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) (Brewer and
Bronzert, 1977; Lewis and Oppelt, 1980). Other classification systems have also been used. They are either based on electrophoretic mobility or on lipoprotein families. A lipoprotein family is a polydisperse system characterized by the unique presence of a single apolipoprotein or its constituent polypeptides. An apolipoprotein is the lipid-free portion of the lipoproteins which may consist of a single polypeptide chain or nonidentical polypeptides (Lewis and Oppelt, 1980). The nomenclatures for different classification systems are shown in Table 1.

B. Chemical compositions and physical properties

Serum lipoproteins, in addition to apoproteins, contain different classes of lipids which include triacylglycerols, phospholipids, free cholesterol and cholesterol esters (Brewer and Bronzert, 1977; Lewis and Oppelt, 1980). Different combinations of lipid components give each lipoprotein specific size and density. The summarized physical and chemical properties of lipoproteins are shown in Table 2.

The apoproteins of the human serum lipoproteins have been intensively studied in the past decade. At the present time, there are six well known apoproteins. These include apoA, B, C, D, E and F (Koren, McConathy and Alaupovic, 1982; Lewis and Oppelt, 1980; Rall, Weisgraber and Mahley, 1982). Most of them are well characterized. Their properties are listed in Table 3.

The apoproteins generally are amphipathic in nature and, thus, facilitate binding of lipids (Rall, Weisgraber and Mahley, 1982; Scanu, Byne and Mihovilovic, 1982). Amphipathic helical lipid-binding regions have been identified in all of the apoproteins whose secondary structure
<table>
<thead>
<tr>
<th>Classification system</th>
<th>Nomenclatures</th>
</tr>
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<tr>
<td>Hydrated density</td>
<td></td>
</tr>
<tr>
<td>Chylomicrons</td>
<td>VLDL</td>
</tr>
<tr>
<td></td>
<td>LDL (LDL₁)</td>
</tr>
<tr>
<td></td>
<td>(LDL₂)</td>
</tr>
<tr>
<td></td>
<td>HDL (HDL₁)</td>
</tr>
<tr>
<td></td>
<td>(HDL₂)</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td></td>
</tr>
<tr>
<td>Omega-Lp</td>
<td>Pre-Beta-Lp</td>
</tr>
<tr>
<td></td>
<td>Beta-Lp</td>
</tr>
<tr>
<td></td>
<td>Alpha-Lp</td>
</tr>
<tr>
<td>Lp-A (A-I, A-II)</td>
<td>Lp-B</td>
</tr>
<tr>
<td></td>
<td>Lp-B</td>
</tr>
<tr>
<td></td>
<td>Lp-A (A-I, A-II)</td>
</tr>
<tr>
<td>Lp-B</td>
<td>Lp-C (C-I, C-II, C-III)</td>
</tr>
<tr>
<td></td>
<td>Lp-C (C-II)</td>
</tr>
<tr>
<td>Lp-C (C-I, C-II, C-III)</td>
<td>Lp-E</td>
</tr>
<tr>
<td></td>
<td>Lp-D</td>
</tr>
<tr>
<td></td>
<td>Lp-E</td>
</tr>
</tbody>
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a. The information is summarized from Lewis and Oppl, 1980; Brewer and Bronzert, 1977; Zubay, 1983; Cardin, Holdsworth and Jackson, 1984.
b. Major apoprotein in the type of lipoprotein.
Table 2. PHYSICAL AND CHEMICAL PROPERTIES OF HUMAN SERUM LIPOPROTEINS

<table>
<thead>
<tr>
<th></th>
<th>Chylomicron</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
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<tbody>
<tr>
<td>Density (g/ml)</td>
<td>&lt;0.94</td>
<td>0.94-1.006</td>
<td>1.006-1.063</td>
<td>1.063-1.21</td>
</tr>
<tr>
<td>Diameter (nm)</td>
<td>100-1000</td>
<td>30-80</td>
<td>20-30</td>
<td>7.5-20</td>
</tr>
<tr>
<td>Mean Mol. Wt.</td>
<td>504 x 10^6</td>
<td>19.6 x 10^6</td>
<td>2.3 x 10^6</td>
<td>(0.15-0.36) x 10^6</td>
</tr>
<tr>
<td>Svedberg Unit(s)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sf (1.063 g/ml)</td>
<td>400-5000</td>
<td>12-400</td>
<td>0-12</td>
<td>sediment</td>
</tr>
<tr>
<td>F (1.20 g/ml)</td>
<td>-</td>
<td>43-770</td>
<td>20-43</td>
<td>sediment-9.0</td>
</tr>
<tr>
<td>Composition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (%)</td>
<td>0.5-2</td>
<td>7-12</td>
<td>11-25</td>
<td>33-57</td>
</tr>
<tr>
<td>Lipid (%)</td>
<td>98-99.5</td>
<td>88-93</td>
<td>75-89</td>
<td>43-67</td>
</tr>
<tr>
<td>Triglyceride (%) total lipid</td>
<td>88^b</td>
<td>56^b</td>
<td>13-29</td>
<td>13-16</td>
</tr>
<tr>
<td>Phospholipid (%) total lipid</td>
<td>8</td>
<td>20</td>
<td>26-28</td>
<td>43-46^b</td>
</tr>
<tr>
<td>Free cholesterol (%) total lipid</td>
<td>1</td>
<td>8</td>
<td>9-10</td>
<td>6-10</td>
</tr>
<tr>
<td>Cholesterol ester (%) total lipid</td>
<td>3</td>
<td>15</td>
<td>34-48^b</td>
<td>29-31^b</td>
</tr>
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</table>

a. The information is from Brewer and Bronzert, 1977; Martin, Hayes and Rodwell, 1983; Okazaki and Hara, 1984; Zubay, 1983.
b. Major lipid.
<table>
<thead>
<tr>
<th>Apoprotein</th>
<th>Molecular wt.</th>
<th>Function</th>
<th>Miscellaneous</th>
</tr>
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<tr>
<td>A-1</td>
<td>28,000-28,331</td>
<td>LCAT&lt;sup&gt;b&lt;/sup&gt; activator</td>
<td>contain 245 amino acids &amp; no CHO</td>
</tr>
<tr>
<td>A-II</td>
<td>17,000-17,400</td>
<td>LCAT inhibitor ?</td>
<td>two identical chains with 77 amino acids each, joined by a disulfide bond at residue 6</td>
</tr>
<tr>
<td>B</td>
<td>250,000-270,000</td>
<td>lipoprotein biosynthesis/secretion; binding by cell surface receptors</td>
<td>insoluble in aqueous buffers except in the presence of detergents or denaturants; structure remains elusive</td>
</tr>
<tr>
<td>C-I</td>
<td>6,625-7,000</td>
<td>LCAT and lipase activator ?</td>
<td>contain 57 amino acids</td>
</tr>
<tr>
<td>C-II</td>
<td>9,110-10,000</td>
<td>LPL&lt;sup&gt;c&lt;/sup&gt; activator</td>
<td>contain 79 amino acids</td>
</tr>
<tr>
<td>C-III</td>
<td>8,764-10,000</td>
<td>? lipase inhibitor</td>
<td>contain 79 amino acids and Gal, GalNac, NeuNac</td>
</tr>
<tr>
<td>D</td>
<td>20,000-35,000</td>
<td>? LCAT activator</td>
<td>also known as cholesterol ester transfer protein</td>
</tr>
<tr>
<td>E</td>
<td>32,000-39,000</td>
<td>? cholesterol transport &amp; binding by cell surface receptors</td>
<td>contain 290 amino acids, also known as arginine rich lipoprotein</td>
</tr>
<tr>
<td>F</td>
<td>28,000</td>
<td>---</td>
<td>minor lipoproteins in human serum, occur mostly in HDL and LDL</td>
</tr>
</tbody>
</table>


b. LCAT = Lecithin - Cholesterol Acyltransferase.

c. LPL = Lipoprotein Lipase.
are known (Gotto, Jr, 1983). These apoproteins provide serum lipoproteins with recognition sites for cell-surface receptors and also supply the co-factors for enzymes involved in their metabolism (Owen and McIntyre, 1982; Owen, McIntyre and Gillett, 1984; Mahley et al., 1984). The distribution and the concentration of these apoproteins in major serum lipoproteins are shown in Table 4.

The structures of the major lipoproteins of normal plasma show great similarity (Brewer and Bronzert, 1977; Oeswein and Chun, 1981; Owen and McIntyre, 1982; Gotto, 1983; Eisenberg, 1984). Generally, they are spherical or nearly spherical and contain an apolar core of triglyceride and cholesterol ester. Surrounding the core, there is a monolayer which consists of apoproteins, phospholipids and free cholesterol. It is this layer that makes lipoproteins hydrophilic (Beckelbaum et al., 1984; Hrisoho, 1984). The fluid mosaic model has been suggested as the way of molecular organization in serum lipoproteins (Brewer and Bronzert, 1977; Hrisoho, 1984).

The function of plasma lipoproteins is to transport lipids for cellular metabolism. Exogeneous lipids, such as dietary lipids are mainly transported as chylomicrons(CM) in the blood. The CM exchange the lipids they carry with body tissue through the action of LPL (activated by apo C-II). Phospholipids and free cholesterol are shifted into HDL for further metabolism (Hrisoho, 1984). The remnant CM is one of the sources of nacent HDL particles. Nacent HDL particles can also be secreted from liver and intestine. They may also originate from secretion of lipid-laden macrophages (Witztum, 1984). Endogeneous lipids, i.e.
Table 4. PERCENT APOPROTEIN DISTRIBUTION IN HUMAN SERUM LIPOPROTEINS

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>% protein</th>
<th>ApoA-I</th>
<th>ApoA-II</th>
<th>ApoB</th>
<th>ApoC-I</th>
<th>ApoC-II</th>
<th>ApoC-III</th>
<th>ApoD</th>
<th>ApoE</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL</td>
<td>12</td>
<td>37</td>
<td>4</td>
<td>6</td>
<td>40</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>25</td>
<td>Trace</td>
<td>99</td>
<td>Trace</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td>50</td>
<td>71</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. The information is from Peeters, 1983.
lipids of hepatic origin, are transported starting from secretion of VLDL in the liver. After its secretion, it undergoes gradual delipidation and releases its triglycerides into extrahepatic tissues. Meanwhile, it undergoes mass transfer with HDL to remove redundant surface materials. Thus, VLDL gradually acquires higher and higher density and, finally, it becomes a LDL particle containing mostly cholesterol esters in its core (Havel, 1984). The LDL, then, degrades in the liver or in peripheral cells by turning its cholesterol ester into an intracellular cholesterol pool through hydrolysis (Brewer and Bronzert, 1977; Hrisoho, 1984; Owen and McIntyre, 1982). The pathway for the degradation of LDL includes "receptor-mediated endocytosis" of LDL receptor-independent pathway (Goldstein and Brown, 1982).

Lipoprotein particles play an important role in regulating plasma cholesterol concentration, an important factor involved in coronary heart disease (Thompson and Bortz, 1978). According to Witztum (1984):

In normal man, LDL carries over two-thirds of the total plasma cholesterol content. LDL particles serve to deliver cholesterol to numerous nonhepatic tissues as well as to return cholesterol to the liver.

If the pathways run for clearance of LDL from plasma are not balanced, i.e. the receptor-mediated pathway is suppressed, elevated plasma levels of LDL results. Consequently, the plasma cholesterol concentration become elevated. This elevation of plasma cholesterol could cause cholesterol deposition in the cells and in the extracellular matrix of the artery and, therefore, may be directly involved in atherosclerosis (Witztum, 1984). In the case of HDL, according to Witztum (1984):
The "nascent" HDL particles of diverse origin could easily serve as an acceptor of free cholesterol from a variety of sources including macrophages or other somatic cells. All such nascent HDL-containing apo AI and free cholesterol bound to a phospholipid bilayer are postulated to serve as a substrate for the LCAT reaction, which serves to esterify the membrane-bound free cholesterol. Once esterified, cholesterol becomes more nonpolar and moves to a more central, nonpolar region of the particle producing the spherical, "mature" HDL particle. There is considerable evidence linking the rate of catabolism of triglyceride-rich particles, and hence generation of nascent HDL particles and levels of "mature" HDL particles in plasma. In part, this could explain the reported protective effect of elevated HDL levels. In part, HDL levels may also reflect the net transfer of cholesterol from non-hepatic tissues to liver via transfer of cholesteryl esters from HDL to IDL and LDL for subsequent return to liver, via transfer of cholesterol directly to liver from HDL, or even by hepatic uptake of intact HDL particles.

C. Isolation and characterization

A diversity of methods have been used for studying serum lipoproteins. These methods utilize different characteristics of serum lipoproteins as a basis for either qualitative or quantitative analysis (Ferreri, 1984).

Based on density differences, ultracentrifugation has traditionally been used for separating serum lipoproteins. This method may be used as sequential ultracentrifugation (Havel, Eder and Bragdon, 1955); density gradient separation (Terpstra, Woodward and Sanchez-Muniz, 1981); zonal centrifugation (Danielsson, Ekman and Johansson, 1978) or analytical ultracentrifugation (DELalla and Gofman, 1954). Generally, this method is very time consuming, may involve expensive equipment and require a large amount of sample for the separation. Although an air-driven ultracentrifuge has been introduced to shorten the time of analysis to 2.5 hr
(Rosseneu et al, 1982), the quality of the separation is not comparable to the results obtained in conventional preparative ultracentrifuge (Shireman and Williams, 1983).

Electrophoresis, which is based on size and charge separation, has also been used for the isolation. Preparative as well as analytical systems have been reported (Ferreri, 1984). Supporting media used for electrophoresis include paper, agarose-gel, starch-gel and polyacrylamide-gel (Lewis and Opplt, 1980). Electrophoresis patterns may be used for qualitative identification of disorders involving serum lipoproteins. However, since different lipoprotein may show different stainability (Parra et al., 1982), the method usually is not recommended for quantitative purposes. When electrophoresis is used as a preparative system, it may involve tedious, time- and effort-consuming procedures (Ferreri, 1984). Therefore, it is used for this purpose only under unusual situations, e.g. minimization of contaminating proteins.

A rapid precipitation method utilizing polyanions for separation has been introduced (Burstein, Scholnick and Morfin, 1970). This method involves interaction between polyanions, e.g. heparin, dextran sulfate, sodium phosphotungstate and lipoproteins with the help of divalent cations, e.g. manganese or magnesium from the MnCl₂ or MgCl₂ (Burstein and Legmann, 1982). It can be done directly for isolation or applied after electrophoresis for quantitation (Neuback et al, 1977). Technically, it is simple to perform and can yield large quantities of lipoproteins. However, the uncertainty to the identity as well as quality of the fractions isolated may limit its routine use (Ferreri, 1984).
Gel filtration (or permeation) chromatography for separation of molecules based on size and shape differences has been applied as a separating system for serum lipoproteins. Agarose gel is the mostly widely used media for separation (Rudel et al., 1974). In recent years, HPLC methods utilizing combination of columns of different pore sizes have been reported to be successful for separating individual classes of lipoproteins (Okazaki and Hara, 1984; Busbee et al, 1981; Vercaemst and Rosseneu, 1983). Generally, this method is simple and offers satisfactory separation, but requires expensive equipment. The method also requires ultracentrifugation for initial collection of total lipoproteins. Microtechniques of subsequent characterizations may also be necessary because of dilution from elution (Ferreri, 1984). Characterization of serum lipoproteins has been accomplished by analysis of chemical composition, e.g. cholesterol content (Hainline, Karon and Lippel, 1982; Havel, Eder and Bragdon, 1955), analytical ultracentrifugation (Bickerstaffe and Desmond, 1982; DE Lalla and Gofman, 1954) and immunological assay (Lewis and Opplt, 1980) in combination with the isolating method described previously.

II. Membrane lipoproteins in milk system

Membrane, in its simplest form, can be visualized as proteins embedded in or attached to a lipid bilayer (Owenm McIntyre and Gillett, 1984). The composition of a membrane is similar to the surface coat of plasma lipoproteins. The lipid components of a cell membrane include mainly phospholipids and cholesterols. Membrane proteins can be grouped into integral membrane proteins (transmembrane or embedded and
projecting from either side of the membrane) or peripheral membrane pro-
teins (adsorbed to the membrane surface or bound to a transmembrane protein) (Brown, 1984). According to Brown (1984): "For integral mem-
brane proteins, the predominant interactions with the lipid bilayer are nonpolar." \( \alpha \)-helix and \( \beta \)-barrel are:

two types of secondary structure that can serve as trans-
membrane channels by segregating charged or polar amino acid side chains into the center of the pore while hydrophobic side chains are in contact with the hydrocarbon portion of the lipid. (Brown, 1984)

For peripheral membrane proteins, the primary interaction to the lipid bilayer are electrostatic attraction from their charged amino acid residues to the ionic portion of the lipid. The structure model for the binding of peripheral proteins to membrane is proposed to be an amphipathic helix, which is also the one postulated for lipid-protein binding of plasma lipoproteins. According to Brown (1984):

Important features of a lipid-binding helix are the presence of at least one ion pair spanning a distance of not more than five amino acid residues in the linear sequence and a high average hydrophobicity for the nonpolar face. This nonpolar face then is interwoven with the hydrocarbon tails of the lipid components while the polar, charged face interacts with the ionic head groups of the lipid.

In milks, the membranes exist mainly as milk fat globule membrane (MFGM). This material is composed of a mixture of proteins, phospholipids, glyco-proteins, triglycerides, cholesterol, enzymes and other minor components (Anderson and Cawston, 1975). The membrane acts as a natural stabilizing agent which enables milk fats to disperse in the
aqueous phase (Mcpherson and Kitchen, 1983). Although reports concerning bovine milk membrane materials are numerous, information concerning membrane materials from human milk is relatively scarce.

The composition of the membrane lipid classes in human milk has been reported (Table 5) by Bracco et al., 1972. This is the only data concerning lipid constituents of human MFGM that has been indicated reliable (Jensen, Hagerty and McMahon, 1978; Lammi-Keefe and Jensen, 1984). The protein moiety of MFGM in human milk has also been studied. By using sodium dodecyl sulfate - two dimensional gel electrophoresis and staining with Coomassie Blue, at least 35 detectable membrane proteins were found (Imam, Taylor and Tokes, 1984). Among these proteins, a few have been characterized (Imam, Laurence and Neville, 1981; Shimizu and Yamauch, 1982; Imam, Laurence and Neville, 1982; Imam, Taylor and Tokes, 1984). The characterized proteins are mainly glycoproteins.

A human MFGM coat fraction which is a dense layer sandwiched between the MFGM and the outer shell of the fat droplet with finely filamentous texture has been isolated and studied (Freudenstein et al., 1979). It is found to be intimately associated with small amounts of phospholipids, gangliosides and carbohydrates.

The isolation or characterization of specific lipoproteins in human milk, either of membrane or serum origin, has not been reported. However, studies concerning lipoproteins in bovine milk have been conducted. In bovine milk, lipoproteins are found to occur in both cream and skim milk fractions (Jensen, 1973).
<table>
<thead>
<tr>
<th>Lipids</th>
<th>% fat globule membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocarbons</td>
<td>trace</td>
</tr>
<tr>
<td>Sterol esters</td>
<td>trace</td>
</tr>
<tr>
<td>TG</td>
<td>58.2</td>
</tr>
<tr>
<td>DG</td>
<td>8.1</td>
</tr>
<tr>
<td>MG</td>
<td>0.6</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>7.3</td>
</tr>
<tr>
<td>Sterols</td>
<td>0.653</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>23.4</td>
</tr>
</tbody>
</table>

(This table is adapted from Bracco et al, 1971)
A. Lipoproteins in cream

In cream, lipoproteins are mainly associated with MFGM. Studies of lipoproteins are complicated. First of all, the exact structural organization of the MFGM has not been resolved (McPherson and Kitchen, 1983). How the components orient within the membrane matrix is still unknown. Secondly, the membrane itself is a lipid-protein complex. It can be separated into different heterogeneous lipoprotein subfractions by various techniques, including continuous resuspension and centrifugation, detergent release, differential centrifugation and density gradient centrifugation (McPherson and Kitchen, 1983). These subfractions differ in buoyant density, lipid composition and polypeptide components. They are either from fractionation of the entire membrane material (Chien and Rechardson, 1967; Swope and Brunner, 1970; Kitchen, 1977) or from released material after detergent treatment (Alexander and Lusena, 1961; Hayashi, Erickson and Smith, 1965). However, whether the detergent released lipoproteins are intact individual components or simply are different fragments of the membrane lipid-protein complex, resulting from the asymmetric organization of the membrane, has not yet been elucidated (McPherson and Kitchen, 1983). Certain models have been proposed to designate the structure of the MFGM (Chien and Rechardson, 1967; Swope and Brunner, 1970; Hayashi, Erickson and Smith, 1965). First of all, MFGM contains a structure matrix composed of water-insoluble lipoprotein complexes to which the water-soluble lipoproteins adsorbed (Hayashi, Erickson and Smith, 1965). Secondly, MFGM is a complex lipoprotein system composed of five or more lipoprotein particulate systems.
Some of them are located on the outer layer but some in the inner (Chien and Rechardson, 1967). Finally, MFGM is a heterogeneous system consisting of layers of protein or lipid-protein complexes, or both, to which lipoprotein micelles of varying sizes are adsorbed (Swope and Brunner, 1970). These models clearly indicate the difficulties in specifying a membrane lipoprotein. For this reason, any study concerning the isolation of lipoproteins from MFGM must clearly specify which fractions are being dealt with and which method is used for isolation. An operational definition may be also required (Lewis and Opplt, 1980).

A homogeneous water-soluble low-density lipoprotein has been isolated from disrupted microsomes in bovine buttermilk (Berlin et al., 1967). Milk microsomes were obtained by sedimentation and disruption through a high-pressure cell (8,000 psi). This lipoprotein floated in salt solution of density 1.063 and had a molecular diameter of 232 A. It is unique in its high phospholipid content and extremely low cholesterol value (Berlin et al., 1967; Jensen, 1973; Anderson and Cawston, 1975). The gross composition of it is: 12.87% protein, 87.13% total lipid (52.02% phospholipid and 35.11% neutral lipid). The relationship between this LDL and serum LDL was not studied.

B. Lipoproteins in skim milk

Another lipoprotein is clearly present in the skim milk fraction of bovine milk (Jensen, 1973). It was obtained by ultracentrifugation in the density range of 1.063 – 1.21 g/ml (Patton and Keenan, 1971). This lipoprotein accounts for 42% of the lipid phosphorus in milk, the other 58% occurring in the MFGM. It is suggested that this component is not a
shed from MFGM but may originate from Golgi vesicle membranes or plasma membrane of the lactating cell (Patton and Keenan, 1971). It is also reported to be similar to the one that has been isolated from bovine buttermilk microsomes (Jensen, 1973).

III. Relationship between human milk and human blood plasma

Milk is closely related to blood. The components are either directly transferred from blood or indirectly derived from precursors in the blood (Linzell and Peaker, 1971; Ribadeau-Dumas, 1983; Mcpherson and Kitchen, 1983). The lipids, for example, are largely from lipolysis of VLDL and chylomicrons in blood through actions of lipoprotein lipases (Patton, 1973). Large protein molecules, e.g. blood serum albumin and ferritin can also find their way into milk (Jensen and Pitas, 1976). According to Ribadeau-Dumas (1983):

Although most of the proteins in milk are synthesized in the secretory cells of the mammary gland, breast milk also contains virtually all the proteins found in the blood, albeit in small quantities.

Under certain conditions, particularly mastitis infection, milk may contain a large amount of constituents transferred directly from blood (Anderson and Needs, 1983).

The antigenic property of human milk and its relationship to blood plasma has been studied. By using immunological analysis, at least 30 antigens have been demonstrated in human colostrum. Prolonging the lactation period decreases the number of detectable precipitates. This change has been reported to be due to quantitative variation rather than
qualitative differences (Hanson and Johansson, 1970). Some of the milk antigens have been found to relate to blood plasma proteins. While others are strictly "milk-specific" (Hanson, 1961). Details of the serological relationship of human blood plasma and human milk proteins are listed in Table 6. The techniques used for demonstrating this relationship were double diffusion and comparative immune electrophoretic techniques.

The serum-related proteins in human milk were reported in amounts less than 0.01 mg/ml (Hanson and Johansson, 1970). They may be identical, such as transferrin or may have distinct dissimilarities to those present in blood, e.g. Immunoglobulin A (Hanson and Johansson, 1970; Hanson et al, 1977).

Although human milk samples have been found to contain components serologically related to β-lipoproteins in blood plasma, no detail or further information about the lipoproteins has been given.

The importance of the existence of serum lipoproteins in the milk system, both human and bovine milk, has relevance to the spontaneous lipolysis of milks (Downey, 1980). For industrial milks, the spontaneous lipolysis will result in rancid off-flavors. For breast milks, the increased levels of free fatty acids due to lipolysis have been linked to breast milk jaundice in human newborns (Berkow, et al, 1984). Such lipolysis may be from the action of lipoprotein lipases (LPL) which are specifically activated by serum apolipoproteins (Jensen and Pitas, 1976).
Table 6. THE ANTIGENIC RELATIONSHIP BETWEEN HUMAN MILK AND BLOOD PLASMA PROTEINS\

<table>
<thead>
<tr>
<th>Blood Plasma</th>
<th>Precolostrum</th>
<th>Colostrum</th>
<th>Mature milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prealbumin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Albumin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid Seromucoid</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>$\alpha_1$-Antitrypsin(3.5S)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P-Lipoprotein</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Transferrin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>$\beta_{1A}$-Globulin(C3)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>/+/-</td>
<td>/+/-</td>
<td>-</td>
</tr>
<tr>
<td>Immunoglobulin A</td>
<td>/+/-</td>
<td>/+/-</td>
<td>/+/-</td>
</tr>
<tr>
<td>Immunoglobulin M</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>Immunoglobulin G</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Immunoglobulin D</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Properdin b</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C1b</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C2b</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C3b</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

a. This table is adapted from Hanson and Johansson, 1970.
Numerous studies to examine the role of serum lipoproteins in milk lipolysis have been conducted (Downey, 1980; Clegg, 1980; Bitman et al, 1983; Berkow et al, 1984). Serum lipoproteins, or at least serum proteins that can activate LPL are suspected to be present in bovine and human milk. However, actual isolation or in depth identification of the serum lipoproteins in milk system has not been reported. In some studies, the approach has been to employ lipoproteins isolated from blood serum for addition to the milk systems where resulting changes are observed. The study by Anderson (1979), utilized a competitive enzyme-linked immunoassay to demonstrate the relative amount of lipoprotein lipase activator (HDL) in bovine milks. It was reported that the activator varied not only in whole milk but also in skim milk and milk serum. Most of it was found in skim milk.

According to Bitman et al (1983), leakage of serum components into human milk may occur, especially when the milk is mechanically expressed. A study of lipolysis by adding human blood serum into human milk system was therefore conducted (Berkow et al, 1984). It was found that addition of such serum had no effect on spontaneous lipolysis of milk fat at either -20°C or -70°C storage. The investigators suggested this might be because of the inaccessibility of apoprotein C-II (specific apoprotein that activates lipoprotein lipases) to the lipase, as no mechanical agitation was employed prior to freezing.
SCOPE OF INVESTIGATION

This study was conducted to study serum-related lipoproteins in human milk. The objectives included:

I. Determination of the lipoprotein profile of human milk by using electrophoretic techniques with differential staining.

II. Verification of the kind of serum lipoproteins present in human milk by application of immunodiffusion techniques.

III. Measurement of the distribution of serum lipoproteins in different fractions of human milk system.

IV. Examination of the effects of freezing and thawing on properties of serum lipoproteins in human milk.

V. Investigation of the possible variation in serum lipoprotein profile between milks from different mothers.
MATERIALS AND METHODS

I. Samples

A. Human milk samples

Breast milk was obtained from individual donors as described by Vitale (1982) and used fresh or after frozen storage at -15°C. The donors were requested to transfer the expressed milks to sterile plastic tubes (50ml). The milk was collected daily from the donors and stored during transport on ice in an insulated carrying case. Freezing was done in liquid nitrogen or by domestic freezer. Frozen milks were thawed by a microwave technique. The tubes were placed in a beaker with cold tap water and temperature probe was inserted in the water. The samples were then microwaved to 40°C over 1-2 min. Pooled milk samples was obtained by mixing fresh or thawed individual milks.

Human milk cream and human skim milk samples were obtained by centrifuging whole milks in either a Micro Hematocrit Centrifuge (IEC Model MB, International Equipment Company, MASS.) for 15 min or at a maximum speed in a Clinical Centrifuge (International Model CL, International Equipment CO., MASS.) for 25min at room temperature.

B. Milk-fat-globule membrane (MFGM)

The procedure by Patton (1982) utilizing non-ionic detergent, Triton
X-100, for releasing MFGM was followed. Milk sample (30ml) was transferred to a plastic centrifuge tube and warmed to 37°C. It was then separated by centrifugation at 3000 x gav. at room temperature for 15 min. The skim milk and cream layers were separated. The cream layer was resuspended in 15 ml of 0.85% saline solution with a spatula and gently swirled for ~30s by hand. The tube was then filled with saline, mixed with a spatula and centrifuged as before. The washing–centrifuging–decanting process was repeated thrice after the initial removal of skim milk. After the second washing the cream layer was transferred by spatula to a clean tube. After the final centrifugation, the lower wash solution was aspirated from each tube and the globule layer was transferred to graduate cylinder. The washed globules were resuspended in saline to their original milk volumes and to contain 0.2% of a 10% (V/V) Triton X-100 (Rohm & Haas, PA.) in saline. The mixture was then gently agitated by inverting the capped tubes for 2 min to allow the detergent to act. The entire procedure up to this step was done at room temperature. The detergent-treated material was immediately cooled to 5°C or lower in an ice bath. It was then transferred to polyallomer tubes (1" x $\frac{3}{2}$") and centrifuged at 2–6°C and 50,000 x gav. for 1hr. A small pellet of membrane at bottom of the tube was collected and freeze dried.

C. Whole milk fat extracted (WMFE)

A method used for delipidation of plasma lipoprotein for preparing apoprotein (Cardin, Holdsworth and Jackon, 1984) was used for fat extraction. Five tenth of lyophilized whole human milk was weighed into an orange top centrifuge tube (Corning 50ml/Centrifuge Tube, Screw Cap,
Corning Glass Works, N. Y.). The tube was then filled with ether: ethanol (3:1 V/V) to 50ml and shaked well to completely mix the sample and solvent. The mixture was then allowed to stand at -20°C for 1hr. The protein was then pelleted in Clinical Centrifuge for 10min at room temperature. The solvent addition-incubation-centrifugation sequence was repeated 3 times. The pelleted protein was then washed with anhydrous ether (The tube was filled to 50ml with ether.) It was then centrifuged in the Clinical Centrifuge for 10 min at room temperature. The supernatant ether was removed by aspiration and the sediment air dried at room temperature to remove residual ether. The residue was then reconstituted with physiological saline and exhaustively dialyzed against physiological saline. The dialyzed protein was finally lyophilized and stored at -20°C.

D. Solubilized MFGM and WMFE

MFGM and WMFE prepared previously were solubilized individually in Triton-X-100 according to procedures reported by Bjerrum (1981). Four miligrams of each sample was weighed into a 4ml vial. Sufficient amount of 0.038M Tris + 0.1M glycine buffer (PH 8.75), 1.9ml in this case, and 20% Triton-X-100 (V/V), 0.1ml in this case, were added to make a final protein concentration of 2mg/ml and Triton X-100 concentration of 1.0%. The mixture was then sonicated twice for 10 seconds (Branson Ultrasonic Cleaner, Branson Cleaning Equipment Co., Shelton, Conn.). The mixture was then transferred into a 1.5 ml centrifuge tube and centrifuged at 15,000rpm at 40°F in an Eppendorf centrifuge (Type 5414 Centrifuge,
Brinkman Instrument Inc.) for 1.5 hr. The supernatant obtained was then transferred to a 4ml vial and kept on ice until use.

E. Lipase treated milk samples

Porcine pancreas lipase, 300 u/mg, lyophilized, was purchased from Boehinger Mannheim Biochemical, Indianapolis, IN. Lipase treated human milk samples were prepared by adding 1 mg lipase to 1 ml of whole milk. The solution was mixed well and then incubated at 25°C for 1.5 hr with occasional mixing. The reaction was then stopped by placing the mixture in an ice bath. The resulting solution was then used as lipase treated whole milk (LTWM). The lipase treated skim milk (LTS M ) was obtained by centrifuging LTWM in the Hematocrit Centrifuge for 15 min and collecting the infranatant portion.

F. Ultracentrifugally isolated fractions

Isolation of lipoprotein fractions from either whole milk or skim milk followed the method of Rudel et al. (1974). The solvent density of milk sample was raised to 1.2 by adding solid KBr (~0.2006g KBr/ml of whole milk, ~0.1745g KBr/ml of skim milk). The well mixed solution (2.5ml or less) was then distributed into pre-scaled ultracentrifuge tubes (Ultra-Clear Centrifuge Tubes, 1/2 x 2 in. Beckman Instruments, Inc. Spinco Division, CA.) For each batch run, a separate tube was prepared with a stain by adding 0.1 ml of 0.1% (w/v) Sudan Black B in ethylene glycol per ml of sample. The Sudan Black solution was prepared by adding 0.1g of the dye to 100 ml ethylene glycol at 65°C. The mixture was vigorously stirred for 2 min and filtered (Terpstra, Woodward and
Sanchez-Muniz, 1981). The tubes were then filled with d 1.23 solution, which was prepared by mixing KBr solution of d 1.3048 and NaCl solution of d 1.0063 according to the method of Havel, Eder and Bragdon (1955), to maximum capacity (5ml) of the tubes. The tubes were then centrifuged in a SW 50.1 Swinging-Bucket rotor for 48 hours at 15°C and 40,000rpm in a Beckman Model L Preparative Ultracentrifuge. The centrifuge was permitted to stop without applying the brake. The tubes were then carefully removed from the rotor and the patterns were inspected. Fractions were collected through aspiration from non-stained tubes according to patterns obtained from stained tubes. The density of each isolated fraction was then measured by pycnometry. The fractions were then subjected to exhaustive dialysis against distilled water and, finally freeze-dried.

G. Human blood serum samples

Blood samples were drawn from non-fasting donors by nurses in Student Health Center at The Ohio State University. Fourteen milliliters of blood from each subject were collected into sterile tubes containing EDTA. The blood sample was then transported, ice chilled, to the laboratory. Upon arrival, the blood was transferred into a centrifuge tube and centrifuged in a Clinical Centrifuge for 20 min at room temperature. The blood serum was then aspirated into a separate test tube for further use. Ultracentrifugal separation of blood sample was done immediately after the serum was collected.

II. Electrophoresis - Disc Polyacrylamide Gel(PAG) Electrophoresis

Electrophoretic separation of human milk samples was done by using a
gel system composed of three gel layers. Samples were pre-stained with lipid stain before electrophoresis. (Naito and Wada, 1980)

1. Chemicals

Acrylamide, N. N'-Methylenebisacrylamide and TEMED were purchased from Aldrich Chemical Company, Inc. Wis. and were all electrophoresis-grade. Trizma base and glycine were from Sigma Chemical Co., Mo. Sudan Black B (SBB) was from Manufacturing Chemists, OH. Riboflavin, ammonium persulfate, HCl and ethylene glycol were of analytical grade.

2. Composition of gel solutions and buffers

The composition of stock gel solution, dyes and buffers is shown in Table 7.

3. Gel tubes

Glass tubes with dimensions: I. D. = 5.0mm and length = 75.0mm were used. The tubes were cleaned with nitric acid solution and rinsed thoroughly with ample amounts of tap and distilled water. They were then soaked in 0.5% Tween 40 (ICI Americas Inc. DE) for 2 hr, followed by air-drying in an upright position.

4. Gel preparation

Composition of working solutions for different layer of gel is shown in Table 8.

(1). Separating gel

The separating-gel working solution was prepared as shown in Table
Table 7. COMPOSITIONS OF STOCK SOLUTIONS FOR PAG ELECTROPHORESIS

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Stock Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Tris</td>
<td>18.3g</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.23ml</td>
</tr>
<tr>
<td>IN HCl</td>
<td>ca. 24ml</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>9.6g</td>
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<tr>
<td>Bisacylamide</td>
<td>0.252g</td>
</tr>
<tr>
<td>Riboflavin</td>
<td></td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td></td>
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<tr>
<td>Distilled Water (to final volume)</td>
<td>100.0ml</td>
</tr>
<tr>
<td>Sudan Black B</td>
<td></td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td></td>
</tr>
<tr>
<td>PH</td>
<td>8.9</td>
</tr>
</tbody>
</table>
One milliliter of this solution was delivered by a 1 ml syringe to each gel tube which had been placed in a leveled rack with the bottom end sealed with parafilm. The solution was then cautiously layered with distilled water to form a sharp interface without interrupting the existing surface (~40μl overlay). The tubes were allowed to remain undisturbed in the rack for polymerization to occur within 45min. Completion of gelation was judged by observing the reappearance of the water-gel solution interface. Water and unpolymerized monomers of acrylamide were then carefully drained by inverting tubes on tissue paper. The last drop of water was removed from the gel tube by carefully inserting a twisted tissue paper and dabbing the inside dry without touching the gel surface.

(2). Spacer gel

The spacer-gel working solutions were prepared as shown in Table 8 and 0.1 ml spacer-gel solution was transferred over the first gel in the

<table>
<thead>
<tr>
<th>Table 8. COMPOSITION OF WORKING SOLUTIONS FOR DIFFERENT LAYER OF GEL.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Separating gel(3.6%)</td>
</tr>
<tr>
<td></td>
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<tr>
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<tr>
<td>Spacer gel(2.5%)</td>
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<tr>
<td></td>
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<tr>
<td>Sample gel(3.33%)</td>
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<tr>
<td></td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Solution J(working SBB)</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
tube and overlay of water was placed on top of the gel solution. A daylight fluorescent lamp (Preparation light, 105-120VAC, 50/60 Hz model, Ames Company, Division Miles Laboratories, Inc. IN.) was used for polymerization. The tubes, with spacer gel solution facing directly toward the preparation light, remained undisturbed for 30min. Completion of photopolymerization was judged by observing reappearance of the meniscus between water-gel solution and the change of the gel solution from a clear to a cloudy white gel. Water overlay was removed as described previously.

(3). Sample gel

The working sample-gel solution was prepared as described in Table 8. A volume of 30 ul milk sample was measured into each tube followed by 0.3 ml of the gel solution. The open end of the gel tubes was covered and mixed well by gently inverting the tubes about 10 times to ensure complete mixing of sample gel and sample. Finally, the tube was overlayed with reservoir buffer and exposed to fluorescent light for 45min. With the formation of a sharp buffer-sample gel interface indicate completion of gelation. The tubes were then drained and were ready for electrophoresis.

5. Electrophoretic run

Electrophoresis was done by using stock solution I as the buffer in a Canalco 400 Chamber with a Model 150 Power Source (Research Products Division, Miles Laboratories, Inc. IN.) A constant current of 3-4mA per
tube was used. The electrophoretic run was stopped when the lipoprotein band had migrated to about 1 cm from the anodal end of the gel tube.

6. Data Recording

Following electrophoresis, the tubes were removed from the cell, cleaned with distilled water and photographed using transmitted light for elucidating electrophoretic patterns.

7. Protein and Carbohydrate(CHO) staining of gels

Some gel tubes were run without pre-staining with SBB. When making sample gel, 2.35 parts of ethylene glycol were substituted for stock solution J. These gels were treated in the manner as other tubes for polymerization and electrophoresis. Immediately after turning off the current supply, these gels were subjected to the following post-staining procedures:

(1). Protein staining

Protein staining solution

This solution was made up by dissolving 0.1g Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA.) in 10ml pure ethanol. The mixture was then made up to 250 ml with 12% (w/v) TCA solution. It was then filtered before use.

Precedures

The method developed by Chrambach et al. (Narayan, 1975) was followed for protein staining with certain modifications. The fragile gels were removed from glass tubes by injecting distilled water along the wall between glass and gel. They were then placed in 12% TCA solution
for 1 hr before transfer to the protein stain solution for 2 hr. After staining, the gels then were rinsed with distilled water and then returned to the 12% TCA solution for photography.

(2). Carbohydrate staining (PAS staining method)

Carbohydrate staining was done by following method of Fairbanks, Steck and Wallach (1971) with modifications.

Schiff's reagent

This reagent was prepared by following procedures listed in Pharma-cia (1980-1).

a. Dissolve 1 g of basic fuchsin in 200 ml of boiling distilled water. Stir the solution for 5 min and cool to 50°C.

b. Filter the solution and add to the filtrate 20 ml of 1N HCl.

c. Further cool the solution to 25°C and add 2 g sodium metabisulphite.

d. The flask was then wrapped with aluminum foil and let the solution stirred in the dark for 12-24 hr.

e. Add 2 g of activated charcoal, shake for 1 min by hand.

f. Filter the solution and store it at room temperature.

CHO destaining solution

This destaining solution contains 50 ml of 1N HCl, 5.0 g of Potassium metabisulphite in 950 ml of water. Ten percent acetic acid (V/V) solution was also used as destaining solution.

Procedures

Gels were removed from glass tubes as stated previously. They were then fixed in 12% TCA solution for 1 hr followed by 10%(v/v) acetic acid solution for overnight. Gels were then put into 0.5%(w/v) periodic acid solution (Aldrich Chemical Company, Inc. Wis.) at room temperature for
2hr. They were placed into Schiff's reagent at room temperature until all of the gel turned purplish. Destaining was done by transferring the gels into CHO destaining solution until the background became colorless. Gels were then stored in 10% acetic acid for photography.

III. Immunological Analysis

A. Reagents

1. Buffer

Barbitone acetate buffer (Oxoid Limited, London, England) - Prepared by dissolving 16.5 g in 500 ml hot distilled water, cooling and making up to 1 liter with distilled water. Ionic strength 0.1, pH 8.6.

2. Agar Solution

I. D. Agar Tablets (Oxoid Limited, London, England) - Prepared by adding 1 tablet of agar to 12.5 ml of barbitone acetate buffer, and making up volume to 50 ml with distilled water. Merthiolate (Thimerosal, Sigma Chemical Co., St. Louis, Mo.) was added (0.01%) as a preservative. The agar was then dissolved by heating in a 100°C water bath. After the solution turned fully clear, it was centrifuged for 2-3 min before cooling if there is undissolved materials and then cooled to 55-60°C for plate preparation.

3. Antisera

Anti-Human Whole Serum antiserum; Anti-Human-α-Lipoprotein antiserum and Anti-Human-β-Lipoprotein antiserum from goat were obtained from Miles Scientific, Division Miles Laboratories, Inc., Napervill,
III. Anti-h-apolipoprotein AI antiserum, Anti-h-apolipoprotein AII antiserum and Anti-h-apolipoprotein B antiserum from sheep were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. All of the antisera were in liquid form.

B. Procedures
1. Double Diffusion Tests

Dissolved agar solution was pipetted onto leveled, dried and clean micro slides (3" x 1", Thickness 0.97-1.07mm) using 3ml per slide. Upon standing for a short time, the agar solution solidified and formed a gel. Then, wells were cut into the agar by using a metal punch. After removing gel from the wells, sample solutions and antisera were applied (about 10ul per well). The slides with samples and antisera were then incubated in a moist chamber for 2-3 days at room temperature. Precipitin lines were observed and photographed using approach described by Ouchterlony (1968). For chemical characterization of the precipitin lines, staining was also conducted. Agar slides were first wetted with distilled water and wrapped by a filter paper from the underside of the glass. The wrapped slides were then placed in an excess amount of 0.85% saline solution. The saline was changed once every 12hr. This washing was completed in two days. After washing, plates (still covered with filter paper) were rinsed rapidly with distilled water and air dried at room temperature. After drying, the paper was moistened and gently removed from the plates. (Arquembourg, Salvaggio and Bickers, 1970)
(1) Protein staining

Procedures from Schwick and Storiko (1964):

Dissolve 0.5% Light Green SF (Sigma Chemical Co., St. Louis, Mo) in 5% TCA solution. Stain slides for at least 1 hr. Wash with 5% TCA solution until background color has been removed. Air dry the slide.

Procedures using Coomassie Brilliant Blue R-250 (CBBR-250):

Dissolve 0.1 gm CBBR-250 (Bio-Rad Laboratories, Richmond CA.) in 10 ml ethanol and add 12% TCA solution to make final volume of 250 ml. Filter the solution before use. Dried slides were stained in this solution for at least 1 hr. The slides were destained with 12% TCA solution until background was clear.

Thiazine Red (National Aniline Division, Allied Chemical and Dye Corporation, N. Y.) staining (Speck, 1984):

Immerse dried slides for 10 minutes in each of the following baths: 0.1% Thiazine Red in 1% acetic acid, 1% acetic acid, 1% acetic acid, and 1% acetic acid containing 1% glycerol. Drain excess fluid from the slide, and air dry the slide.

Procedures using Nigrosin (MCB Manufacturing Chemists, Inc., Gibbstown, N. Y.):

Dip slides in 0.125% Nigrosin solution for at least 1 hr. Rinse the slides with distilled water. Destain the slides in 6% acetic acid until background became clear. Rinse the slide with distilled water and air dry the slide.
(2) Lipid staining

Dissolve 0.5% Oil Red O (Allied Chemical, New Jersey) in 50% ethanol.
Filter the solution before use. Stain the slides for at least 2hr.
Wash with 50% ethanol. Air dry the slide.

Stock solution - Sudan Black B 100mg
Isobutyl alcohol 100ml
After gel slides have been washed and dried, they were dipped in a solution of ethylalcohol : distilled water : stock solution (25ml : 15ml : 2ml) for 20min. The slides may then be cleared in 50ml ethyl alcohol and then rinsed with 50ml distilled water.

Procedures using Phosphine GN (Basic Yellow 5, Allied Chemical, Morristown, N. J.):
Dissolve 1 gm phosphine GN in 100ml ethylene glycol, the mixture was then diluted to 200ml with distilled water (Final concentration of phosphine = 0.5%). Stain dried agar plate in this mixture for 15min.
Wash the plate with distilled water. The plates were then dipped in 50% ethylene glycol in water solution for destaining. Leave the plate in 50% ethylene glycol until background was clear. Rinse slides with distilled water. Examine slides under UV light.
(3) Lipid-Protein double staining (Schwick and Storiko, 1964)

Place the slide in 0.5% Oil Red O in 50% ethanol for at least 2 hr, and wash with 50% ethanol. The slide was then immediately put into 0.5% Light Green SF solution in 5% TCA for at least 1 hr. It was then washed with 5% TCA and air dried.

(4) CHO staining

PAS staining method:
Procedures as described previously.

Toluidine Blue staining:
Dried gel slides were dipped in 0.2% Toluidine Blue (Allied Chemical, Morristown, N. J.) in water for 10 min. They were then clarified by washing in distilled water.

2. Immunelectrophoresis

(1) Agar gel only (single step)

Agar solution was prepared in the same way as described previously. Three milliliters of agar solution per slide were casted and solidified as before. Wells and trough were then cut on the agar plate. The agar in the wells was removed by suction. Sample solution (8 ul/well) was then delivered into the wells. The gel slides were then installed in the electrophoresis chamber (Gelman Deluxe electrophoresis chamber, Gelman Instrument Company). The chamber was filled with barbitone acetate buffer (Ionic strength 0.1, PH 8.6) prepared as described before. Filter paper wetted with buffer solution was used to connect agar gel and buffer solution. The electrophoresis was run at constant voltage (50 V)
using Bromophenol Blue (0.005%) as tracking dye (total run took about 4hr). After tracking dye moved ~0.5cm toward the end, the system was disconnected and the plate was removed from the electrophoresis chamber. The agar gel in the trough was then removed. Antibody solution was then delivered into the trough (30ul/trough). The gel slides with electrophoretic separated samples and antibodies were then incubated in a moist chamber for diffusion to take place. Record results after 72 hr incubation at room temperature. The gels were then washed, dried and stained as described previously.

(2) Disc-PAG followed by agar (two steps)

Disc-PAG electrophoresis was run as described previously. After completion of run, gels were removed intact from the glass tube. They were then sliced horizontally or longitudinally with razor blade according to electrophoretic pattern obtained from duplicate gels run with characterizing stains. The sliced gel was then mounted on micro slide or petri dish. Enough agar solution was then delivered to fill the empty space in micro slides or petri dishes. After agar solidification, trough (on micro slide) and wells (on petri dish) were cut into the agar. They were cut 1cm away from the sliced PAG gel. The agar slides with PAG gel were then incubated in a moist chamber for about 1.5hr. The gels in the wells and trough were then removed. Antisera were filled almost to the brim of the wells (10ul each) and troughs (30ul each). The gel slides and petri dishes were then incubated in a moist chamber at room temperature for 4-5 days. After immunoprecipitation has occurred, lines were
observed and photographed. For staining, PAG gel was carefully removed from slide and petri dish. The slide was then wrapped with filter paper, washed and stained as described before. The gel in the petri dish was washed by direct addition of saline. Saline was changed as before. After two days of washing, the gel was stained in the petri dish in the same manner as for washing. Results were then observed.
RESULTS

I. Electrophoretic Analysis of Serum Lipoproteins in Human Milk.

The construction of the gel system used for this study is shown in Figure 1. A normal blood serum sample gives the lipoprotein pattern shown in Figure 2 (Naito and Wada, 1980). When the gel system is applied to individual human milk samples, the typical pattern of separations as shown in Figure 3 is obtained. Milks from different mothers all give the same electrophoretic profile. Although differences in the intensities of bands separated among different samples may be observed. Milks from one individual donor sampled at different stage of feeding (i.e. foremilk, middle milk and hindmilk), may also show differences in band intensities. Foremilk generally showed stronger bands than middle and hindmilk. In fresh milk samples, the lipid dye may in some way be preferentially bound by the fat so that the lipoprotein bands showed relatively low intensity in the samples of higher fat contents. As shown in Plate I, fresh skim milk showed stronger bands than fresh whole milk sample. When fresh cream sample was used, the bands were stained very lightly that were barely visible. However, this interference does not seem to occur in frozen-thawed milk samples. If milk samples were frozen-thawed several times, the band intensities increased. In contrast to the phenomenon observed for fresh samples, the frozen-thawed
FIGURE 1. DIAGRAMMATIC SKETCH OF DISC-PAG ELECTROPHORETIC SYSTEM

1. sample gel (3.3%)
2. stacking gel (2.5%)
3. separating gel (3.6%)
FIGURE 2. DIAGRAMMATIC SKETCH OF DISC-PAG ELECTROPHORETIC PATTERN OF NORMAL BLOOD SERUM LIPOPROTEINS

(This figure is adapted from Naito and Wada, 1980)
FIGURE 3. TYPICAL DISC-PAG ELECTROPHORETIC PATTERN OF HUMAN MILK LIPOPROTEINS

A, B – bands obtained
PLATE I.

DISC-PAG ELECTROPHORETIC PATTERNS OF FRESH HUMAN MILK SAMPLES

1, 2 - fresh whole human milk
3, 4 - fresh human skim milk
whole milk sample showed stronger bands than the corresponding skim milk sample (Plate II). In consequence, the frozen-thawed whole milk sample also showed stronger bands than fresh whole milk samples.

Comparison of the electrophoretic patterns from human milk samples and blood serum sample (Plate III) revealed two bands in human milk corresponding to pre-β-lipoprotein present in blood serum, and the other to α-lipoprotein. The band from human milk corresponding to α-lipoprotein sometimes resolved into two bands, indicating that more than one component occupies this band area. Slight variation of the length of different gel layer may be the reason for resolving of these components. As shown in Plate IV, increasing the length of sample gel increases the separation among the bands. At least three bands were observed which stained with Sudan Black B nearby the position of α-lipoprotein. It was observed that these α-lipoprotein-like bands diffuse very quickly through the gel after electrophoresis. The one band corresponding to pre-β-lipoprotein remained at the original position for a longer time, which is also true for the components from blood serum. The α-lipoprotein-like bands in human milk that stained blue with SBB always disappeared quickly after the electrophoretic run but left a turbid band at that position. In the case of blood samples, the α-lipoprotein band also diffuse rapidly after completion of the run but no turbid bands at the position have been observed. Because of this instability, it is important that electrophoretic patterns be recorded as soon as the electrophoresis is completed. The band left behind in the
PLATE II. DISC-PAG ELECTROPHORETIC PATTERNS OF FROZEN-THAWED HUMAN MILK SAMPLES

1. frozen-thawed 6 times whole milk from donor x
2. skim milk from frozen-thawed 6 times whole milk (from donor x)
3. skim milk from frozen-thawed 5 times whole milk (from donor y)
4. frozen-thawed 5 times whole milk from donor y
PLATE III. DISC-PAG ELECTROPHORETIC PATTERNS OF BLOOD SERUM AND HUMAN MILK SAMPLES

1. human blood serum
2. frozen thawed whole milk
3. skim milk from frozen-thawed whole milk
4. cream from frozen-thawed whole milk
PLATE IV. EFFECT OF DIFFERENT LENGTH OF SAMPLE GEL ON THE ELECTROPHORETIC LIPOPROTEIN PATTERNS OF HUMAN MILK

1. sample gel solution 0.2 ml
2. sample gel solution 0.3 ml
3. sample gel solution 0.3 ml (duplicate)
4. sample gel solution 0.4 ml
\(\alpha\)-lipoprotein area after diffusion was found to pick up PAS-carbohydrate staining; so did the band in the pre-\(\beta\)-lipoprotein position (Plate V).

II. Identification of Lipoproteins in Human Milk by Immunodiffusion

In order to study the serum-related lipoproteins in human milk, it is necessary to examine their presence in detail and study their distribution between the fractions of milks. A micro double diffusion plate test was chosen for this purpose. The arrangement of the double diffusion plate is shown in Figure 4. Antigens and antisera were applied accordingly. The antisera used were commercially available anti-human \(\alpha\)-lipoprotein antiserum and anti-human \(\beta\)-lipoprotein antiserum. According to the product specifications, these are monospecific agents against human blood serum. Using these antisera to test fresh human milk samples, a precipitating reaction was observed (Plate VI). The precipitation occurred both for human whole milk against anti-human \(\alpha\)-lipoprotein and human whole milk against anti-human \(\beta\)-lipoprotein. However, the precipitation from anti-H-\(\alpha\)-lipoprotein and anti-H-\(\beta\)-lipoprotein against fresh milk sample did not occur consistently in every sample. Some fresh milk samples gave very faint precipitation or did not show any reaction at all. It is noticed from Plate VI that even though fresh whole milk sample showed reactions against the two antisera used, fresh skim milk did not contain any components producing the same kind of reaction.
PLATE V. DISC-PAG ELECTROPHORETIC PATTERNS OF HUMAN MILKS POST STAINED WITH PAS-CHO STAINING

1. skim milk from frozen-thawed whole milk
2. frozen-thawed whole milk
FIGURE 4. DIAGRAMMATIC SKETCH OF THE PLATE USED FOR DOUBLE DIFFUSION ANALYSIS

5, 10 - antiserum well
1, 2, 3, 4, 6, 7, 8, 9 - antigen (or sample) well
PLATE VI. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING FRESH HUMAN MILK SAMPLES AGAINST ANTI-HUMAN-LIPOPROTEIN ANTISERA

5 - anti-h-\(\alpha\)-lipoprotein antiserum
10 - anti-h-\(\beta\)-lipoprotein antiserum
1, 2, 6, 7 - fresh whole milk
3, 4, 8, 9 - fresh skim milk
In human milk banks, expressed milks are usually kept in frozen storage and then thawed for subsequent use in feeding (Berkow et al, 1984). For this reason, it was considered worthwhile to examine how freeze-thawing cycles affect the milk samples. It is noteworthy that freeze-thawing enhances the precipitation reaction between milk samples and the antisera used. Some milk samples do not show any precipitation reaction when it is fresh but show strong precipitation after it is frozen-thawed. Similarly fresh skim milk does not give any precipitation reaction while skim milk obtained from a frozen-thawed sample does. For different samples, the number of freeze-thawing cycles required for skim milk to show the precipitation is different. Plate VII and Plate VIII show results of immunodiffusion for two milk samples from two different mothers. Both milks were frozen-thawed twelve times. The sample on Plate VII was obtained through breast pump. When it was fresh, this milk gave very faint precipitation reaction against anti-H-\(\alpha\)- and anti-H-\(\beta\)-lipoprotein antisera. After it was frozen-thawed once, it gave a distinct precipitation reaction which was identical to the pattern shown on Plate VI. After it was frozen and thawed three times, the skim milk started to react with the antisera and produced precipitation bands. The band first appearing is the one indicated with small arrows on Plate VII. As the number of freeze-thawing cycles further increased, the precipitation band that fuses with the band obtained from whole milk sample became evident as an independent band from the one indicated by small arrow. It was observed that frozen-thawed whole milk did not give the precipitation band indicated by small arrow. The sample on Plate
PLATE VII. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING FROZEN-THAWED HUMAN MILK SAMPLES AGAINST ANTI-HUMAN-LIPOPROTEIN ANTISERA

5 - anti-h-\(\alpha\)-lipoprotein antiserum
10 - anti-h-\(\beta\)-lipoprotein antiserum
1, 2, 6, 7 - frozen-thawed 12 times whole milk
3, 4, 8, 9 - skim milk from frozen-thawed 12 times whole milk
PLATE VIII. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING FROZEN-THAWED HUMAN MILK SAMPLES AGAINST ANTI-HUMAN-LIPOPROTEIN ANTISERA

5 - anti-h-α-lipoprotein antiserum
10 - anti-h-β-lipoprotein antiserum
1, 2, 6, 7 - frozen-thawed 12 times whole milk
3, 4, 8, 9 - skim milk from frozen-thawed 12 times whole milk
VIII was drip milk which was dripping from one of the breasts while the baby was feeding at the other breast. This sample, when fresh, did not give any precipitation band. The clear precipitation bands from the whole milk did not appear until it was frozen-thawed twice. The skim milk did not show any clear reaction even when the milk sample was frozen-thawed up to nine times. Then, there is a faint band appeared, formed from skim milk against anti-H-\(\alpha\)-lipoprotein antiserum. It became very distinct after milk was frozen-thawed 11 times (as indicated in Plate IX-A. with small arrow). There were no bands observed from skim milk against anti-H-\(\beta\)-lipoprotein even when the cycles of freeze-thawing were increased up to 13 times. The reason for the appearance of the precipitation line indicated on Plate VII (small arrow) in skim milk sample but not in whole milk sample is suspected to be from the interaction among the milk components. The electrophoretic patterns shown in Plate X illustrate this point. In whole milk samples, components are more attached or aggregated to each other. The association, however, is weak and due to centrifugal force, they may be released to the bulk aqueous phase and exist in a free state. Therefore, skim milk, stained by a protein staining technique, shows more protein bands than whole milk sample.

In order to compare the properties of the precipitation line obtained from reacting milk sample against anti-H-lipoprotein antiserum with that obtained from blood serum, milk samples and blood serum were applied to the same double diffusion plate. The results in Plate XI are consistent with the view that precipitation lines obtained from human
PLATE IX. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING FROZEN-THAWED HUMAN MILK SAMPLES AGAINST ANTI-HUMAN-LIPOPROTEIN ANTISERA

5 - anti-h-α-lipoprotein antiserum
10 - anti-h-β-lipoprotein antiserum
1, 2, 6, 7 - frozen-thawed 11 times whole milk
3, 4, 8, 9 - skim milk from frozen-thawed 11 times whole milk
PLATE X. DISC–PAG ELECTROPHORETIC PATTERN OF FROZEN–THAWED HUMAN MILK
POST STAINED WITH PROTEIN STAINING

1. skim milk from frozen–thawed 18 times whole milk
2. frozen–thawed 18 times whole milk
stain – Coomassie Brilliant Blue R–250
PLATE XI. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING FROZEN–THAWED HUMAN MILK SAMPLES AND HUMAN BLOOD SERA AGAINST ANTI–HUMAN LIPOPROTEIN ANTISERA

A
1, 6 - frozen-thawed whole human milk
2, 7 - milk cream from frozen-thawed whole milk
3, 8 - human blood serum
4, 9 - skim milk from frozen-thawed whole milk

B
5 - anti-h-\(\alpha\)-lipoprotein antiserum
10 - anti-h-\(\beta\)-lipoprotein antiserum
milk samples occur as a result of reaction of fusion. However, the precipitation lines obtained from milk samples do not fuse with those obtained from blood. Different appearance of the precipitation lines obtained from milk and blood are also observed. Blood serum gives sharp precipitation line but milk samples give fuzzy lines. The location of the precipitation line between antigen and antibody well is found different between the ones obtained from milks and from blood sera.

To study the electrophoretic property of the components in human milk that form precipitates against anti-H-lipoprotein antisera and compare the movement to that of blood serum, a single step immunoelectrophoresis run was applied. The results obtained are shown schematically in Figure 5. The precipitates obtained from human milk against both antisera were found to stay at the position of the wells. Alpha-lipoprotein from human blood serum moved away from the well. Beta-lipoprotein from human blood serum also stayed at the location of well. Again, sharp bands of precipitation were observed for blood serum and fuzzy ones for milk samples.

An attempt to identify the electrophoretic bands stained by Sudan Black B in Disc-PAG electrophoresis was accomplished by using a two-step immunoelectrophoresis. When this method was validated by using blood serum samples, the anti-H-\(\alpha\)-lipoprotein antiserum only reacted to the \(\alpha\)-lipoprotein band separated from Disc-PAG but not to the pre-\(\beta\) or \(\beta\)-lipoprotein bands. The anti-H-\(\beta\)-lipoprotein antiserum reacted both to pre-\(\beta\)- and \(\beta\)-lipoprotein bands but not to the \(\alpha\)-lipoprotein band. The two-step immunoelectrophoresis method was then applied to test milk
FIGURE 5. RESULT OF SINGLE STEP IMMUNOELECTROPHORETIC ANALYSIS USING BLOOD SERUM AND FROZEN-THAWED HUMAN MILK AGAINST ANTI-HUMAN-LIPOPROTEIN ANTISERA

A - 1. whole human milk  
   2. human blood serum  
   3. anti-h-\(\alpha\)-lipoprotein antiserum

B - 1. whole human milk  
   2. human blood serum  
   3. anti-h-\(\beta\)-lipoprotein antiserum
samples and the results obtained by reacting antisera to the longitudinally sliced gels are shown on Plate XII. A fuzzy-looking precipitation line which tailed across the whole gel was observed from both anti-H-\(\alpha\)- and \(\beta\)-lipoprotein antisera. When human skim milk was used for the test, no precipitation was observed. When reacting the sliced gel against anti-human whole serum antiserum, more than one precipitation lines were observed at the position corresponding to \(\alpha\)-lipoprotein (Plate XIII). This confirms the postulate, made previously, that more than one component occupies this band area. The fuzzy, tailing precipitate laying across the gel was still observed. For skim milk sample, the two sharp lines but not the fuzzy, tailing one were observed when the same test was applied. It was suspected that the tailing of the precipitate could be from the fast diffusion of the component inside the polyacrylamide gel. A set of gels was therefore sliced horizontally and embedded in agar to react with antisera. The regions sliced are the ones indicated schematically on Figure 6. The results obtained are shown on Plate XIV and XV. In the \(\alpha\)-lipoprotein region, distinct precipitates may be seen to have occurred against both \(\alpha\)- and \(\beta\)-lipoprotein antiserum. In the pre-\(\beta\)-lipoprotein region, only a zone of cloudiness was found around the well of anti-\(\alpha\)-lipoprotein antiserum. When a skim milk sample was examined in the same way, the same results were observed but the precipitates were much weaker so that photography could not register them.

Although precipitates are found when reacting human milk samples against anti-H-lipoprotein antisera, differential staining would still
PLATE XII. RESULT OF TWO-STEP IMMUNOELECTROPHORESIS USING LONGITUINALLY SLICED GEL AGAINST ANTI-HUMAN-LIPOPROTEIN ANTISERA

Sample - frozen-thawed whole human milk
A - anti-h-α-lipoprotein antiserum
B - anti-h-β-lipoprotein antiserum
PLATE XIII. RESULT OF TWO-STEP IMMUNOELECTROPHORESIS USING LONGITUDINALLY SLICED GEL AGAINST ANTI-HUMAN WHOLE SERUM ANTISERUM

sample - frozen-thawed whole human milk
antiserum - anti-h-whole serum antiserum
FIGURE 6. GRAPHICAL PRESENTATION OF THE HORIZONTAL SLICING OF THE POLYACRYLAMIDE GEL

A. disc-PAG electrophoretic pattern of human milk (stained)
B. disc-PAG electrophoretic pattern of human milk (nonstained)
1. sliced region (pre-\(\beta\)-lipoprotein region)
2. sliced region (\(\alpha\)-lipoprotein region)
PLATE XIV. RESULT OF TWO-STEP IMMUNOELECTROPHORESIS USING HORIZONTALLY SLICED GEL AGAINST ANTI-HUMAN ANTISERA (α-LIPOPROTEIN REGION)

- Sample: frozen-thawed whole human milk
- Center well: sliced gel
  1 - anti-h-whole serum antiserum
  2 - anti-h-α-lipoprotein antiserum
  3 - anti-h-β-lipoprotein antiserum
  4 - anti-h-apolipoprotein AI antiserum
  5 - anti-h-apolipoprotein AII antiserum
  6 - anti-h-apolipoprotein B antiserum
PLATE XV. RESULT OF TWO-STEP IMMUNOELECTROPHORESIS USING HORIZONTALLY SLICED GEL AGAINST ANTI-HUMAN ANTISERA (PRE-β-LIPOPROTEIN REGION)

- Sample: frozen-thawed whole human milk
- Center well: sliced gel
- 1: anti-h-whole serum antiserum
- 2: anti-h-α-lipoprotein antiserum
- 3: anti-h-β-lipoprotein antiserum
- 4: anti-h-apolipoprotein AI antiserum
- 5: anti-h-apolipoprotein AII antiserum
- 6: anti-h-apolipoprotein B antiserum
be needed in order to specify the nature of the precipitates. When the precipitation lines obtained from blood serum against anti-human-lipoprotein antisera were stained, the line obtained from anti-\(\alpha\)-lipoprotein antiserum stained well with Light Green SF but only weakly with Oil Red O. Presumably, this is because of the relatively low lipid content in HDL particles. The line obtained from anti-\(\beta\)-lipoprotein antiserum picks up Light Green SF as well as Oil Red O strongly. When the precipitates obtained from human milk samples against both anti-human-\(\alpha\) and \(\beta\)-lipoprotein antisera were stained, none of the precipitates were found to pick up the protein and lipid stain. In order to circumvent the possible error from different stainability of dyes to different molecules, several different dyes including Light Green SF, Coomassie Brilliant Blue R-250, Thiazine Red, Nigrosin for protein staining; Oil Red O, Sudan Black B, Phosphine GN for lipid staining were tested. None of them were capable of staining the precipitation lines obtained from human milk samples against anti-\(\alpha\)-lipoprotein antisera. Carbohydrate staining including Toluidine Blue and Basic Fuchsin were also tested. Although the background picks up both stains because of the nature of the agar, after prolonged destaining, the precipitation lines can still be observed as white lines. These precipitation lines can usually be visualized as white lines after saline washing, drying and staining. Both undried and dried agar plates have been used for staining but the same nonstaining properties are always observed.

In order to study the nature of the precipitates, several treatments including fat extraction (WMFE), lipid digestion (lipase treated
samples) and heat treatment (heated samples) were conducted on milk samples. For fat extracted samples, it was found that no precipitates occurred when using Triton-solubilized whole milk fat extracted against anti-human-α- and β-lipoprotein antisera. If WMFE dissolved directly in barbitone acetate buffer (pH 8.6) was used, a weak precipitation line was obtained when reacting against anti-human-α-lipoprotein antiserum at concentration of 10.8 mg/ml buffer (Plate XVI. A-1, A-2). At this concentration, an even weaker precipitate was also observed when the sample reacts with anti-human-β-lipoprotein antiserum (diluted with distilled water 1:1). The precipitate was so weak that photograph cannot record it (Plate XVI, B-6, B-7).

When milk samples were treated with lipase and used for double diffusion, the lipase treated whole milk samples gave nearly the same double diffusograms as the untreated samples except that for lipase treated samples, the cloudy zone of precipitates around the well was larger than untreated ones (Plate XVII). When skim milk samples obtained from lipase treated whole milks were used for testing (Plate XVIII), it was observed that precipitation lines were produced against both anti-human-α- and β-lipoprotein antisera. Skim milks obtained from untreated milks only show precipitation lines against anti-human-α-lipoprotein antiserum but not anti-human-β-lipoprotein antiserum. However, because the precipitates obtained from skim milks of untreated sample against anti-β-lipoprotein antiserum (Plate XVIII-3, XVIII-4) and from skim milks of lipase treated samples against anti-α-lipoprotein
PLATE XVI. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING FAT EXTRACTED HUMAN MILK SAMPLES AGAINST ANTI-HUMAN-LIPOPROTEIN ANTISERA

5 - anti-h-α-lipoprotein antiserum (4ul)
10 - anti-h-β-lipoprotein antiserum (diluted with water 1:1; 4ul)
1, 6 - WMFE (4ul/well)
2, 7 - WMFE (6ul/well)
3, 8 - MFGM (4ul/well)
4, 9 - MFGM (6ul/well)
PLATE XVII. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING LIPASE TREATED AND UNTREATED WHOLE HUMAN MILKS AGAINST ANTI-HUMAN-LIPOPROTEIN ANTISERA

5 - anti-h-α-lipoprotein antiserum (6 ul)
10 - anti-h-β-lipoprotein antiserum (6 ul)
1, 2, 6, 7 - untreated frozen-thawed whole milk
   (1, 2 - 4 ul/well; 6, 7 - 8 ul/well)
3, 4, 8, 9 - lipase treated frozen-thawed whole milk
   (3, 4 - 4 ul/well; 8, 9 - 8 ul/well)
PLATE XVIII. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING SKIM MILK SAMPLES FROM LIPASE TREATED AND UNTREATED WHOLE HUMAN MILKS AGAINST ANTI-HUMAN-LIPOPROTEIN ANTISERA

- 5 - anti-h-α-lipoprotein antiserum (3ul)
- 10 - anti-h-β-lipoprotein antiserum (3ul)
- 1, 2, 6, 7 - skim milk from lipase treated frozen-thawed whole milk (6 ul/well)
- 3, 4, 8, 9 - skim milk from untreated frozen-thawed whole milk (6 ul/well)
antiserum are very faint, they can not be recorded clearly through photography.

Whole milks and skim milks were heated in a boiling water bath for 30 minutes and used for testing. The results obtained are shown on Plate XIX and XX. It is evident that, after this treatment, whole milk samples still show precipitation lines against both anti-α- and θ-lipoprotein antisera (Plate XIX-A and Plate XX-A). However, the zone of cloudiness around the antigen wells have disappeared for heat treated whole milks. In the case of skim milk, when the heated and unheated samples were reacted against anti-α-lipoprotein antiserum (Plate XIX, B), the precipitation lines from heated samples became very weak and can no longer exhibit the fusion to the same extent. The zone of precipitates around antigen wells have been indicated by small arrows on Plate XIX, B. When samples were tested against anti-θ-lipoprotein antiserum (Plate XX, B), the precipitation lines from heated samples totally disappeared, so did the zone of precipitates around the antigen wells. Similar precipitation lines around the antigen wells were also observed (small arrows Plate XIX, B).

A set of tests were also conducted by using heated and unheated milk samples against heated antisera. The anti-human-α- and θ-lipoprotein antisera were heated in boiling water for 30 minutes. No precipitation lines between the antigen wells and antiserum wells were observed for heated antiserum against unheated milk samples. However, a zone of cloudiness or precipitates around the antigen wells was still evident but the intensity was reduced (Figure 7 and 8). When both milks and
PLATE XIX. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING HEATED AND UNHEATED MILK SAMPLES AGAINST ANTI-HUMAN-α-LIPOPROTEIN ANTISERUM

5, 10 - anti-h-α-lipoprotein antiserum
1, 2 - untreated frozen-thawed whole human milk
3, 4 - heated frozen-thawed whole human milk
6, 7 - untreated skim milk from untreated frozen-thawed whole human milk
8, 9 - heated skim milk from unheated frozen-thawed whole human milk
PLATE XX. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING HEATED AND UNHEATED HUMAN MILKS AGAINST ANTI-HUMAN-β-LIPOPROTEIN ANTISERUM

5, 10 - anti-h-β-lipoprotein antiserum
1, 2 - frozen-thawed whole milk
3, 4 - heated frozen-thawed whole milk
6, 7 - skim milk from frozen-thawed whole milk
8, 9 - heated skim milk from unheated frozen-thawed whole milk
FIGURE 7. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING HEATED AND UNHEATED HUMAN MILK SAMPLES AGAINST HEATED ANTI-HUMAN-\(\alpha\)-LIPOPROTEIN ANTISERUM

1, 2 - frozen-thawed whole milk
3, 4 - skim milk from frozen-thawed whole milk
5, 6 - heated frozen-thawed whole milk
7, 8 - heated skim milk from frozen-thawed whole milk
FIGURE 8. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING HEATED AND UNHEATED HUMAN MILK SAMPLES AGAINST HEATED ANTI-HUMAN-β-LIPOPROTEIN ANTISERUM

1, 2 - frozen-thawed whole milk
3, 4 - skim milk from frozen-thawed whole milk
5, 6 - heated frozen-thawed whole milk
7, 8 - heated skim milk from frozen-thawed whole milk
antisera were heated, no precipitation were observed, neither between
the antigen and antibody wells nor in the zones around the wells.

Regardless of the treatments used, the precipitation lines obtained
from reacting milks and antisera were not capable of picking up either
protein or lipid stains.

III. Study of the Distribution of Lipoproteins in Human Milks

In order to study the distribution of lipoproteins in human milk,
different fractions from milk samples were isolated. First of all, milk
fat globule membranes (MFGM) were obtained by using Triton X-100 treat-
ment. The MFGM obtained was then solubilized again in Triton X-100 and
reacted against anti-human-lipoprotein antisera. The Triton solubilized
MFGM was found to have no reaction at all against anti-human-lipoprotein
antisera. However, when the Triton solubilized MFGM was examined by
disc-PAG electrophoresis, it gave an electrophoretic pattern in which
three bands stained with lipid dye (Plate XXI, A, B, C). Bands B and C
were found to stain strongly for both protein and carbohydrate (PAS-
CHO). When MFGM was examined directly by dissolving it in barbitone
acetate buffer but without being solubilized by Triton, it was found to
give precipitation lines against both anti-\(\alpha\)- and \(\beta\)-lipoprotein anti-
sera (Plate XVI, A-3, 4; B-8, 9) at a concentration of 5.4 mg/ml buffer.
However, the precipitation lines obtained from reacting with anti-\(\beta\)-
lipoprotein antiserum were too weak to be recorded through photography.
The solubilization of MFGM in barbitone acetate buffer required vigorous
shaking to completely dissolve the material, especially at higher con-
PLATE XXI. DISC-PAG ELECTROPHORETIC PATTERN OF TRITON X-100 SOLUBILIZED MILK FAT GLOBULE MEMBRANE PRESTAINED WITH SUDAN BLACK B

sample - milk fat globule membrane isolated from frozen-thawed human milk
centrations (e.g. greater than 10 mg/ml buffer). The precipitation lines thus obtained were found not to pick up either protein or lipid stains.

Centrifugal studies on human milk samples was also conducted in order to examine the distribution of lipoproteins as well as to isolate the lipoproteins. In these experiments, a blood serum sample was included as a standard which contained Sudan Black B to indicate the completeness of the centrifugal separations. When the initial density of the solution was adjusted to greater than 1.21, it was observed that after 26 hours of centrifugation of the blood serum standard, there was still some lipid-staining proteins which did not float to the top of the solution (Plate XXII-3). The centrifugation time was, therefore, increased to 48 hours. After 48 hours of separation, there was no blue colors observed below the top layer for blood sample which indicated the completeness of the separation (Plate XXIII-1). For whole milk samples, a single layer stained dark blue at 26 hours of centrifugation was found to separate into two layers (Plate XXIII-3) at 48 hours of centrifugation. The existence of very high density lipoprotein (density of greater than 1.21) in whole milk sample was observed.

For examination of any freeze-thawing effect on the ultracentrifugal pattern of human milk, frozen-thawed milk samples were used for the ultracentrifugation. The results obtained on Plate XXIV show that increasing frequencies of freeze-thawing decreases the density of the very high density lipoproteins.

Ultracentrifugal studies using skim milk samples were also conducted. Adjusting the initial density to a value greater than 1.21 produced
PLATE XXII. PATTERNS FROM ULTRACENTRIFUGATION FOR BLOOD SERUM AND HUMAN MILK (26 HOURS)

Speed: 40,000 rpm; Rotor: SW 50.1; Temp: 15°C

1. frozen-thawed (3 times) whole human milk (stained)
2. human blood serum (nonstained)
3. human blood serum (stained)
PLATE XXIII. PATTERNS FROM ULTRACENTRIFUGATION FOR BLOOD SERUM AND HUMAN MILK (48 HOURS)

Speed: 40,000 rpm; Rotor: SW 50.1; Temperature: 15°C

1. human blood serum (stained)
2. human blood serum (nonstained)
3. frozen-thawed whole human milk (stained)
PLATE XXIV. EFFECT OF FREEZE-THAWING TREATMENT ON THE ULTRACENTRIFUGAL PATTERNS OF HUMAN MILKS

Speed: 40,000 rpm; Rotor: SW 50.1; Time: 40 hr; Temperature: 15°C

1. frozen-thawed 2 times whole milk (stained)
2. frozen-thawed 2 times whole milk (nonstained)
3. frozen-thawed 7 times whole milk (stained)
4. frozen-thawed 7 times whole milk (nonstained)
5. frozen-thawed 15 times whole milk (stained)
6. frozen-thawed 15 times whole milk (nonstained)
patterns of separation which were similar to that of twice frozen-thawed whole milk samples (Plate XXV-2). By examining the freeze-thawing effect on the patterns of separation for skim milk samples, it was found that the trend was similar to that of whole milk samples. In other words, increasing the frequency of freeze-thawing decreases the density of these very high density lipoproteins.

Another ultracentrifugation experiment at an initial density of less than 1.21 g/ml was also run to further examine the distribution of lipoproteins in skim milk. Procedures described by Terpstra, Woodward and Sanchez-Muniz were followed with some modifications. The changes of the procedures included a reduction in the amount of distilled water used for overlay (1.8 ml instead of 2.4 ml), an increase in the time of the centrifugation (39 hours instead of 7 hours), a reduction in the speed of centrifugation (40K rpm instead of 50K rpm) and a decrease in the temperature of the centrifugation (15°C instead of 20°C). Because of the imposing changes in the procedures, a blood serum sample was included to aid in examining the effectiveness of the separation. The results obtained are shown on Plate XXVI. The ultracentrifugal pattern of blood serum obtained agrees well with that reported by Terpstra, Woodward and Sanchez-Muniz (1981). The pattern obtained from skim milk shows that lipoproteins distribute not only in the density region of about 1.04 but also in the region between 1.06-1.14 (graphical presentation of the pattern on Figure 9).

In order to study the immunological properties of the different layers of milk samples corresponding to the blue-staining layers in the
PLATE XXV. PATTERNS FROM ULTRACENTRIFUGATION FOR HUMAN SKIM MILKS

Speed: 40,000 rpm; Rotor: SW 50.1; Time: 48 hr; Temp: 15°C

1. blank (distilled water + 0.25 ml SBB solution)
2. skim milk from frozen-thawed once whole milk (stained)
3. skim milk from frozen-thawed once whole milk (nonstained)
PLATE XXVI. PATTERNS FROM ULTRACENTRIFUGATION FOR HUMAN BLOOD SERUM AND HUMAN SKIM MILK (INITIAL DENSITY ADJUSTMENT < 1.21 g/ml)

Speed: 40,000 rpm; Rotor: SW 50.1; Time: 39 hr; Temp: 15°C

1. skim milk from frozen-thawed 3 times whole milk (SBB stained)
2. blood serum (nonstained)
3. blood serum (nonstained)
4. blood serum (SBB stained)
FIGURE 9. GRAPHICAL PRESENTATION OF THE ULTRACENTRIFUGAL PATTERN OF HUMAN SKIM MILK (INITIAL DENSITY ADJUSTMENT < 1.21 g/ml)

Speed: 40,000 rpm; Rotor: SW 50.1; Time: 39 hr; Temp: 15°C

<table>
<thead>
<tr>
<th>density scale</th>
<th>color description</th>
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<tbody>
<tr>
<td>+ 1.04</td>
<td>grayish blue</td>
</tr>
<tr>
<td></td>
<td>transparent</td>
</tr>
<tr>
<td>+ 1.06</td>
<td>dark blue particles</td>
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<tr>
<td></td>
<td>grayish</td>
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<tr>
<td></td>
<td>light blue</td>
</tr>
<tr>
<td>+ 1.14</td>
<td>dark blue</td>
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sample - skim milk from frozen-thawed 3 times whole milk
control sample after ultracentrifugation, the layers were first collected through aspiration, as shown graphically in Figure 10. The results obtained by exposing these layers to anti-human-lipoprotein antisera are shown on Plate XXVII. Only layer 1 gives precipitation lines as those obtained from original whole milk samples. A similar approach was also used for skim milk at initial density of greater than 1.21. At lower concentration (5.8 mg/ml barbitone acetate buffer), precipitation lines occurred both against anti-α- and -β-lipoprotein antisera. However, they were very faint so that photography could not record them clearly (Plate XXVIII). By progressively increasing the concentration, the precipitation lines became very pronounced at concentration of 12 mg/ml buffer (Plate XXIX). An attempt to stain them for visualization of protein with Light Green SF was made. Only at concentration of 12 mg/ml buffer, were protein bands appearing. For the top layer reacting against anti-α-lipoprotein antiserum (Plate XXVIII, A-3, A-4), the stained lines were located at the inner side of the originally existing precipitates facing toward the antiserum well as indicated by the arrow on Plate XXIX. For the top layer reacting against anti-β-lipoprotein antiserum (Plate XXVIII, B-6, B-8), the stained lines were located very close to the antigen well. The results are shown schematically in Figure 11. Although stained bands are visible on the plate, those precipitates which did not pick up stain are also clearly visible as white precipitates on the plate.
FIGURE 10. GRAPHICAL PRESENTATION OF THE ISOLATED LAYERS OBTAINED FROM ULTRACENTRIFUGALLY SEPARATED HUMAN MILK SAMPLE

Speed: 40,000 rpm; Rotor: SW 50.1; Time: 48 hr; Temp: 15°C

A. ultracentrifugal pattern of frozen-thawed whole milk (stained)
B. ultracentrifugal pattern of frozen-thawed whole milk (nonstained)
1, 2, 3 - layers isolated
PLATE XXVII. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING ISOLATED LAYERS FROM ULTRACENTRIFUGALLY SEPARATED WHOLE MILKS

5 - anti-h-α-lipoprotein antisera
10 - anti-h-β-lipoprotein antisera
1, 6 - Layer 1.
2, 7 - Layer 2.
3, 8 - frozen-thawed whole human milk
4, 9 - Layer 3.
PLATE XXVIII. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING ISOLATED LAYER FROM ULTRACENTRIFUGALLY SEPARATED HUMAN SKIM MILK (LOWER CONCENTRATION)

5 - anti-h-α-lipoprotein antiserum (4 ul)
10 - anti-h-β-lipoprotein antiserum (diluted with water 1:1, 4 ul)
1, 2, 6, 7 - Top layer isolated from skim milk (from frozen-thawed whole milk), concentration 5.8 mg/ml buffer; vol. 6 ul/well
3, 4, 8, 9 - Freeze dried skim milk (from frozen-thawed whole milk), concentration 40 mg/ml buffer; vol. 6 ul/well
PLATE XXIX. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING ISOLATED TOP LAYER FROM ULTRACENTRIFUGALLY SEPARATED HUMAN SKIM MILK (HIGHER CONCENTRATION)

A
5 - anti-h-α-lipoprotein antiserum (6ul)
10 - anti-h-β-lipoprotein antiserum (6ul)
1, 2, 7, 9 - double distilled water (6ul/well)
3, 4, 6, 8 - top layer isolated from skim milk (from frozen-thawed whole milk), concentration 12 mg/ml buffer; vol. 6 ul/well

B
FIGURE 11. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING ISOLATED TOP LAYER FROM ULTRACENTRIFUGALLY SEPARATED HUMAN SKIM MILK AGAINST ANTI-HUMAN-LIPOPROTEIN ANTISERA (PROTEIN STAINED; HIGHER CONCENTRATION)

5 - anti-h-α-lipoprotein antiserum (6ul)
10 - anti-h-β-lipoprotein antiserum (6ul)
1, 2, 7, 9 - double distilled water (6ul/well)
3, 4, 6, 8 - top layer isolated from skim milk (from frozen-thawed whole milk), concentration: 12mg/ml buffer; vol: 6ul/well
protein stain - Light Green SF
IV. Immunological Identification of Human Milk Lipoproteins by Using Monospecific Apolipoprotein Antisera

In order to further enhance the specificity of the immunological test used, commercially prepared antisera from marker apolipoprotein namely anti-\(h\)-apolipoprotein Al antiserum, anti-\(h\)-apolipoprotein AlI antiserum and anti-\(h\)-apolipoprotein B antiserum were employed to test the existence of lipoproteins in human milk. When these antisera were used to react with frozen-thawed whole milk and skim milk, no lines of precipitation were observed before and after staining. When they were used to test the isolated top layer from ultracentrifugally separated skim milk, at lower concentration (5.8 mg/ml) used, a halo of precipitates was found to occur circling the antigen well in each case (Plate XXX, XXXI, XXXII) before staining. When these antisera were tested against blood serum sample, there was no noticeable precipitations before staining. They have to be stained in order to be clearly visualized. The gel plates obtained from reacting top layer of skim milk against anti-apolipoprotein antisera were therefore subjected to protein staining (Light Green SF). No stained lines were observed from reaction against anti-\(h\)-apolipoprotein AlI. However, there were lines, staining faintly green, observed from reaction against anti-\(h\)-apolipoprotein Al. These lines were located about half-way between the two wells. They were very faintly stained, and without cautious examination, they could be easily overlooked. There were also green-stained zones found from reaction against anti-\(h\)-apolipoprotein B. The area was located right around the antigen well and on the side facing toward the antiserum well.
PLATE XXX. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING ISOLATED MILK FRACTIONS AGAINST ANTI-HUMAN-APOLIPROTEIN AI ANTISERUM

5 - antisera diluted with water 1:1, vol: 4 ul
10 - antisera diluted with water 1:2, vol: 4 ul
1, 2, 6, 7 - isolated top layer from ultracentrifugally separated human skim milk, concentration: 5.8 mg/ml buffer; vol: 6 ul/well
3, 4, 8, 9 - milk fat globule membrane
concentration: 5.4 mg/ml buffer; vol: 6 ul/well
PLATE XXXI. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING ISOLATED MILK FRACTIONS AGAINST ANTI-HUMAN-APOLIPOPROTEIN AII ANTISERUM

5 - antiserum diluted with water 1:1, vol: 4 ul
10 - antiserum diluted with water 1:2, vol: 4 ul
1, 2, 6, 7 - isolated top layer from ultracentrifugally separated human skim milk, concentration: 5.8 mg/ml buffer, vol: 6ul/well)
3, 4, 8, 9 - milk fat globule membrane,
   concentration: 5.4 mg/ml buffer, vol: 6ul/well)
PLATE XXXII. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING ISOLATED MILK FRACTIONS AGAINST ANTI-HUMAN-APOLIPOPROTEIN B ANTISERUM

5 - antisera diluted with water 1:1, vol: 4 ul
10 - antisera diluted with water 1:2, vol: 4 ul
1, 2, 6, 7 - isolated top layer from ultracentrifugally separated human skim milk (concentration: 5.8 mg/ml buffer; vol: 6 ul/well)
3, 4, 8, 9 - milk fat globule membrane (concentration: 5.4 mg/ml buffer, vol: 6 ul/well)
Again, the stained zone was not very distinct and not easily defined. When milk fat globule membrane was used for testing, no halo of precipitates or lines or zones staining for protein were observed before and after staining at concentration of 5.4 mg/ml buffer. In order to confirm these results, higher concentration of samples were used again for testing. At concentration of 12 mg/ml buffer for top layer of skim milk and 15 mg/ml buffer for milk fat globule membrane, halos of precipitates were observed again for the top layer reacting against each kind of antiserum. This time, using the high concentration, halos of precipitates were also found to occur for milk fat globule membrane against each kind of antiserum. However, the halos obtained from milk fat globule membrane were much closer to the antigen well compared to those obtained from top layer of skim milk. After these plates were subjected to Light Green SF protein staining, there were no stained lines observed from reaction against anti-human-apolipoprotein AII for both top layer of skim milk and milk fat globule membrane (Figure 11 and Figure 12). However, clearly stained lines were obtained from reaction of top layer of skim milk at concentration of 12 mg/ml buffer against anti-h-apolipoprotein AI (Figure 12) and B antisera (Figure 13). For milk fat globule membrane, no stained lines were observed after reaction against anti-h-apolipoprotein B, however, lines stained very very faintly with Light Green SF against anti-apolipoprotein AI were observed. Again, they were so faint that the plate have to be carefully examined in order for them to be found.
FIGURE 12. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING HUMAN MILK FRACTIONS AGAINST ANTI-HUMAN-APOLIPOPROTEIN AI ANTISERUM (PROTEIN STAINED)

1, 2 - isolated top layer from ultracentrifugally separated human skim milk, concentration: 6mg/ml buffer, vol: 1 - 6ul, 2 - 8ul
6, 7 - isolated top layer from ultracentrifugally separated human skim milk, concentration: 12mg/ml buffer, vol: 6 - 6ul, 7 - 8ul
3, 4, 8, 9 - milk fat globule membrane, concentration: 15mg/ml buffer, vol: 3, 8 - 4ul/well, 4, 9 - 8ul/well
5, 10 - antiserum (diluted with water 1:1), vol: 5 - 4ul, 10 - 6ul
stain - Light Green SF
FIGURE 13. RESULT OF DOUBLE DIFFUSION ANALYSIS (PLATE TEST) USING HUMAN MILK FRACTIONS AGAINST ANTI-HUMAN-APOLIPOPROTEIN B ANTISERUM (PROTEIN STAINED)

5, 10 - antiserum (diluted with water 1:1), vol: 5 - 4ul, 10 - 6ul
1, 2, 6, 7 - isolated top layer from ultracentrifugally separated human skim milk, concentration: 12mg/ml buffer, vol: 1, 6 - 6ul/well, 2, 7 - 8ul/well
3, 4, 8, 9 - milk fat globule membrane, concentration: 15mg/ml buffer, vol: 3, 8 - 6ul/well, 4, 9 - 8ul/well
stain - Light Green SF
DISCUSSION

The primary objective of this study has been to closely examine the serological and physical properties of serum-related lipoproteins in human milk systems using well established methods for studying human blood serum lipoproteins.

Although the existence of serum-related lipoproteins in human milks has been reported (Hanson, 1960; Hanson and Johansson, 1970), they have received very little study. In order to conduct an in-depth study on the properties of these constituents in the human milk system, several considerations need to be taken into account. First of all, the human milk itself is a very complex system. It contains more than 200 recognized constituents (Blanc, 1981). Among these, more than 50 kinds are protein in nature including more than 30 kinds of enzymes (Bezkorovainy, 1977; Blanc, 1981; Anderson, Powers and Tollaksen, 1982). Secondly, the blood serum-related proteins in the human milk have been reported to be present in amounts less than 0.01 mg/ml (Hanson and Johansson, 1970). Finally, human milk fat globule membranes, which originate from the plasma membrane of the mammary cell (Nickerson and Akers, 1984), is essentially a lipid-protein complex (Anderson and Cawston, 1975). It could contain intact lipoprotein moieties that are not related to those existing in blood. Because of these complexities, when choosing methods
for studying the serum-related lipoproteins in human milk systems, the sensitivity and the specificity of the methods chosen should be strongly emphasized.

I. Electrophoretic Analysis of Serum Lipoproteins in Human Milks

Electrophoresis has been utilized both for separation and characterization of proteins. This utilization depends on several properties of protein molecules, such as their charges and sizes. There are several variations of PAG-disc electrophoresis; the method chosen for use in milk samples was the one introduced by Naito and Wada (1980). The system consists of three gel layers. Sample solution is loaded in the sample gel. The stacking gel has the function of aligning different proteins of similar molecular size, configuration and charge into concentrated bands. These concentrated bands are subsequently separated into discrete bands in the separating gel. This system has been adapted to separate plasma lipoproteins to aid in classifying the various dyslipoproteinemias in clinical laboratories (Muniz, 1977; Miles Laboratories, Inc., 1981; Naito and Wada, 1980). Because of the high resolution and great flexibility reported, it was chosen for preliminary screening of the lipoprotein profile of human milks.

From the results obtained, it is evident that human milk contains components which show similar electrophoretic mobility to pre-β-lipoproteins and α-lipoproteins of blood sera in polyacrylamide gels. Differences in the stained intensity of these bands among different milk samples are also noticed. The differences could be from the quantitative
variation of the milk components in different milk samples (Blanc, 1981). It is also observed that the intensity of the bands obtained seems to be influenced by the fat content of the milk sample used. The possible cause for this influence may be explained as follows. Fat globules, with an average diameter of 2-3 μm (Blanc, 1981; Ruegg and Blanc, 1981), are much larger than blood serum lipoprotein particles (0.1-1 μm for the largest chylomicron, Table 2.). Because of the molecular sieving effect of the polyacrylamide gel system (Naito and Wada, 1980), the fat globules may remain stationary in the sample gel layer during electrophoresis. They pick up and bind lipid dyes tenaciously. Therefore, increasing fat content increases dye retaining in the sample gel. The dye available for lipoprotein staining is thus reduced. For frozen-thawed milk samples, components of the fat globule membranes may be released into the bulk aqueous phase because of the destabilization of the hydrophobic bonding (Neville, 1984). These components may then be able to penetrate into the stacking gel or the separating gel. Due to their lipid-protein nature, they can carry along more lipid dyes into the separating gels. Therefore, for frozen-thawed milk samples, staining does not seem to be affected by fat content to the same extent as fresh milk samples.

Although the electrophoretic patterns show that there are components with similar mobility to pre-§- and κ-lipoproteins of blood sera, one can not conclude definitely that these components are identical to those in blood. They could simply be some other kinds of lipoproteins having the same electrophoretic mobility as blood serum lipoproteins.
Particularly, the gel system used in this study has very high porosity. Many components in the milk system may occupy the same band area as indicated by the results obtained. Therefore, more studies are needed to further identify the bands obtained.

II. Identification of Lipoproteins in Human Milk by Immunodiffusion

Immunodiffusion has been accepted as a standard technique for use as an analytical tool because of its specificity, resolution and simplicity. This test utilizes the principle of diffusion to bring two reactants, i.e. antigen and antibody, in contact with one another and produce an antigen-antibody reaction. Such reactions are known to be highly specific (Kawai, 1981). Therefore, the technique provides excellent opportunities for qualitatively and quantitatively studying the composition of biological products (Ouchterlony, 1968).

By applying the double diffusion analysis to test milk samples, precipitation reactions were observed. The precipitation lines obtained from anti-\(\alpha\)- or -\(\beta\)-lipoprotein antiserum are located half-way between the antigen and antiserum well. Since the traversed distance of a precipitate from its diffusion center is governed mainly by the relative concentrations of the reactants and the diffusion coefficients of the reactants (Ouchterlony, 1968), the results obtained could indicate a compromise between the factors. In other words, the antigens that cause the precipitation might have off-setting concentrations or diffusion coefficients to the antisera used.
Different samples giving different responses to the antisera used were observed. This could be due to the differences of the amount of the antigens present in milk samples. The results also indicate that most of the antigens giving precipitations to the antisera used are associated with the cream portion of the milk samples. Freeze-thawing treatment influences the relative distribution of the antigens in the cream and skim portions. These observations may suggest that the components which give precipitations are closely related to sites of the hydrophobic bondings. They could be membraneous materials. Because of the destabilization from the freeze-thawing treatment, these components are either released into the bulk aqueous phase or reorient themselves to expose their antigenic sites. Therefore, the intensity of the precipitation reactions in frozen-thawed samples and skim milks is enhanced.

From the results obtained by using the pumped milk and the drip milk, it is evident that these two milks have very different responses to the antisera used. The differences may result from the quantitative differences between the two milk samples. Drip milk has been known to contain different amounts of milk components from the expressed milks. It has a much lower fat content than expressed milks (Hamosh et al, 1984). However, since the frequency of the freeze-thawing cycles needed for the drip milk to achieve the same precipitation reactions as those occurring in the pumped milk is very different, qualitative differences in the way of the molecular attachment between the two samples can not be excluded.
The multiple lines of precipitation generated from skim milks (obtained from repeated freeze-thawing treatment of whole milks) may be interpreted as follows. First of all, the lipoproteins used for making the antisera are heterogeneous in nature. They may contain different antigenic determinants on their surface. The antisera produced by immunizing animals with such antigens will consist of different populations of antibody molecules (Kaiwai, 1981). These antibodies may not cause precipitation reactions for components in blood except lipoproteins themselves. They may react with milk components to cause precipitations. Secondly, freeze-thawing treatment could cause breakage of the membrane materials. The fragments obtained may contain different antigenic determinants. Because of their size or shape differences, they may have different diffusion coefficients. Therefore, they travel in the gel at different rates and form precipitations at different locations. Finally, the rings or zone of precipitation around the antigen wells obtained from frozen-thawed milk samples are observed. According to Schwick and Skoriko (1964), they may be non-specific precipitations obtained from using the lipemic or aged samples.

Precipitin patterns normally observed in double diffusion plate test in which two antigen solutions are compared using antisera as analytical agents include the pattern of fusion, the pattern of intersection and the pattern of partial intersection. These patterns and the interpretation of each of them are shown in Figure 14. From the results obtained by comparing a blood serum sample and a milk sample, a reaction of partial intersection seems to occur for both antisera used. However,
Pattern a (of identity or fusion) develops when the compared antigens (B) are identical serologically and are used in equal concentrations against their specific antiserum (As/B). A skewed pattern results when the same antigens are compared but one of them is less concentrated than the other (b). When two serologically different antigens are compared using an antiserum that contains antibodies to both of them, each antigen-antibody system precipitates independently of the other, so that the resulting precipitin bands cross in the pattern of nonidentity or intersection (c). An antigen that is similar enough to another to be capable of precipitating some antibodies in the antiserum against the latter (the homologous antigen) will form a precipitin band which is arrested at its juncture with the band formed by the homologous antigen. The latter band, however, continues to grow, forming a "spur" (pattern of partial identity or partial intersection) whose length is inversely proportional to how closely related the cross-reacting antigen is to the homologous antigen and whose curvature and faintness are directly related to this relationship (d). A double spur forms if two antigens are compared which are different but are related to a third antigen, and antiserum to this third antigen is employed (e).

This information is adapted from Crowle (1973), p. 259, Fig. 4.5.
since the precipitation lines formed by milk samples disappear at an area close to the precipitation lines formed by blood sera and the appearances of the precipitation lines are so different, the reaction of partial identity can not hold a complete explanation for the phenomena observed. According to Ouchterlony (1968), the types of patterns demonstrated in Figure 14 are based on concentration balanced systems. In an imbalanced system, the concentration differences may cause eventual dissolution of precipitates because of the excess of the reactants. Precipitin bands can also be dense enough to present a physical barrier for the diffusion of reactants (Crowle, 1973). Therefore, the pattern of intersection may be falsely interpreted as a pattern of partial intersection. In order to further elucidate the relationship between the antigens from the two samples, other types of analyses need to be employed.

Immunoelectrophoreses which separate reactants from each other by their electrophoretic mobility before they are exposed to immunoprecipitation can circumvent the problem of being unable to resolve the coexisting antigen-antibody systems that form precipitation at a close area in double diffusion analyses. They can also demonstrate the electrophoretic mobility of the existing reactants (Crowle, 1973). From the results obtained by using single step immunoelectrophoresis, it is evident that the components in the milk system causing precipitations against the antisera used have very different properties from those in the blood serum. Not only the appearances of the precipitations are different but also the electrophoretic mobilities. However, the precipitations formed
from the milk sample against both anti-lipoprotein antisera are very similar in both aspects. A preliminary conclusion may be made at this point that the components in milk systems causing the precipitations observed are not identical to those in the blood. Whether the precipitates formed by milk samples against both antisera used are from the same components in the milk system need further investigation.

By using the two-step immunoelectrophoresis to identify the electrophoretic bands stained by Sudan Black B in disc-PAG electrophoresis, it is observed that the components in milk systems causing the precipitations diffuse very quickly in the polyacrylamide gel. Therefore, they formed tailing precipitation lines. This may indicate their relatively low molecular weight in nature. In addition, strong precipitations formed by reacting milk samples against both anti-α- and -β-lipoprotein antisera are from the same band area (α-lipoprotein region, Plate XIV). It is therefore confirmed that the components in milk systems forming the precipitations against both antisera are different from those in blood sera. The lipid stained bands evident in disc-PAG electrophoretic pattern from milk samples may contain mostly other lipoprotein particles besides those related to blood sera. Serum-related lipoproteins may still be present at these band area. However, they could be in too low concentration to form visible precipitations or the interfering substances present may have caused the precipitations interrupting the complexing of lipoproteins with antisera.

According to Crowle (1973), a frequent problem of interpretation of results from immunodiffusion tests relates to a nonspecific
precipitation developed which is not directly a result from aggregation of antigen by antibody. "Some nonspecific precipitates are readily identified, but even yet are not adequately explained." These nonspecific precipitations could be from protein-protein interactions, such as acidic proteins in the antigen solution reacting with basic proteins in the antiserum solution or vice versa, protein-nonprotein interactions, e.g. proteins and detergents or nonprotein precipitations, e.g. tissue lipids. From the results obtained in this study, the precipitates formed by milk samples are very different from those by blood serum. In addition, the precipitates formed by milk samples against either anti-\( \alpha \)-lipoprotein or anti-\( \beta \)-lipoprotein antiserum are very similar in their behavior. An assumption was therefore made that these precipitates could be a type of nonspecific precipitations. They are formed by the same components in milk samples against both antisera used. In order to prove this assumption, several different tests were made to study the nature of the precipitates.

First of all, differential staining was used to specify the nature of the precipitates. It was found that the precipitates did not pick up lipid, protein and carbohydrate dyes. It is reported by Crowle (1973) that bands that would not take up any of the commonly used protein stains are occasionally observed. The reason for this nonstainability has not been well explained. However, differences in antigen-antibody ratio, the presence of nonprotein antigen, or different affinities of a given stain for different kinds of antibody have been suggested. Since
different stains have been examined in this study, the last cause is not likely to exist.

In order to further examine the nature of the precipitates, milk samples with fat extraction treatment were therefore utilized. Since there is consideration that, after fat extraction, hydrophobic proteins may form aggregations and lose their antigenicity (Bjerrum, 1981), the fat extracted sample was first solubilized in Triton-X 100 for the examination. No precipitates were observed for Triton solubilized samples. Since the protein will normally be delipidated by the action of the detergent, proteins whose antigenicity is dependent upon the presence of lipid may lose their reactivity (Bjerrum, 1981). For this reason, the results obtained may indicate the participation of the lipid particles in forming the precipitates. Because of the delipidation from the detergent, the protein particles lose their reactivity to the antisera used. By dissolving the fat extracted whole milk samples directly in buffer solution, very weak precipitations were obtained. This could be from the residual lipids remaining in milk samples after fat extraction (Cardin, Holdsworth and Jackson, 1984). The intensity of the precipitation lines obtained from reacting to anti-β-lipoprotein antiserum seems to be reduced more than those from anti-α-lipoprotein antiserum. This may suggest that the lipid moiety is more involved in the precipitations formed from reacting to anti-β-lipoprotein antiserum.

Initially, lipase treatment was intended as a treatment to cause digestion of lipids to test if these were necessary for the precipitations. From the results obtained, it is observed that precipitations
were enhanced by lipase treatment. The possible explanation for this phenomenon is that only enough lipase used for hydrolyzing the triglycerides located in the membranes. The lipid particles in the central core of the globules were therefore released into the bulk aqueous phase along with the membraneous material. For this reason, the liberated lipids are more available for enhancing the precipitation reactions. The precipitates were, therefore, enhanced. The skim milks obtained from lipase treated whole milks also show precipitations because of the release of the membraneous materials into the bulk aqueous phase.

The results from studies of heat denaturation of the reactants revealed certain differences in the response of whole milk and skim milk. The differences are: a. The precipitation lines from heated whole milk were not affected either in intensity or in position. However, the turbid zone around the antigen well disappeared. b. The precipitation lines for skim milk were greatly diminished by heating but with an additional band appearing. Two possible explanations can be assigned to the phenomena observed. Firstly, proteins may play some roles in causing the precipitations formed from skim milk and after heat denaturation they no longer show the same kind of precipitation reaction. Therefore, the intensity of the precipitates is greatly reduced. There are also components not sensitive to heat participating in the reactions. Therefore, residual precipitates are still observed. Secondly, for whole milk samples, the antigens that caused precipitation reactions are more protected from heat denaturation. Therefore, after heat treatment, the milk samples still give the same precipitation lines as unheated milks.
The zone of precipitates around antigen wells are generated by protein molecules because they disappeared after heat treatment.

By using unheated milk samples to react with heated antisera, precipitation lines disappeared but the zone of cloudiness did not. This suggests that the zone of cloudiness are generated by protein molecules in milk samples reacting with nonprotein molecules in the antiserum solutions. The disappearance of precipitation lines indicates that antibody molecules did participate in forming the precipitation lines according to the statement made by Crowle (1973) which says:

If a precipitate has been observed to form between antiserum and a tissue extract and there is question as to whether it is due to antibodies in the antiserum, the antiserum can be boiled and then should no longer precipitate the tissue extract. Any antiserum "precipitin" surviving this test probably is not antibody.

From the results obtained through all of these tests, it is obvious that the precipitation lines obtained against anti-human-lipoprotein antisera are produced by antibody(ies) in the antisera against certain components in milk samples. The components are tightly associated with hydrophobic bonding. They are different from lipoprotein particles in human blood serum but may also be lipid associated. These components contain also protein moieties. However, the complex between these components and antibodies causes the products to be nonstainable. The precipitates from anti-α-lipoprotein and -β-lipoprotein antisera seem to be from the same components in milk systems. However, the way these components bind to the antibodies in the two antisera seems not to be identical.
III. Study of the Distribution of Lipoproteins in Human Milks

The distribution of lipoproteins in human milks can be visualized more clearly by studying the properties of different milk fractions. From the results obtained by using the isolated milk fat globule membranes, it is evident that the components causing the precipitations against anti-lipoprotein antisera are present in this fraction. The behavior of these components, when reacting to Triton X-100, is similar to that of fat extracted whole human milk. Although Triton solubilized milk fat globule membrane did not show precipitation lines, examination of the electrophoretic patterns reveals distinct lipoprotein bands to be present. Each lipoprotein particle seems to be carbohydrate associated. Whether these lipoprotein particles are different fragments from original membranes or they simply are different protein classes associated with triton molecules (as shown in Figure 15) needs further studies.

From the results obtained through ultracentrifugation, it is observed that by adjusting the density to greater than 1.21 g/ml, milk samples exhibit a layer of very high density lipoprotein that is not present in the blood serum sample. When the milk samples are subjected to freeze-thawing cycles, this high density lipoprotein layer moves toward a lower density region. This suggests that these lipoprotein particles release their protein portion rather than the lipid portion as a result of the freeze-thawing effect. Presumably, the bonding for the released proteins to the lipoprotein is weaker than the lipid bonding. Therefore, the linkage can be broken by the freeze-thawing more easily.
FIGURE 15. SOLUBILIZATION OF THE BIOLOGICAL MEMBRANES BY NON-IONIC DETERGENTS

The membrane model is taken from ref. 32. Depending on the ratio of detergent/membrane lipids, different steps of solubilization may be obtained (ref. 113). In step 1, when a small amount of detergent is present, the molecules of detergent are incorporated into the membrane without breaking it. In step 2, the membrane is solubilized into micellar solution containing mixed protein-lipid-detergent micelles in equilibrium with detergent micelles and free detergent molecules. Finally (step 3), when enough detergent is added, pure protein-detergent micelles may be obtained in equilibrium with detergent-lipid and detergent micelles. The micellar solution of step 3 may be used for isolation of individual membrane proteins.

This information is adapted from Gulik-Krzywicki (1975), Fig. 2.
This may also explains the observed heterogeneity of the lipoprotein particles.

By examining the ultracentrifugal pattern of skim milk with initial density adjusted to less than 1.21 g/ml, it is again confirmed that, in addition to the particles corresponding to blood serum lipoproteins, milks also contain lipoprotein particles which have different properties. The origin of these skim milk lipoprotein particles could be from fragments of milk fat globule membranes, secretory vesicles and several other sources, such as endoplasmic reticulum (Patton and Keenan, 1975).

By using the isolated ultracentrifugal layers to react with anti-human-α- and -β-lipoprotein antisera, the components in milk samples that cause precipitations are observed to be present in the top layers of skim and whole milks. In other words, these components float at the density region of less than 1.21 g/ml. A concentration effect seems to be present also in the immunological assay because the top layer isolated from skim milk did not show pronounced precipitations unless higher concentration was used. This phenomenon agrees well with that reported by Crowle (1973) saying that low concentration or dilution will progressively weaken the immunoprecipitates.

After staining the immunodiffusion plates obtained by using higher concentration of top layer (skim milk) against anti-human-lipoprotein antisera, both stained and non-stained lines were observed. This suggests several possibilities. First of all, serologically, the anti-lipoprotein sera used may not have been as pure as originally thought (Crowle, 1973). When more than one precipitation lines (the stained ones
and the nonstained ones) develop in a monospecific test, supposedly involving only a single precipitation system, the purity of the antiserum must be suspect. Secondly, the stained bands may be the ones that are truly developed from serum-related lipoproteins. This statement is from the observation of the similarity between the locations and curvatures of the stained bands from milk fractions (Figure 11) and those from blood sera (Plate XI). According to Crowle (1973), the precipitin band curvature and location often reveal important qualitative and quantitative properties of the reactants. The basic principles of this statement are shown in Figure 16. Since the isolation of the top layer concentrated the sample nearly ten times, the true concentration of the serum-related lipoproteins may be less than 0.01% (w/v) in the skim milks. The calculations were based on the assumption that the isolated top layer consisted of only the serum-related lipoproteins. Since there could be other components present in addition to the serum-related ones, the concentration of the serum-related lipoproteins could be much less than 0.01% in the skim milk phase. In addition, the stained lines or zones obtained in this study are not very sharp and distinctly visible. The fuzzy bands tend to arise when an immunologic excess of antibodies or antigens is used (Crowle, 1973). Therefore, it may be concluded that the concentration of the serum-related lipoproteins in the milk fraction is much less than those in the human blood serum sample (normal blood serum lipoprotein concentrations - chylomicrons: 15 ± 15 mg/100ml; \( \beta \)-lipoprotein: 278 ± 67 mg/100ml; pre-\( \beta \)-lipoprotein: 88 ± 41 mg/100ml; \( \alpha \)-lipoprotein complex: 171 ± 39 mg/100ml, Opplt, 1980). Because they
FIGURE 16. RELATIONSHIP BETWEEN PRECIPITIN BAND CURVATURE AND DIFFUSION COEFFICIENTS OF REACTANTS IN DOUBLE DIFFUSION PLATE TESTS

The curvature of a precipitin band between reactant sources in double diffusion plate tests depends on the relative diffusion coefficients of antigen and antibody, even when these two are not used in exactly equivalent proportions. In diagrams a and d, antigen and antibody have equal diffusion coefficients (i.e., probably equal molecular weights), and they form straight precipitin bands. When they are used at equivalence, their band forms midway between them (a); when antigen concentration initially exceeds that of antibody, the band is formed closer to the antibody source (d). The precipitin band formed by an antigen with a higher diffusion coefficient (lower molecular weight) than antibody curves toward the latter at serologic equivalence (b). This curvature is accented if antigen excess is employed (c). The opposite effects prevail when antigen has a lower diffusion coefficient than antibody (c, f).

This information is adapted from Crowle (1973), p. 269, Fig. 4.9.
exist in such a low concentration they are therefore easily overlooked. Besides, the reactions resulting in the nonstainable precipitates are very pronounced, and may obscure the reaction between antisera and serum-related lipoproteins and the observation of the results as well.

IV. Immunological Identification of Human Milk Lipoproteins by Using Monospecific Apolipoprotein Antisera

From the results obtained previously, it is realized that there are milk-specific components reacting with anti-human-lipoprotein antisera in addition to serum-related ones to form precipitations. In order to further confirm the existence of the serum-related lipoproteins as well as to increase the specificity of the tests used, antisera prepared against each specific apoproteins were therefore used. The concentrations of milk fractions were tested by trial and error. At the lower concentration used, halos of precipitates are observed around the wells. These halos are presumably formed as a result of an extremely imbalance in the concentrations of the antigen and antibody used. It behaves like a radial single diffusion test. Because the antigen concentration is too low, antibody diffuses over the antigen well before they can reach the equivalence and form precipitation. The antigen, then, diffuse radially into the antibody covered area and form a disc of precipitation that stops growing when the equivalence has reached (Crowle, 1973). For this reason, higher concentration of the antigen solutions were again examined. After staining, green stained lines were observed from the reaction of milk fractions against anti-apolipoprotein AI and B antisera.
There was only a single precipitation line observed for each system which testifies to the purity and specificity of the antisera used. From the results, it is evident that the apolipoprotein AI and B are present in human milks. They are mainly distributed in the top layer of ultracentrifugally separated skim milks (density < 1.21 g/ml). Although there is no stained lines visible from reactions involving anti-apolipoprotein AII antiserum, apolipoprotein AII may still be present in the top layer because a halo is obviously formed in the test performed. It may be because the concentration is too low that precipitates are not stable enough to survive the washing and staining (Crowle, 1973). For milk fat globule membrane, at concentration of 15 mg/ml (concentrated more than forty times compared to the original milk sample), halos were also observed. Since they were not very distinct and were very close to the antigen wells, they could indicate a very low concentration of the reactants present compared to those in the top layer of skim milk. Whether they are from contamination during the isolation process or they originally are in the fat globule membranes needs further studies.

Apolipoprotein AI and AII are the major protein components of chylomicrons and HDL particles. In these two lipoprotein classes, the concentration of apolipoprotein AI is much greater than apolipoprotein AII. Apolipoprotein B is the major protein component of chylomicrons, VLDL and LDL particles (Table 1 and Table 4). These lipoproteins can all float in the solution of density 1.21 g/ml (Table 2). The results obtained from this study agree well with these principles. In other words, the antigens responding to anti-apolipoprotein AI, AII and B antisera
are located at the density range indicated. Concentrations of these antigens shown from the immunological tests also demonstrate that the amount of apolipoprotein A1 is greater than AII. Since leakage has been indicated as a major pathway for serum components to be transferred into milk systems (Bitman et al, 1983), it seems reasonable to assume that these apolipoproteins may be from pre-\(\beta\)-, \(\beta\)-; \(\alpha\)-lipoproteins and chylomicrons as well.
SUMMARY

This study has dealt with the examination of serological and physical properties of serum-related lipoproteins in human milk systems. Because of the possible role they may play in the lipolysis of banked milks and the lack of study on their properties in the milk system, several approaches have been designed to conduct an in-depth examination. Sensitivity and specificity of the tests used have been the major concern because of the complexity of the human milk itself and the small amount of serum-related proteins in the system reported.

The major approach used in this study is the immunological analysis using antisera generated against human blood serum α-lipoprotein, β-lipoprotein, apolipoprotein AI, AII and B. Antigens against these antisera are found to present in human milks. They are mainly located in the skim milk especially in the ultracentrifugal fraction of density less than 1.21 g/ml. They account for less than 0.01% (w/v) in the skim milk used. Because of the very low concentration present, they are easily overlooked by using whole milk samples as the specimens. Concentrating the samples is necessary for accurate survey of these components.

Electrophoresis and ultracentrifugal analyses demonstrated that human milks contain lipoprotein particles not present in blood serum. In the former analysis, a band having the same electrophoretic mobility as
the pre-β lipoprotein and the other as the α-lipoprotein in blood serum were observed. In the latter analysis, a layer of very high density lipoprotein (d > 1.21 g/ml) was found to exist in addition to those corresponding to the blood serum. These milk specific lipoproteins did not show any serological relationship to those in the blood serum. They were observed to be greatly affected by the freeze-thawing process.

Immunological testing of whole milk samples revealed the presence of milk specific components reacting very strongly with anti-human-α-lipoprotein and -β-lipoprotein antisera to form precipitates in addition to those formed from serum-related lipoproteins. These components associate mainly with membraneous materials. They have the same electrophoretic mobility as human blood serum α-lipoproteins in disc-PAG but show very different serological characteristics from blood serum lipoproteins. They form precipitates which are not stainable by protein, lipid and carbohydrate dyes used. Their behavior in the immunodiffusion tests can be affected by the freeze-thawing treatment, the fat extraction, the Triton-solubilization and lipase treatment but not greatly by heat treatment. From different milk sources, such as pumped milk and drip milk, they show different characteristics when subjected to freeze-thawing. Since they react with antibodies in the antisera used (anti-α- and -β-lipoprotein) in addition to those serum-related, a relatively low specificity and purity of these antisera was confirmed. For more specific examination, use of antisera prepared against marker apolipoproteins is required.
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