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SERUM LIPOPROTEIN ELECTROPHORESIS *

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Abstract.

A simple and rapid method is described for separating the serum lipoproteins into clear, discrete, and reproducible bands by electrophoresis on cellulose acetate. Lipoproteins were fractionated into chylomicrons, beta, pre-beta, alpha-lipoprotein and albumin bound fatty acids. Quantitation was accomplished by staining the membranes with Oil Red O and scanning with The Beckman Analytrol. Sera of patients with primary or secondary lipidemias show definite patterns reflecting changes in lipid metabolism

Introduction

Lipoproteins are conjugates complex of specific protein and lipids, which are phopholipids, cholesterol, cholesterol esters, triglycerides (Neutral fats), fatty acids, sterols, carotenoids and fat soluble vitamin (A, D, E, K). They are generally distributed in living matter, cell nuclei, mitochondria, cell membranes, chloroplasts, egg yolk, milk and in the blood stream. (5)

Lipoproteins presented in the plasma have large molecules, ranged from approximately 20,000-10,000,000 molecular weight units and contain from 40-95% lipid respectively. They can be transferred across the membranous boundaries of cell, and the large molecules of them can be soluble in water by the hydrophilic portion, such as protein and phospholipids, and on the outside in contact with water, while the hydrophobic portion, such as the triglycerides, cholesterol are in the interior, sheltered from contact with water molecules. (10, 13)

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Oncley et al found that the molecules of lipoprotein was spherical in shape and 185° A in diameter. Each of them is different in many properties, for example, solubility in water and ethanol-water mixtures, size and shape of molecules, electrostatic reaction and lipid content.

Composition of Lipoproteins

When plasma lipids are extracted with suitable lipid solvent. They can be separated into small groups of triglyceride, phospholipid, and cholesterol in nearly the same proportion. Small amount of unesterified long-chain fatty acid about 5% of total fatty a in normal plasma as shown in the table below:

Lipids of the blood plasma in man (8)

	mg/100 ml	
	mean	range
Total lipid	570	360–820
Triglycerides	142	80–180
Total phospholipid	215	123–390
Lecithin	–	50–200
Cephalin	–	50–130
Sphingomyelins	–	15–35
Total cholesterol	200	107–320
Free cholesterol (non-esterifeid)	55	26–106
Free fatty acids (non-esterifeid)	12	6–16

Hillyard & others (1955) studied the human serum lipoproteins by means of Ultracentrifugation, and analyzed each fraction for protein, phospholipid, free and esterified cholesterol, and triglycerides. The result was shown in the table below,

Percentage Composition of Lipoproteins in Man (8)

	Fraction		
	A	B	C
Density	1.063	1.063–1.107	1.107–1.220
Lipids :			
Phospholipid	21	29	20
Cholesterol free	8	7	2
Cholesterol ester	29	23	13
Triglyceride	25	8	6
Total lipid	83	67	41
Protein	17	33	59

Fraction A consists of B–lipoprotein, density below 1.063

Fraction B consists of a_2–lipoprotein, density between 1.063–1.107

Fraction C consists of a_1–lipoprotein, density between 1.107–1.220

From this study, the B - lipoprotein (Fraction A) has higher fat and lower protein, molecular weight about 1,300,000 in contrast, the lower fat and higher protein of a - lipoprotein, it has molecular weight about 200,000.

Therefore, the lipoprotein which have higher fat and lower protein must have lower specific gravity called Low - density lipoprotein (Sp. Gr. $<$ 1.063), and lipoprotein with higher protein and lower fat content, called High - density lipopretein (Sp. gr. $>$ 1.220).

Isolation

Plasma lipoproteins, stable, or unstable, are a heterogeneous group of compounds that can be separated into smaller groups by various ways :

1. Salting out
2. Ethanol salt fractionation
3. Precipitation by antibodies and non-specific polyanions
4. Chromatography
5. Ultracentrifugation
6. Electrophoresis.

The most significant methods are Ultracentrifugation and electrophoresis.

Ultracentrifugation (8, 10)

The lipoprotein classes may be isolated by floatation in the preperative ultracentrifuge by selection of the proper solvent density. The lipoproteins show different

rate of floatation when centrifuge in salt solution, and the migration can be measured and recorded photographically.

Lipoproteins are classified as having low density if they show floatation in salt solution of density 1.063. Those with densities between 1.063 and 1.21 are called high-density lipoproteins (HDL). The low-density fraction (LDF) is further subdivided on the basis of their floatation rate in Svedberg floatation unit, Sf. (1 Sf unit = 10^{-13} cm/second/dye/gm at 26° C). The subgroup Sf 0-12 is the highest of the low density fraction and is found in all plasma of all person. Another subgroup Sf 12-20 was first characterized by Gofman and was found in increased concentration. More recently, Gofman has included this subgroup in a broader low-density subgroup (Sf 12-400). This broad group, we shall see, represent basic low-density lipoproteins to which variable amounts of triglycerides have become attached.

Another two subgroups of plasma lipoproteins are Chylomicrons, which the density less than water, 0.96, and the Albumin-bound free fatty acids, consisting of 99% protein and 1% lipid.

Composition of the lipoproteins in plasma of man (8, 10)
(adapted from Obson & Vester, 1960 and Hoffman, 1970)

Fraction	Source	Density	Sf	Av. Conc., mg/100 ml	Electrophoretic Zone	Composition % Total lipid						
						Protein (%)	Total lipid (%)	Triglyceriee	Phospholipid	Cholesterol Ester	Cholesterol Free	Free fatty acids
Chylomicrons	Intestine	0.96	10^4–10^5	0–10		1	99	88	8	3	1	–
Low density lipoprotein												
LDF 1, VLDLP	liver	0.06–1.006	20–400	120	B	7	93	56	20	15	8	1
LDF 2		1.006–1.019	12–20	40	B	11	89	29	26	34	9	1
LDF 3		1.019–1.063	0–12	280	B	21	79	13	28	48	10	1
High density Lipoprotein												
HDL 1	liver	1.063	2		a_1							
HDL 2		1.063–1.125		40	a_1	33	67	16	43	31	10	–
HDL 3		1.125–1.210		240	a_1	57	43	13	46	29	6	6
Albumin–FFA	Adipose tissue					99	1	0	0	0	0	100

LDF = Low density fraction

HDL = High density lipoproteins

VLDLP = Very low density lipoproteins.

Electrophoresis

Electrophoresis of lipoproteins, though providing a measure of the net electrical charge carried by a given lipoprotein under the conditions of electrophoresis. The method use barbital buffer as a solvent and the support media can be starch medium, agar or agarose, filter paper and cellulose acetate.

There are 2 differences between lipoprotein add protein electrophoresis. The first is less amount of serum or plasma sample for protein electrophoresis than that of lipoprotein electrophoresis. And the second is the dye for staining. In lipoprotein staining we use fat soluble dye, such as oil red O (Sudan II), Sudan III, sudan IV, Sudan black or Fat red 7 B

Types of Lipoproteins (5, 7, 8, 10)

By the method of electrophoresis, lipoproteins are separated into 4 groups ;

(1) **Alpha lipoprotein** migrate the greatest distance from the origin with alpha-globulin and are composed of 20 % cholesterol and 80 % phospholipids. The concentration in plasma is about 3 % of plasma protein or 35 % total plasma lipoprotein, the density ranged from 1.063 - 1.210. Hydrated a - lipoprotein, contains 15 % water and molecular weight is about 165,000-400,000.

Oncley, Scatchard and Brown studied a - lipoprotein by the method of light-scattering and viscometry and found that it is oval in shape, about 300×50 °A (5, 7)

a -lipoprotein is stable substance consisting of higher phospholipid and protein than the other lipoproteins. It is soluble in fat solvent to form a protein and small amount of phospholipid, but it can not be precipitated by polyanions.

In comparison with B-lipoprotein, they are composed of nearly the same amount of fatty acid, the ratio of sphingomyelin and lecithin is 0.2 by weight but there are more esterified cholesterol and phospholipid in a - lipoprotein (7)

(2) **Beta lipoprotein** migrate with B-globulin. There are about 5% ofplasma protein or 75% tatal plasma lipid, the density is between 1.006-1.063 or 1.03 in average. By ultracentrifugation, the most part is separated in Sf 0-12 fraction.

There are no a - but B-lipoprotein in newborn infant plasma, which composed of 60 % cholesterol.

B-lipoprotein consists of 20-25 % by weight, 8 % cholesterol, 35 % esterified cholesterol, 22 % phospholipid, 10 % triglyceride and small amount of fatty acid. The ratio of sphingomyelin and lecithin is 0.4. B -lipoprotein is oval in shape, about 15×350 °A, and molecular weight of $1.3\text{-}32\times10.^{6}$

B - lipoprotein forms cholesterol and glycerides with cold ether or n-heptane.

Robert and Szezo found that some of hormones estrogen, estriol, progesterone and fat soluble carotenoid are carried by the B-lipoprotein.

(3) **Pre-beta lipoprotein** migrate slighty ahead of the B-fraction and has the density of 0.06 to 1.006 or VLDLP (Sf 20-400).

Pre - B - lipoproteins are composed of 85% lipid, mostly triglycerides and 2-15% protein. (7)

(4) **Chylomicrons** will not move at all in the electric field. They have the least density of 0.96% and Sf less than 400 chylomicrons can be seen under dark field or electron microscope, they are spherical in shape, 0.1-5.0 microns, but circulating chylomicrons are not larger than 1 micron (7, 8)

Chylomicrons are composed of triglycerides, arounded with phospholipid, cholesterol and small amount of esterified cholesterol, and the protein content is about 0.5-2.5% by weight.

In general, the method of electrophoretic separation of lipoproteins can be done by 2 ways, paper and cellulose acetate methods.

(1) **Lipoprotein by paper electrophoresis** Straus and Wurm separated lipoproteins and fixed them by heating at 107-120°C, then stained lipid with fat red 7B decolorized the background in sodium hypochlorite. The evaluation can be done by densitometry or photometrically.

Less and Match (11) found that the lipoprotein resolution was better when using buffer containing 1 : 100 albumin solution (w/v) due to reduction of adsorption of protein by the filter paper. The lipoproteins are separated into 3 fractions; a - ,pre-B and B-lipoprotein.

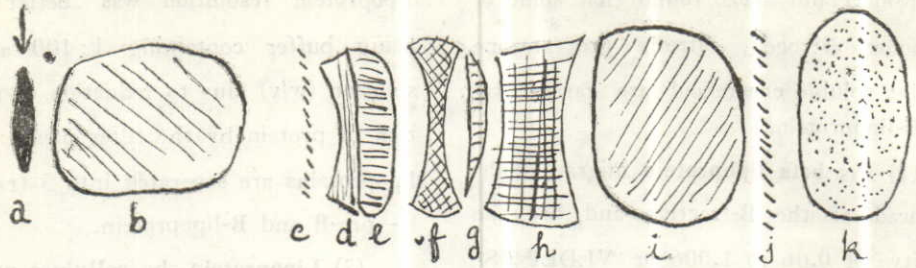
(2) **Lipoprotein by cellulose acetate :** This method is convenient, less time consuming and more distinct than the paper electrophoresis method.

Many workers had studied about the lipid staining solution. Colfs and Verheden used sudan black as a staining, and then the other used Schiff's staining.

Chin and blankenhorn separated 5 - 10 microliter plasma by running electrophoresis for 90 minutes, then stained overnight with oil red 0 dye solution and cleared with glycerol and evaluated them by scanning.

Another workers used cellulose acetate (Sephraphore III, Gelman instrument Co.) as a supporting media. The time of electrophoresis was about 15 - 20 minutes at 200-250 voltages. The lipoproteins are separated into 4 bands, chylomicrons, beta, pre-beta and alpha-lipoprotein.

Raymond E. Becking Jr. and Ralph D. Ellefson (3) used cellulose acetate (Gelman Sephraphore III) for separating 0.75 microliter of serum at 300 volt for 45 minutes. They compared the separated fraction on cellulose acetate with ultracentrifugation method and classified them as follow :



Arrow = Point of application

a = Chylomicron

b = Gamma-lipoprotein

c = plasma "fibrinogen" lipoprotein

d = beta-lipoprotein 2

e = beta-lipoprotein 1

f = very low density pre beta lipoprotein 2

g = high density pre beta lipoprotein

h = very low density pre beta lipoprotein 1

i = alpha-lipoprotein 2

j = alpha-lipoprotein 1

k = albumin-bound fatty acids

Disorders of lipoproteins (4, 7)

Disorders of lipoproteins is due to abnormality of lipid transport or metabolism. There are three types of abnormal lipid metabolism.

1. Dyslipidemia or Dyslipoproteinemia which have no lipid except fatty acid in the plasma.

2. Hypolipoproteinemia

3. Hyperlipoproteinemia

Lipoprotein deficiency States (4, 7)

These are :-

1. Abetalipoproteinemia due to poor absorption of lipid in infant stages. They always have mental retardation. The laboratory findidgs of plasma are marked lowering of cholesterol and glyceride. The beta-lipoprotein fraction cannot be seen by the electrophoretic method.

2. Hypobetalipoproteinemia. The lipid content in the plasma are lowered in phospholipid, cholesterol and glycerol. By electrophoresis, beta-lipoprotein fraction is below normal.

3. Alpha-lipoprotein deficiency. (Tangier disease). The laboratory findings are lowering in plasma phospholipid and cholesterol, but moderately high glyceride. Electrophoretic finding is absent of high-density alpha-lipoprotein.

Hyperlipoproteinemia (4, 7, 14)

Frederickson et al classified hyperlipo proteinemia by electrophoresis to 5 types as follow :-

Type I The serum is milky with characterized by marked increase in the cholesterol and glyceride. The electrophoresis shows excess pre-beta lipoprotein.

Type II Familial hypercholesterolemia, clear serum with markedly elevated cholesterol and normal to elevated of glyceride. The beta-lipoprotein is increased by electrophoretic method when the pre-beta is increased or normal.

Type III There are moderately elevated of cholesterol and variable to elevated glyceride (familial endogenous hyperlipemia) which produce turbidity of serum. Electrophoresis shows increase in "Floating" B-lipoprotein.

Type IV The serum is turbid with usually elevated glyceride of endogenous or "carbohydrate-induced" and slightly elevated cholesterol. The electrophoresis shows hyperpre-beta lipoprotein.

Type V This is a mixed type of exogenous and endogenous origins. There is hypertriglyceridemia. The electrophoresis shows hyper pre B-lipoproteinemia and hyperchylomicronemia.

Secondary hyperlipoproteinemia, similar to Type V by electrophoresis study. For example, Diabetes mellitus, Acute alcoholism and chronic pancreatitis.

Materials and Methods

The method we used in this experiment is of the Fletcher and Styliou (6), but instead of Sephraphore III cellulose acetate we used Beckman Cellulose acetate as for the protein analysis. And because of poor separation when 0.25×3 ul serum was used, so we applied more sample. The 0.25×7 ul. serum showed the best separation. All serum specimens were obtained during postabsorptive period.

Results

The serum lipoproteins are separated into small fractions of chylomicrons, B, pre-B, a-lipoprotein and albumin-bound fatty acids.

From 24 normal serum samples, we got 0% chylomicrons, 30-70% B-lipoprotein (average 48.2%), 2.7-20.8% pre-B-lipoprotein (average 11.4%), 2.0-13.0% a-lipoprotein (average 9.5%) and 9.5-52.6% (average 30.9%) of abumin-bound fatty acids.

The experiment was done on the sera of various conditions. The results obtained are already shown in Figure 1-12

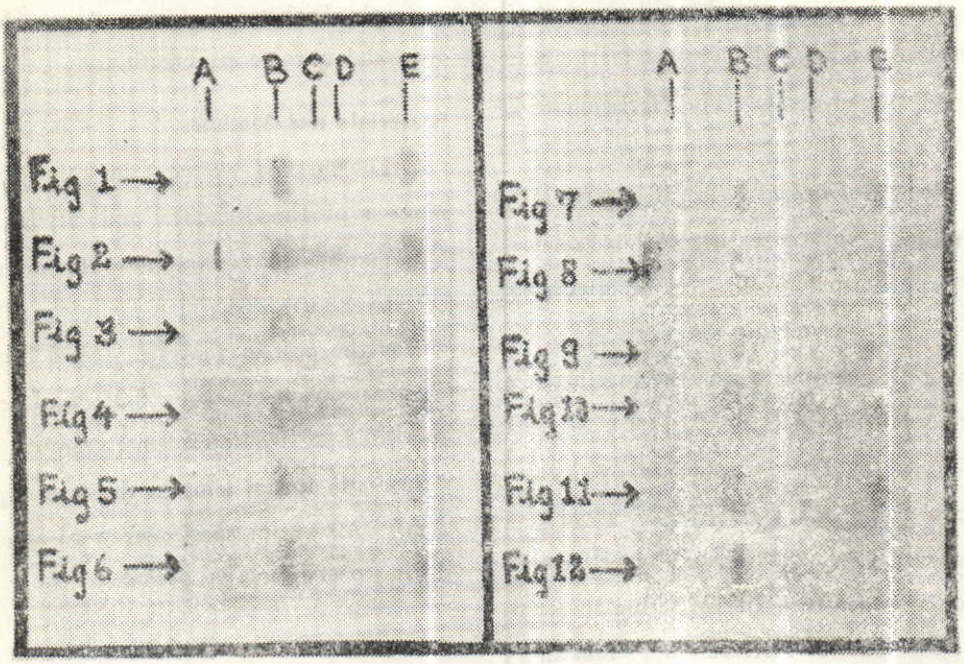


Fig. 1–4 shows serum lipoprotein patterns in normal persons.

Fig. 5–6 shows slightly increased B–lipoprotein and markedly elevated pre–B–lipoprotein in case of Diabetes mellitus.

Fig. 7–8 Jaundice serum with increased or normal B–lipoprotein but decreased or absent of pre–B–lipoprotein.

Fig. 9 is the lipoprotein pattern of cirrhotic serum which has low B–lipoprotein and absent of a–lipoprotein,

Fig. 10–11 show markedly elevated of B– and a – lipoprotein in Multiple myeloma.

Fig. 12 The condition of hypercholesterolemia shows the elevation of B– and a – lipoprotein but lowering of pre – B – lipoprotein.

Discussion

with the improved resolution of the lipoprotein by electrophoresis on the cellulose acetate, this method diluted 1 package of Beckman buffer B-2 to 1,400 ml. instead of 1,000 ml. Decreasing in the ionic strength of the buffer enhances the resolution of lipoprotein in the following ways:

1. Lengthens the overall pattern.
2. Separates " fibrinogen " lipoprotein

from beta-lipoproteins by moving it closer to the origin. But in this experiment we used serum, so there are no fibrinogen shown at all.

3. Separates beta-lipoprotein into 2 components, B_1 and B_2-lipoprotein.

4. Spreads out and separates pre-beta and alpha components.

Stain which is not freshly made or stain which has been used previously gives a poorly-stained pattern. The freshly-made, supersaturated, aqueous-alcoholic solution of oil red 0 give excellent staining. Fifteen minutes of staining give the best result; staining for longer period leads to decreased intensity in chylomicron and alpha components.

Freshly prepared decolorizing solution can not remove all dye from the background of the membrane. Excessive exposure may result in decolorization of lipoprotein, especially in the chylomicron and alpha components. This bleaching stopped quickly by transferring the membrane to the series of acetic acid washes when the desired level of decolorization and intensification is reached. Clearing the membrane at over 80°C may cause the fuse of the membrane to the glass. The presence of glycerol on the glass-plate or in the solution will impair the clearing of the membrane.

In our experiment, we used Beckman cellulose acetate membrane, but Gelman Sparaphore III cellulose acetate gives clearer background.

Evaluation of sample can be done by Densitometry as in the protein study. The comparison between normal serum and different diseases which involve the lipoprotein metabolism, we shall see the different of the components only in chylomicron, B, pre-B and a-lipoprotein. A rather intense band in the position of albumin is found in this method. The band shows marked intensification during the bleaching procedure. The exact nature of this component is being investigated (6); it may represent albumin-bound free fatty acids.

Conclusion

Scanning of the 24 normal serum shows chylomicrons as 0% B-lipoprotein 48.2% (range 30.4-70.3%), pre-B-lipoprotein 11.4% (range 2.7-20.8%), a-lipoprotein 9.5% (range 2.0-13.9%) and albumin bound fatty acids 30.9% (range 9.5-52.6%).

In condition of primary lipidemia, i.e., hypercholesterolemia shows significant elevation of a- and B-lipoproteins but low pre-B-lipoprorein.

Secondary lipidemia, as in Diabetes mellitus related with slightly high B-lipoprotein and marked elevation of pre-B lipoprotein. There are high B-lipoprotein but low or absent of pre-B lipoprotein in jaundice. In Multiple myeloma shows marked elevation of both B- and a-lipoprotein.

The detailed modification of the method being used in this study was also discussed.

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