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UNIVERSITY OF ALBERTA

HUMAN PLASMA LIPOPROTEIN FRACTIONATION BY AGAROSE GEL ELECTROPHORESIS

by

© WILLIAM CANTLON IRWIN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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DEPARTMENT OF BIOCHEMISTRY

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SPRING, 1969

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "HUMAN PLASMA LIPOPROTEIN FRACTIONATION BY AGAROSE GEL ELECTROPHORESIS", submitted by William Cantlon Irwin in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Human plasma lipoproteins were fractionated by preparative ultracentrifugation and further characterized by analytical ultracentrifugation, chemical analysis, electron microscopy, and immunoelectrophoresis. These fractions were then subjected to agarose gel electrophoresis and the following conclusions drawn.

1. Those lipoproteins of $S_f > 400$ do not enter the gel.

2. The S_f0 - 20 lipoproteins are the slowest migrating group that show distinctly after staining. The S_f0 - 10 and 10 - 20 lipoproteins, separated by preparative ultracentrifugation and individually electrophoresed on agarose gel, have slightly different migration rates. However the difference is not sufficient to show as two separate fractions when plasma is electrophoresed.

3. Lipoproteins of S_f20 - 400 are located in the general $alpha_2$ globulin region. There is evidence that lipoproteins of S_f approaching 400 are located at the anode side of the $alpha_2$ band. Lipoproteins with a lower S_f have a lesser migration rate and when elevated levels are encountered in abnormal plasma, may present a continuum back to the S_f10 - 20 area. Thus there appears to be a definite and continuous correspondence between S_f values and electrophoretic mobility for lipoproteins of S_f0 - 400.

4. The farthest migrating group of lipoproteins are found in the α_1-albumin region and consist of a mixture of

$alpha_1$ lipoproteins plus any Sudan Black B sensitive lipids adsorbed to albumin.

The above conclusions are valid only for separations carried out on agarose gels of 0.5 g./100 ml. concentration.

Agarose gel electrophoresis requires less time for completion than does paper electrophoresis and the migration of lipoprotein is more reproducible. Thus this procedure should lend itself particularly well to phenotyping hyperlipidemias and aid in the diagnosis of diseases involving secondary lipoprotein anomalies. In addition, the electropherogram has a clear background which makes it suitable for photometric scanning. Quantitative integration of varying concentrations of Sudan Black B stained plasma lipoprotein reveals that the relationship between integrated area and lipoprotein concentration is not linear. However if the procedural conditions are rigidly adhered to, reproducible results may be obtained which will be a valuable aid in the evaluation of therapeutic measures instigated for correction of primary lipoprotein disorders and, for following the course of diseases producing secondary lipoprotein abnormalities.

TERMINOLOGY GUIDE

Plasma lipoproteins, classified by analytical ultracentrifugation, are related to their respective density ranges in the following table. The interrelationship with other conventional nomenclature, used mainly in respect to electrophoretic procedures, is also given.

Density	Analytical Ultracentrifuge S_f	Other Terminology*
V.L.D.L. $<$ 1.006 g./ml.	$>$400	Chylomicrons
	20 - 400	Pre-β lipoprotein
L.D.L. $>$ 1.006 g./ml.	10 - 20	
L.D.L. $<$ 1.063 g./ml.	0 - 10	β lipoprotein
H.D.L. $>$ 1.063 g./ml. $<$ 1.21 g./ml.		α_1 lipoprotein
V.H.D.L. $>$ 1.21 g./ml.		

V.L.D.L. - very low density lipoprotein.
L.D.L. - low density lipoprotein.
H.D.L. - high density lipoprotein.
V.H.D.L. - very high density lipoprotein.

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*This terminology is meant only as a general guide. The terms take on slightly different meanings when applied to different methods of lipoprotein fractionation.

Generally speaking, β lipoprotein has an electrophoretic migration rate similar to β globulin and α_1 lipoprotein has a migration rate similar to α_1 globulin. The term pre-beta refers to the lipoprotein migrating between beta and alpha globulin. The term β lipoprotein should be reserved for the relatively homogenous group of lipoproteins that have flotation values of S_f 3 - 9, although it is frequently used to designate all the low density lipoproteins (S_f 0 - 20).

The term particle has frequently been used in the literature to designate a specific group of lipoproteins of size large enough to be seen with the light microscope. In this text it has been used indiscriminately with reference to any lipoprotein macromolecule.

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I. INTRODUCTION

Knowledge of lipid-protein relationships in the first half of this century was rather slow in developing. Perhaps the earliest evidence for existence of serum lipoprotein complex was presented by Nerking (1) in 1901. He found that previously unextractable fat became ether-soluble, following the exposure of serum to pepsin.

Hardy (2) observed phosphorus to be present in serum globulin, but did not consider it to be lipid bound. Several years later, in 1913, Haslam (3) described the phosphorus complex as a "lecithin-like body or bodies which would appear closely connected with the globulin, but not part of the molecule".

Rand (4) in 1918 established the presence of cholesterol in serum globulin, and in 1926 it was shown to be associated in small quantities with albumin (5,6).

A major contribution to the lipoprotein concept was made in 1941 when Blix, Tiselius, and Svensson (7) found all major serum protein components fractionated by "free" electrophoresis to contain cholesterol and phospholipid. The alpha and beta globulins were associated with the largest amounts of lipid, although albumin and gamma globulin contained significant amounts of cholesterol as well as phospholipid. The beta globulin boundary was

frequently opalescent presumably due to associated neutral fat.

Quantitative analytical ultracentrifugation of serum lipoproteins developed from the initial observation of Pedersen (8), that the "X-protein" complex of the sedimentation diagram reported by McFarlane (9) in 1935 was extremely sensitive to changes in salt concentration. Pedersen (10) found that by increasing the density of the suspending media, "X-protein" could be made to float rather than sediment, in an ultracentrifugal field. This enabled isolation of "X-protein" and a close relationship between this fraction and beta lipoprotein isolated from serum by ethanol fractionation was suggested.

Gofman, Lindgren and Elliot (11) in 1949 postulated that the albumin boundary asymmetry in the ultracentrifugal pattern of undiluted sera was due to a "pile up" of lipoprotein (X-protein) on the albumin concentration gradient. The basis for this phenomenon was the difference in sedimentation rates on either side of the albumin boundary gradient. A related anomaly occurring in protein mixtures had previously been described by Johnston and Ogston (12).

These observations led Gofman and associates (13) to propose a new method for the estimation of low density lipoproteins utilizing the ultracentrifuge. They adjusted the small particle density of serum to 1.063 g. per ml. and then separated the low density lipoproteins from other serum macromolecular complexes by preparative ultracentri-

fugation. All large molecules of density greater than 1.063 g. per ml. undergo sedimentation whereas the lipoproteins of density less than 1.063 g. per ml. layer at the top of the tube. These lipoproteins are removed and then characterized in the analytical ultracentrifuge. The flotation rates are given in S_f units (Svedbergs* of flotation). The term S_f is reserved by convention to the movement of lipoprotein particles in a sodium chloride solution of density 1.063 g./ml. at 26°C.

Papers published by Gofman and associates (13,14) aroused considerable clinical interest in serum lipoproteins and their relationships to various pathological conditions. Their initial studies involved mainly the S_f0 to 10 and 10 to 20 groups. They found:

(a) elevation of S_f10 - 20 lipoprotein is associated with atherosclerosis.

(b) hypertensive patients with coronary artery disease showed higher S_f10 - 20 levels than individuals with uncomplicated hypertension.

(c) patients with myocardial infarction or angina pectoris had consistently higher levels of these lipoproteins than apparently normal individuals.

(d) diabetic patients with vascular disease had higher levels than diabetic individuals not showing vascular complications.

---

* One Svedberg unit equals 10^{-13} cm./sec./dyne /g.

(e) the presence of the $S_f10 - 20$ class of molecules in high concentration was noted in myxedema, nephrosis, and xanthoma tuberosum.

An extension of these studies to include the $S_f20 - 100$ and $S_f100 - 400$ groups revealed in most instances, changes of even larger magnitude in the above mentioned anomalies (15).

Gofman pointed out that while the total serum level of any one of the lipid constituents, e.g. cholesterol, is determined by the presence and amount of the individual lipoprotein molecules, the converse is not true. Thus the serum total cholesterol is of little value in predicting the level of any one class or group of lipoproteins.

Qualitation and quantitation of individual lipoprotein groups was therefore of clinical importance and would be valuable in diagnosis, prognosis, and evaluation of therapeutic measures instigated for correction of the anomalies. The value of analytical ultracentrifugal analysis, as a diagnostic tool, was limited by its general lack of availability due mainly to its cost. Thus, alternative methods of analysis were investigated.

Smith (16) attempted to clarify the relationship between fractions separated by paper electrophoresis and by the analytical ultracentrifuge, so that the results of the large series of runs by Gofman and associates (17) using the analytical ultracentrifuge would be of value to laboratories not having this equipment. Using the prepara-

TABLE 1

Relationships between Ultracentrifugal and Paper Electrophoretically Separated $S_f0 - 400$ Lipoproteins. [Smith (16)]

Smith Fraction No.	Paper Electrophoretic Component	Ultracentrifugal Component	Mean Ultra-centrifugal Peak Value
1	Deposit on origin	S_f400 plus	Not visible
2	Trail lipids (origin to beta lipoproteins)	$S_f50 - 400$	S_f74
3	Pre-beta* lipid with trail to origin	$S_f20 - 100$	$S_f28.5$
4	"Fast" beta lipoprotein	$S_f10 - 30$	S_f14
5	Beta lipoprotein	$S_f0 - 10$	$S_f6.0$

*Pre-beta refers to that lipoprotein which has a mobility greater than normal beta lipoprotein, but less than $alpha_1$ lipoprotein.

TABLE 2

Lipoprotein Typing by Paper Electrophoresis.

Type	Predominant Paper Electrophoretic Characteristic Change from Normal	Analytical Ultracentrifuge Characteristic Change from Normal
I	Majority of lipid deposited at the origin. All other lipoproteins ↓	S_f100 - 400 ↑
II	Beta ↑↑↑ Pre-beta ↑±	S_f0 - 12 ↑↑↑ S_f20 - 100 ↑±
III	"Broad β" band present pre-beta ↑±	S_f0 - 12 ↓ S_f12 - 100 ↑↑
IV	Pre-beta ↑↑↑	S_f0 - 20 ↓± S_f20 - 400 ↑↑↑
V	Some lipid deposited at origin. Pre-beta ↑↑	S_f20 - 400 ↑

↑ Increased

↓ Decreased

± May or may not be

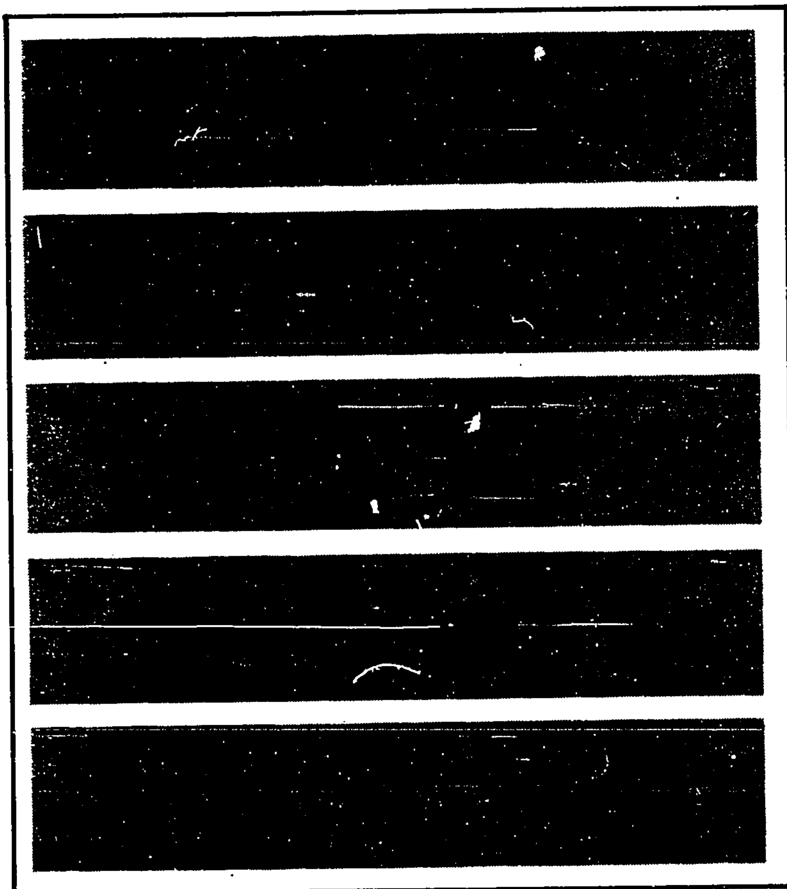

FIGURE 1: Paper electropherograms showing the five types of plasma lipoprotein patterns described by Fredrickson (24).

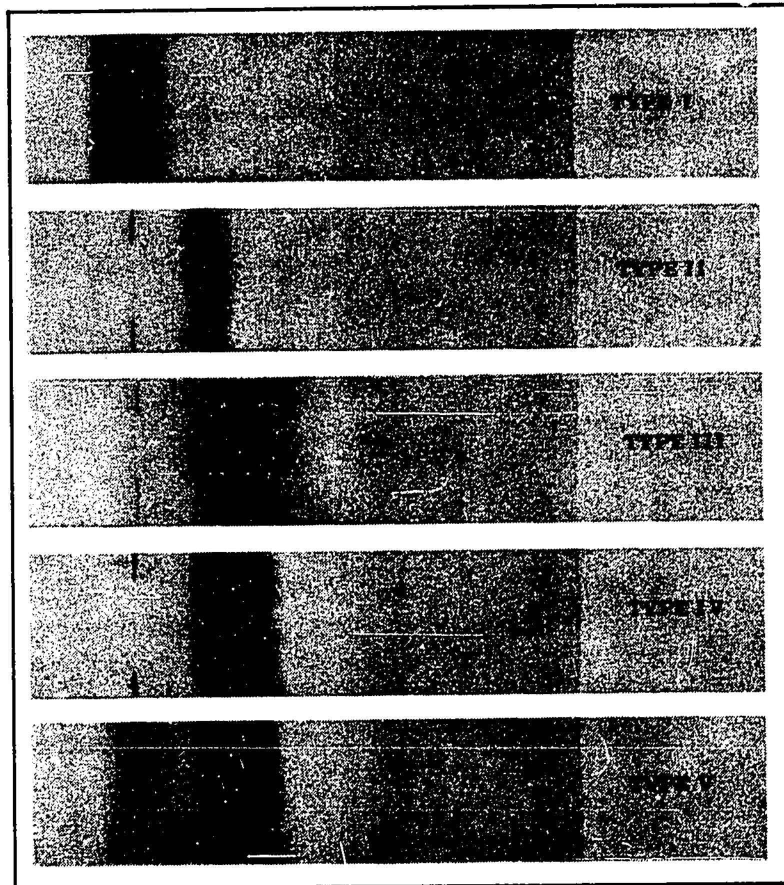

FIGURE 1: Paper electropherograms showing the five types of plasma lipoprotein patterns described by Fredrickson (24).

tive ultracentrifuge, she separated the low density (S_f0 - 20) and very low density (S_f20 - 400) lipoproteins into five groups utilizing varying combinations of density, centrifugal force and time. These fractions were further characterized by analytical ultracentrifugation and the relationship to paper electrophoretic patterns established is shown in table I.

However, by Smith's paper electrophoretic technique, pre-beta lipoprotein overlaps beta lipoprotein. A marked improvement was achieved by Lees and Hatch (18) who obtained sharper separation of serum lipoproteins by the addition of albumin to the buffer. Using this technique, a relatively distinct "pre-beta" fraction was obtained with some sera. Fredrickson and associates (19,20, 21,22,23) using this improved technique of paper electrophoresis developed a system of classifying hyperlipoproteinemias into five different types as shown in table 2.

As is seen lipid disorders have been grouped into types based upon alterations in plasma lipoprotein fractions. Fredrickson has implied that examination of the paper electropherograms combined with cholesterol and triglyceride analysis will yield a tentative phenotype. The types of electropherograms obtained are demonstrated in figure 1 (24). It should be emphasized that by paper electrophoresis, normal fasting plasma shows only beta and alpha lipoprotein, pre-beta lipoproteins are absent as a distinct band and chylomicrons are not seen at the

origin.

These five types of lipoprotein patterns have been observed both where the lipoprotein anomaly is the primary defect and also where the lipoprotein anomaly is secondary to other disorders such as diabetes, hypothyroidism, obstructive hepatic disease, nephrotic syndrome, pancreatitis, alcoholism and certain dysglobulinemias. If the lipoprotein anomaly is secondary in nature, the anomaly disappears as the primary disorder is corrected. When lipoprotein patterns of types I, II and III appear as a genetic or familial trait, they are unique to the family group (22); however types III and IV may be different phenotypic expressions of the same genetic anomaly or anomalies, since both types are frequently found in the same family (25). The genetics of the type V phenotype are not clear (23).

A brief description of the clinical manifestation, prognosis, and therapeutic treatment of the different anomalies follow. As will be seen, these disease entities and possible treatment varies considerably and logically treatment should not be embarked upon until the type is established.

Type I phenotype is generally diagnosed in infancy. These children look healthy but have "bouts of colic" and an unusually prominent abdomen. Frequently, yellow papules are found on the skin and mucosa and the liver and spleen are sometimes enlarged. Lipemia retinalis is

often observed. Blood samples look like "cream of tomato soup". If the abnormality is not detected until the child is old enough to describe his abdominal discomforts, he may undergo laparotomy before the nature of his syndrome has been appreciated. Treatment consists of a moderate restriction of fat. Infants formulae is changed to one containing only skim milk and fat free sources of calories. Adults should restrict their fat intake to 20 to 25 g. per day.

Thus far, there has not been any evidence of accelerated coronary artery disease associated with this anomaly.

Type II familial hyperlipoproteinemia is inherited as an autosomal dominant trait. Although occasionally expression may be delayed until the second decade it can generally be detected by abnormally high beta lipoprotein concentrations in the very young. Tendinous xanthomas located in the Achilles tendons and the extensor tendons of the hands and feet are a characteristic manifestation and accelerated arterial disease especially involving the coronary arteries are common.

Dietary treatment of the type II abnormality consists of limiting the intake of cholesterol rich foods, and substitution of polyunsaturated for saturated fats and prevention of obesity.

Cholestyramine, which binds bile acids and prevents their reabsorption, has been reported to be the most effective drug for lowering the elevated beta lipoprotein and choles-

terol levels in type II hyperlipoproteinemia (22).

The most typical lesion of the type III lipoprotein abnormality is the "palmar striae" found on the palmar surfaces of the hands. The creases and sometimes the tips of the fingers or other areas, especially where rings are worn, contain yellow deposits. These are often subtle changes and are sometimes overlooked by both patient and physician. Although xanthoma tendinosum and xanthoma tuberosum occur in both types II and III hyperlipoproteinemias, the latter is more characteristic of the type III. Xanthoma tuberosum may be found on the elbows, buttocks, and knees. The xanthomas do not generally appear before the age of twenty-five.

The prognosis in type III is uncertain, however the majority have evidence of vascular disease and it seems reasonable to use at least moderate means of control.

Dietary management is accomplished by caloric reduction until an ideal weight is reached and then maintenance of this weight by diets containing 40 to 50 per cent of calories from fats that are high in poly-unsaturates and low in cholesterol.

The drug most effective for treatment of this abnormality is chlorophenoxyisobutyric acid (CPIB). It has been stated that this drug is not effective in treatment of the type II anomaly.

The type IV lipoprotein pattern does not appear to be a phenomena associated directly with ingested fat, but is

generally due to carbohydrate. This pattern can be induced in a normal individual over a period of time by feeding a high carbohydrate diet. Severe forms of this anomaly may show some of the abnormalities associated with type I such as eruptive xanthomas, lipemia retinalis and bouts of abdominal pain. The anomaly has been seen in children, but generally is not seen until adulthood.

There is a definite correlation between type IV anomaly and coronary artery disease, however most of the evidence is retrospective. By this, it is meant that it has been obtained after a myocardial infarct, at which time the patients may have been on a low fat and high carbohydrate diet, which normally increases the amount of $S_f 20$ - 400 species of lipoprotein in plasma.

Dietary treatment of primary type IV hyperlipemia consists of weight control and avoidance of excessive dietary carbohydrates. The most effective drug is CPIB. Also, oral antidiabetic agents may be of value.

The type V pattern is probably a stage of one or more of the first five anomalies mentioned. For example it can be produced in a type IV by the imposition of a heavy load of dietary fat. Also type IV patterns have occurred in type V kindreds, which provides further evidence for interrelationship. A type V pattern can also be produced when a type I anomaly is put on a low fat diet. Symptoms are similar to those seen in type I, however they do not appear until the late teens or the third decade.

Because type V individuals have both a fat and carbohydrate induced abnormality, management becomes complicated. Generally speaking, diets that are not unusually high in either fats or carbohydrates should be used to maintain an ideal weight and a relatively normal lipoprotein pattern. However, if the pre-beta lipoproteins increase markedly it would be advisable to reduce the carbohydrate intake. This means more fat in the diet and a probable increase in $S_f > 400$ lipoproteins. Since the clinical complications associated with increased $S_f > 400$ lipoprotein (seen as increased deposition of lipoproteins at the origin after electrophoresis) are not as serious as those associated with increased pre-beta lipoprotein, the diet where this is the dominant abnormality should probably be adhered to unless clinical problems (e.g. abdominal pain) associated with type I become too severe.

Examination of figure I gives the general impression that the five types are relatively distinct and may be established from the paper electropherograms alone. This is not the case. Type I, with chylormicron at the origin, is relatively easy to ascertain, however the differences between types II, III and IV are more subtle. Type III is described as a "broad beta", of which the majority of the lipoprotein is of a density less than 1.006 g. per ml. (the approximate small particle density of serum). As the migration rate is similar to beta lipoprotein, differentiation of type III from type II is not

possible by paper electrophoresis alone. Thus preparative ultracentrifugation of the plasma and further electrophoretic runs on the supernatant and infranatant is required to show that this is type III (density < 1.006 g./ml.), not type II (density > 1.006 g./ml.)

Also, as mobilities of the lipoproteins vary from run to run, differentiation of type IV from types II and III is difficult if either the beta or pre-beta band is absent. Inclusion of a normal control serum in each electropheretic run is mandatory and is of some help in resolving these difficulties. Further problems arise when lipoproteins of S_f50 - 400 are present in increased amounts, as these lipoproteins smear from the point of application to the beta, pre-beta area and may overlay the beta lipoprotein.

In addition to the aforementioned problems, paper electrophoresis is a lengthy procedure, and densitometric problems inherent in filter paper electrophoresis (26) have discouraged its use as a method for quantitating the amounts of lipoprotein present. It was therefore decided to search for a method of serum lipoprotein fractionation that would: (a) yield as much information as the analytical ultracentrifuge and be less costly and more accessible; (b) require less time for completion than paper electrophoretic separation; (c) provide a more distinct and reproducible separation than paper electrophoresis; (d) be more suitable for quantitation than paper electrophoresis.

Since most laboratories possess electrophoretic and densitometric scanning equipment, an electrophoretic method seemed practical as to expenditure and availability.

A procedure described by Cawley and Eberhardt (27) appeared to qualify as to electrophoretic time required and appeared suitable for "quantitation" by densitometric scanning. Dupont P 40.B 35 mm. safety motion picture film leader was used as a base for the electrophoretic stabilizing medium of Ionagar #2* gel. This is a combination and modification of two procedures. One described by Giri (28), who used a sheet of polyester film as a supporting base material for a stabilizing agar gel medium for protein electrophoresis and the other of Pickett and Sommer (29), who had used 35 mm. motion picture polyester film for histological techniques.

Initial experiments using "Ionagar" as the stabilizing media were encouraging in that sharp bands were obtained which appeared to be beta, pre-beta, and $alpha_1$ lipoprotein fractions. However under the conditions being used, electrophoresis could not be continued much longer than one-half hour without the gel liquefying. Correspondingly, one hour electrophoresis time was required to obtain adequate migration of the beta lipoprotein. Therefore, it was obvious that a more stable medium was required.

---

*"Ionagar" No. 2, Code L12, Consolidated Laboratories Inc., Box 234, Chicago Heights, Illinois.

Agar, according to Araki (30) is a complex of agarose and agaropectin. Agaropectin is a polysaccharide containing sulfate and carboxyl groups, which imparts to agar, ion exchange properties which cause undesirable side effects. Agarose is a linear polysaccharide which consists of alternating residues of D-galactose and 3,6-anhydro-L-galactose.

Hjerten (31), used agarose as an "anticonvection" agent in zone electrophoresis. Burstein and Fine (32), and Rapp (33) found the electrophoretic properties of agarose to be superior to those of agar. The former authors (32) and more recently Rapp and Kahlke (34) published papers on the use of agarose gel electrophoresis for serum lipoproteins. These authors did not attempt extensive identification of the lipoprotein fractions obtained. There were also methodological differences as compared to the procedure reported here.

Preliminary studies of agarose gel electrophoresis were made here and in comparison to Ionagar was found to:

(a) retain its physical consistency beyond the time required to obtain adequate separation of the lipoprotein fractions;

(b) result in a greater relative mobility of the lipoprotein fractions;

(c) give a firmer gel which improves ease of handling;

(d) give a clear gel. Ionagar contained impurities which tended to give a cloudy gel.

Agarose gel electrophoresis is a technique within the scope of the clinical laboratory. Separations obtained using this supporting media were found to be consistent and clear cut. On this basis it was deemed worth while to further characterize the fractionation obtained.

II. EXPERIMENTAL

A. Agarose Gel Electrophoretic Procedure

In this section, the electrophoretic conditions and staining procedures used to study plasma lipoprotein separated on agarose gel are described. It should be noted that two procedures are presented, one called qualitative and the other quantitative. The major difference between the two is in the staining techniques. The qualitative procedure gave peak heights on scanning that were too high for accurate densitometry; thus another method termed "quantitative" had to be devised.

1. Electrophoretic Conditions

Plasma sample: Whole blood was mixed with the disodium salt of ethylenediaminetetra-acetic acid (E.D.T.A.). The cells were removed by centrifugation at room temperature. Plasma electrophoresed the same day as obtained was kept at room temperature. If storage was to be prolonged past the day collected, the plasma was stored at 4°C.

Agarose: "Sea Kem" Agarose manufactured by Marine Colloid Inc. and sold by Bausch and Lomb, Rochester, N.Y. was used throughout the study.

Buffer: LKB Veronal Buffer for paper electrophoresis packaged by LKB-Produkter AB, Stockholm 12, Sweden, was used to prepare a buffer of ionic strength of 0.05 and pH 8.6.

Oven: A model 16, Precision Scientific Co., oven was kept at 80 to 90°C to heat a size 0 (42 mm. plate

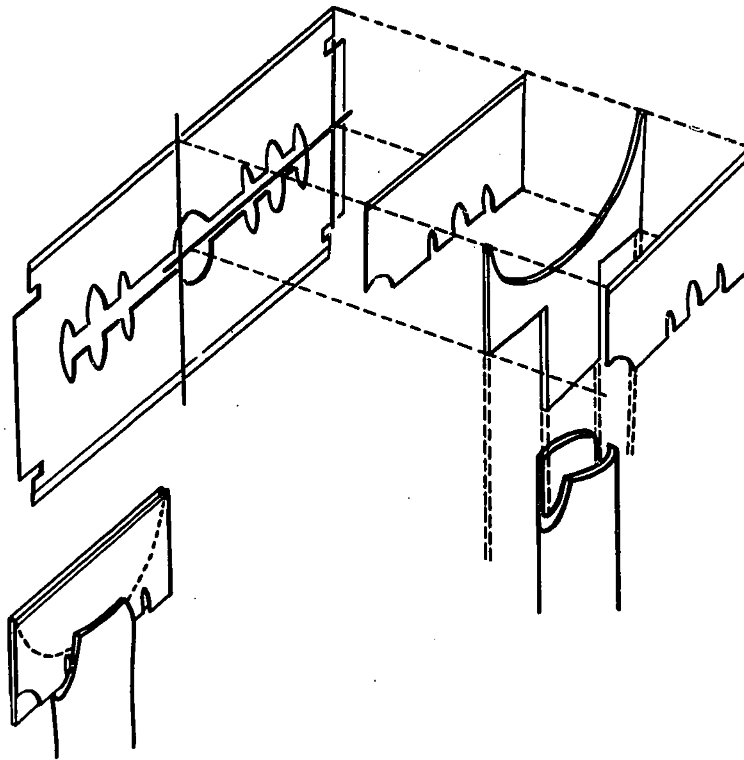

FIGURE 2: Application reservoir cutter. The upper portion of the diagram shows the individual components prior to assembly and the assembled cutter is shown in the lower left hand corner.

diameter) Buchner funnel, a 300 ml. vacuum flask and a 100 ml. graduated cylinder.

35 mm. Film Support for the Agarose Gel: Dupont P. 40B 35 mm. safety motion picture film leader was purchased from Dupont of Canada Ltd., #700 Chinook Professional Building, Chinook Shopping Centre, Calgary, Alberta. A resin coating on one side promotes adherence of the agarose solution and gel to the film. The resin according to the manufacturer's specifications is on the "inner" side of the film. Not all batches of this film are equally suitable for lipoprotein electrophoresis as some batches absorb excessive amounts of Sudan Black B. This may be related to the thickness of the resin coating which is not carefully controlled by the manufacturer.

Electrophoretic Cell: A Model E 800-2 cell from Research Specialties, Berkeley, California, was used. This cell may be cooled by circulating tap water and was used in conjunction with a Model 1910, Research Specialties, Richmond, California, power supply.

Reservoir Cutter: A reservoir cutter was constructed by gluing razor blades, broken into 20 mm. lengths, on either side of a strip of rigid plastic, as shown in figure 2. Epoxy glue was used and the plastic portion separates the blades by 1 mm. A 4-inch length of tygon tubing (3/16 inch bore, 1/16 inch wall thickness) was also attached with epoxy glue as shown.

Agarose gel was prepared by adding 100 ml. of 0.05

ionic strength LKB Veronal Buffer to 0.5 g. of agarose, in a flask. The flask was then placed in a boiling water bath for approximately one hour. The solution was mixed one or two times during the heating period.

The resulting solution was then put through a No. 40 Whatman filter in a Buchner funnel preheated to 80 to 90°C, using just sufficient vacuum to draw the solution through, without creating bubbles. Approximately 33 ml. of the filtered solution was then transferred to a pre-heated (80 to 90°C) 100 ml. graduated cylinder, from which it was poured onto a strip of Dupont P. 40B 35 mm. safety motion picture film leader 54 cm. long. The film must be placed on a very level surface so as to obtain even distribution of the agarose solution.

The gel strip was allowed to set for approximately one hour and was then transferred to a Model E 800-2 Research Specialties electrophoretic cell, cooled by circulating tap water. Slits for the serum (1 x 20 mm.) were cut into the gel using the reservoir cutter previously described. The slit was formed by removal of the gel with the aid of a modified toothpick*. The quality of

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*The flat side of the small end of a Keenan (Kaybee)** flat style toothpick was thinned to a fine edge to facilitate removal of the gel and trimmed so that the width was not greater than that of the reservoir.

**Manufactured by Keenan Woodenware Limited, Owen Sound, Ontario.

the electropherogram is partially dependent on the nature of the walls of the reservoir. Irregularities in the wall will produce poor electropherograms. The "cleanest" cut is obtained when the gel is jabbed with the reservoir cutter, rather than placing it on the surface and pushing it in slowly. Six plasma samples of 30 microlitre each were applied to each electrophoretic strip, at 5 cm. intervals along the 54 cm. length.

After electrophoresis at 600 volts for 30 minutes, the agarose gel strip was placed in methanol for 15 minutes. Failure to carry out this step results in opacity on drying. The agarose gel was then dried under a warm air stream supplied by an "Oster Airjet Hair Dryer".

When densitometry is to be done the above electrophoretic conditions are employed, except that 10 μl., rather than 30 μl. of sample is applied to the gel slot.

2. Staining Conditions

(a) Qualitative

Sudan Black B Stain: 5 g. Sudan Black B (Fisher Cat. #S-668) was added to 400.0 ml. of 95% ethanol and stirred on a magnetic stirrer for ten minutes. 100.0 ml. of distilled water was added with continued stirring. The solution was allowed to sit overnight prior to use. This allows undissolved dye particles to settle out. Aliquots used for staining were returned to the stock bottle immediately after use to minimize oxidation of the dye. This dye was used over a period of weeks, and one must be aware that when staining lipoprotein, the amount

of dye taken up decreases with the age of the dye.

Gel Rinse Solution: 200.0 ml. of distilled water added to 800.0 ml. of 95% w/w ethanol.

Gel Staining and Rinsing Vessels: Plastic trays 90 mm. wide, 185 mm. long, and 30 mm. deep were used for staining and rinsing. The rinsing tray should be either clear or white in color so that one can tell when unbound dye has been adequately removed from the gel.

The electrophoretic strips were divided into equal halves for convenience in handling and then stained for one-half hour in the Sudan Black B solution described above. The strips were laid side by side in the tray, not one above the other. Oxidation of the dye and evaporation of the solvent was reduced by covering the staining tray with "Saran" wrap. Excess dye was removed with several quick rinses (10 to 20 seconds each) in a solvent of the same composition used for the stain. This was followed immediately by a water rinse. The strips were rinsed individually using an "exaggerated" agitation motion. The entire rinse sequence was completed in less than two minutes. The electropherograms were then allowed to air dry.

(b) Quantitative

Sudan Black B Stain: Sudan Black B stain was prepared according to Swahn (35). 1.5 g. Sudan Black B (Fisher Cat. #S-668) was added to a solvent prepared by adding sufficient distilled water to 927 ml. of 95% w/w ethanol to bring the total volume to 1500 ml. This mixture

was brought to a boil with constant mixing, then allowed to cool overnight. It was then filtered twice through a No. 3 Whatman filter paper. Swahn states that on account of evaporation during heating and subsequent filtration, the final concentration of the solution will be 50 to 55 percent ethanol.

Gel Rinse Solution: Sufficient distilled water was added to one litre of 95% w/w ethanol to bring the total volume to two litres.

Gel Staining and Rinsing Vessels: Standard Durrum electrophoretic staining equipment* was used. The electrophoretic strip spacers of the staining rack were bent into vertical positions.

Approximately 1500 ml. of Sudan Black B solution was placed in the staining vessel. The electrophoretic strips were placed in the staining rack in such a manner that the rack spacers did not coincide with any of the separated lipoprotein fractions. The strips were allowed to stain for two hours at room temperature. The staining rack was then transferred to a second vessel containing the rinse solution. The strips were rinsed for thirty seconds with continuous agitation, then transferred to a third vessel containing distilled water to remove the ethanolic rinse solution, and then allowed to air dry.

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*Spinco Division of Beckman Instruments, Palo Alto, California.

B. Preparative and Analytical Ultracentrifugation Studies of Plasma Lipoprotein

As previously stated it is important to obtain more information about the physical identity of plasma lipoprotein fractions obtained by agarose gel electrophoresis. Ideally, this would be done by taking the fractions separated by agarose gel electrophoresis and determing flotation rates by analytical ultracentrifugation. However, removal of lipoproteins from agarose gel is difficult, particularly in quantities sufficient for analytical ultracentrifuge studies. Therefore an attempt was made to obtain the same information by somewhat the reverse process. Fractions were initially isolated by preparative ultracentrifugation, and aliquots were then subjected simultaneously to agarose gel electrophoresis and analytical ultracentrifugation. A description of the methodology follows.

1. Methodology

(a) Preparative Ultracentrifugation

The low and very low density plasma lipoproteins were divided into five different density classes by varying the speed and time of centrifugation and the salt density of the medium. The procedure used was essentially that described by Smith (16) with the following modifications:

(a) A Beckman #21 rotor with adaptors that would accommodate the 1/2 x 3 1/2 inch cellulose nitrate tubes was used, whereas Smith used a 30.2 rotor which is made to accept the 1/2 x 3 1/2 inch tubes. This necessitated some changes in time of centrifuging to obtain the same G-minute

values, as the #21 rotor will not withstand the higher gravitational forces at which the 30.2 rotor is used.

(b) The fractions were isolated from 6 ml. of plasma rather than 9 ml.

(c) A "layering" technique as described below was used for separating fractions 1 and 2 from the remainder of the plasma lipoproteins. Smith used a multiple wash technique which requires much more handling of the fractions and a great deal more time.

(d) A Beckman tube slicer was used in conjunction with a Pasteur pipette to remove the lipoprotein fractions, rather than using the Pasteur pipette alone. This enabled removal of the supernatant without the danger of contamination by mixing with lower layers.

Details of the conditions for isolation of each fraction are as follows:

Fraction #1:

Six ml. of plasma was overlayered with 3 ml. of 1.0071 g./ml.* NaCl and centrifuged at 9,750 r.p.m. for 45 minutes U.T.S. time, at room temperature. The uppermost 1.5 ml. was removed and labelled "fraction #1".

Fraction #2:

The infranatant left from fraction #1 was overlayered with 1.5 ml. 1.0071 g./ml. NaCl and centrifuged at 18,750 r.p.m. for 90 minutes U.T.S. time at room temperature.

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* Density in g./ml. at 25°C.

The uppermost 1 ml. was removed and labelled "fraction #2".

Fraction #3:

The infranatant left from fraction #2 was overlayered with 1.0 ml. of 1.0071 g./ml. NaCl and centrifuged at 18,750 r.p.m. for 20 to 22 hours. The refrigeration control of the centrifuge was set at 47° to 50° fahrenheit. The uppermost 1 ml. was removed and labelled "fraction #3".

Fraction #4:

If the infranatant left from fraction #3 was less than 8 ml. in volume, its volume was adjusted to 8 ml. with 1.0071 g./ml. NaCl. Following this 1 ml. of 1.1317 g./ml. NaCl was added to give a total volume of 9 ml. and a final small particle density of 1.021 g./ml. The solution was mixed well and then centrifuged at 18,750 r.p.m. for 20 to 22 hours at a centrifuge thermostat setting of 47° to 50° Fahrenheit. The uppermost ml. was removed and labelled.

Fraction #5:

One ml. of 1.388 g./ml. NaBr solution was added to the remaining 8 ml. to give a small particle density of 1.063 g./ml. The solution was mixed and centrifuged at 18,750 r.p.m. for 20 to 22 hours at a centrifuge thermostat setting of 47° to 50° fahrenheit. The uppermost ml. was removed and labelled "fraction #5".

(b) Analytical Ultracentrifugation

All fractions were adjusted to a small particle density of 1.063 g./ml. before being spun in the Spinco Model E analytical ultracentrifuge. To make this adjustment,

0.5 ml. of fractions #1,2 and 3 were mixed with 0.4 ml. of 1.1317 g./ml. NaCl solution, and 0.5 ml. of fraction #4 was mixed with 0.3 ml. of this solution. Fractions #1, 2, 3 and 4 were spun at 36,000 r.p.m. and the Schlieren patterns recorded at 2, 2, 4 and 8 minute intervals respectively. Fraction #5 was spun at 52,000 r.p.m. and photos were taken every 8 minutes. All runs were made at 20°C.

Time/distance curves were plotted and the flotation constants calculated from the equation:

$$F = \frac{dx/dt}{60_w 2_x} = \frac{2.303\ d\ (\log x/dt)}{60\ _w 2}$$

Where: F - the flotation constant

x - the distance in cm. from the axis of rotation

w - the angular velocity in radians per second

t - the time in minutes

(c) Agarose Gel Electrophoresis of Lipoprotein Fractions Separated by Preparative Ultracentrifugation

To study the electrophoretic behavior of the lipoproteins separated by preparative ultracentrifugation, fractions 1, 2, 3 and 4 were diluted with an equal volume of lipid-poor plasma and electrophoresis was done. This addition of plasma protein provides an internal marker and prevents the erratic migration that occurs when the fractions are electrophoresed in its absence (16,37). Lipid-poor plasma was prepared by adjusting the density of plasma to

TABLE 3

Average and Range of Main Peak Flotation Rates Obtained for Each of the Five Fractions Isolated by Preparative Ultracentrifugation.

	Flotation rate (S_f) of each fraction				
	1	2	3	4	5
Main Peak Value (Average)	Peak not visible	59.8	31.0	13.0	5.6
Main Peak Value (Range)		58-62	24-45	11-17	4.0-6.3

TABLE 4

Comparison of Smith's (16) Analytical Centrifuge Data on Lipoprotein Fractions Isolated by Preparative Ultracentrifugation (16) with Data from Present Study.

Fraction number and Identification		Flotation rates (S_f) for main peak of each fraction	
		Average	Range
1	Smith Present Study	Peak not visible	
2	Smith Present Study	74 59.8	52 - 101 58 - 62
3	Smith Present Study	28.5 31.0	18 - 42 24 - 45
4	Smith Present Study	14 13.0	12 - 18 11 - 17
5	Smith Present Study	6.0 5.6	4.0 - 7.5 4.0 - 6.3

1.21 g./ml. and centrifuging at 100,000 G for 24 hours. The density adjustment was made according to Havel (36). The lipid layer was removed with the aid of a Beckman tube slicer and the remaining lipid-poor plasma was dialyzed against two changes of 0.9% NaCl containing 0.001 molar E.D.T.A. Dialysis was carried out at 4°C, aided by continuous stirring, and allowed to proceed approximately 24 hours for each change of NaCl solution.

It was necessary to dialyze fraction #5 prior to electrophoresis as the high salt concentration of this fraction distorted the electropherograms. After addition of the lipid-poor plasma, dialysis was carried out at 4°C against 0.9% NaCl solution containing 0.001 molar E.D.T.A., for approximately 17 hours. Electrophoresis and staining was then carried out as previously described in sections II. A. 1 and II. A. 2(a).

2. Discussion

The preparative ultracentrifugation of the plasma lipoproteins apparently yielded fractions similar to those of Smith (16). This is evidenced from the good agreement of the average and range flotation values of the main peaks shown separately in table 3 and compared with Smith's data in table 4.

The flotation rates have not been corrected for concentration effects. Theoretical justification for not doing so is now presented. Generally speaking, increasing concentrations will result in decreasing flotation values.

The reason for this is probably three-fold (38). First, the effective viscosity of the medium surrounding each sedimenting particle will be some function of both the concentration of the particles and their individual contributions to the macroscopic viscosity of the solution. Secondly, the density of the solution through which the molecules sediment is partially a function of concentration of the particles. Finally, the contribution of backward flow of liquid in the opposite direction to the particle movement, due to the liquid flowing back to fill the space formerly occupied by the particle, will cause a decrease in the rate of sedimentation.

Ewing (39) published both uncorrected and concentration correction Schlieren patterns for a solution containing S_f0 - 400 lipoproteins obtained by preparative ultracentrifugation of plasma. A corrected S_f peak of 6 had an uncorrected value of approximately 5.6. The S_f10 - 20 peak was estimated approximately 2 units low from the uncorrected pattern and the S_f20 - 100 peak approximately 10 to 15 units low.

However the differences between uncorrected and corrected S_f values shown above are larger than comparable corrections for values obtained in this study. The reason for this is that the lipoprotein concentrations are much lower in the present study. For example the procedure (40) used by Ewing for isolation of the S_f0 - 400 fraction results in a three-fold concentration of the lipoproteins. Prior to concentration the average total S_f0 - 400 lipoprotein content is in the 550 to 575 mg./100 ml. range

(39,41). Thus the final concentration obtained by Ewing (39) would be approximately 1700 mg./100 ml. However the mobility of any one component will be affected only by those components having a lesser mobility. Thus the flotation value in Ewing's study for the $S_f10 - 30$ lipoprotein peak will be affected by the concentration of the $S_f0 - 10$ lipoprotein plus any lipoproteins with mobilities less than the $S_f10 - 30$ peak. The sum of these would amount to approximately 1200 mg./100 ml. In the present study the $S_f10 - 30$ lipoprotein was separated from the $S_f0 - 10$ lipoprotein prior to analytical ultracentrifugation. Thus the total lipoprotein content was in the region of 200 mg./100 ml.

The flotation value for the $S_f20 - 100$ lipoprotein peak in Ewing's study will be affected by $S_f0 - 20$ lipoprotein plus any lipoproteins with mobilities less than the $S_f20 - 100$ peak. The sum of these lipoprotein concentrations would be in the region of 1400 gm./100 ml. The lipoprotein concentration in the present study of $S_f20 - 100$ and 50 - 400 fractions, with the exception of one abnormal specimen, was in the region of 300 to 400 mg./100 ml.

Thus in the data reported here, the error in S_f values due to concentration effects is minimal, due to the relatively low lipoprotein concentrations of the fractions used for analytical ultracentrifuge studies.

Next the electrophoretic characteristics of the lipoprotein fractions (separated by preparative ultracentrifugation)

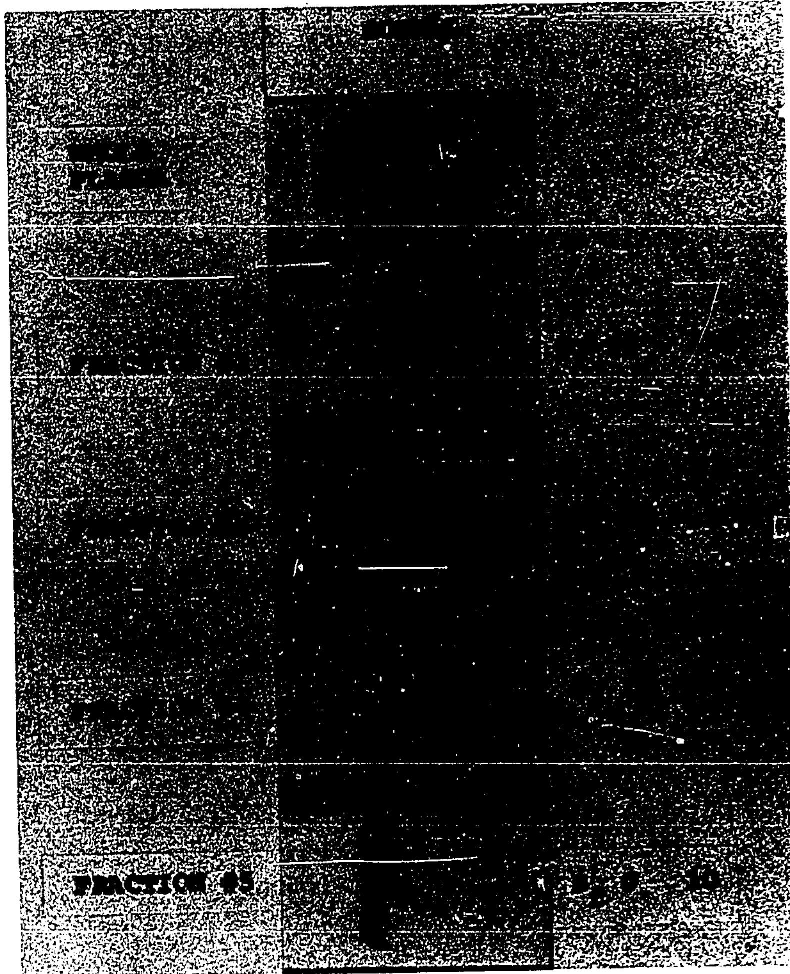

FIGURE 3: Agarose gel electrophoresis of a normal whole plasma and the lipoprotein fractions obtained following preparative ultracentrifugation.

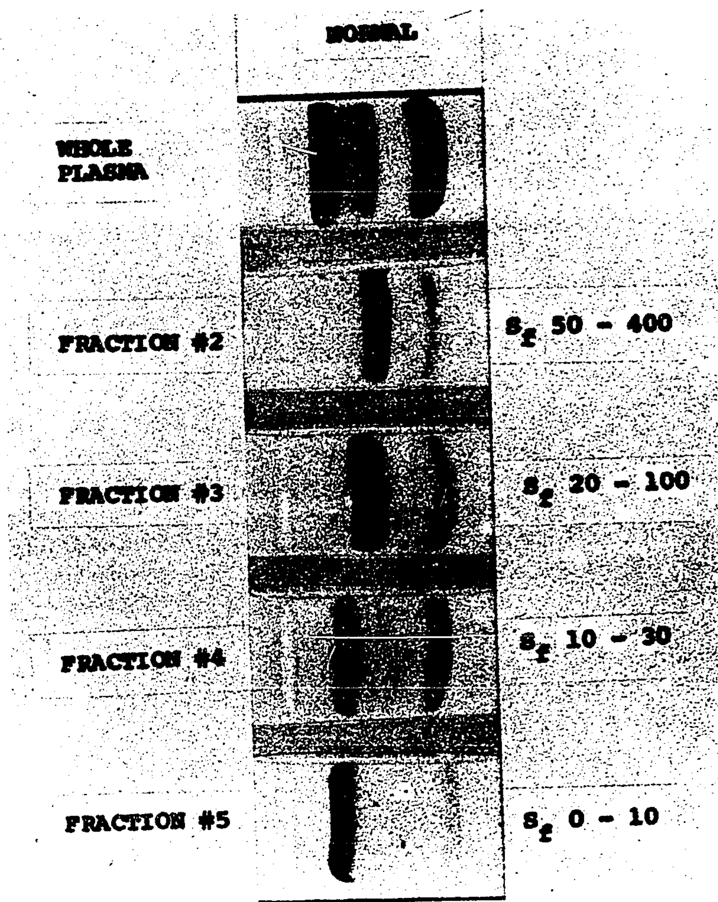

FIGURE 3: Agarose gel electrophoresis of a normal whole plasma and the lipoprotein fractions obtained following preparative ultracentrifugation.

FIGURE 4: Agarose gel electrophoresis of normal plasma and plasma obtained from patients having types II and III lipoprotein abnormalities, along with the respective fractions obtained from them by preparative ultracentrifugation.

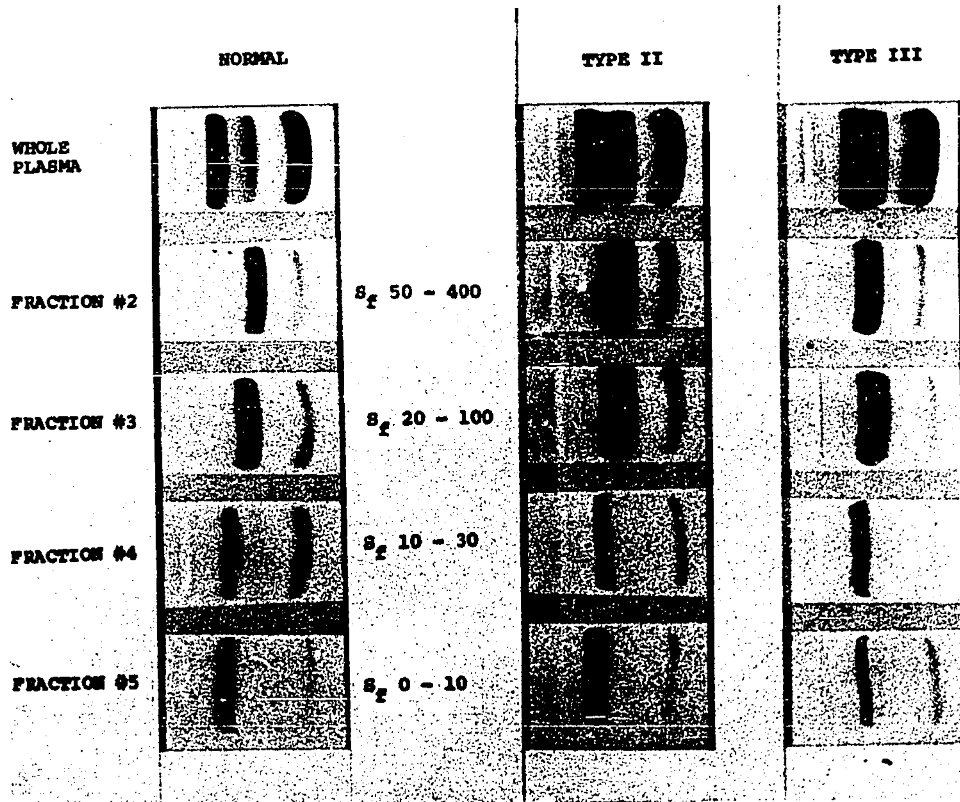

FIGURE 4: Agarose gel electrophoresis of normal plasma and plasma obtained from patients having types II and III lipoprotein abnormalities, along with the respective fractions obtained from them by preparative ultracentrifugation.

were studied on agarose gel electrophoresis. Results obtained on normal plasma are shown in figure 3. Examination of the migration position of the various lipoprotein fractions shows that there is some overlap between the S_f50 - 400 and S_f20 - 100 groups. A similar statement may be made regarding the S_f10 - 30 and S_f0 - 10 fractions. However, in broader terms, if one considers S_f0 - 30 (fractions #4 and 5) and S_f20 - 400 lipoprotein (fractions #2 and 3), there is good separation of these two important sub-classes.

Similar statements generally may be made about the electrophoretic mobility of lipoproteins separated from plasma containing abnormal levels of lipids (fig. 4). However there are two exceptions. Note that in type III abnormality, the S_f0 - 10 fraction has a migration distance equivalent to S_f10 - 30 lipoprotein, which is unusual. This may be an artifact due to the low concentration of S_f0 - 10 lipoprotein found in type III anomalies. The effect of absolute concentration of lipoprotein on electrophoretic mobility was studied further and will be discussed in a later section. The second exception, also in the type III example, involves the two important broad subclassed S_f0 - 30 and S_f20 - 400 lipoproteins which are not well separated, although the separation on the electropherogram itself is better than appears on the photograph.

As may be seen, each electropherogram of the lipoprotein fractions contains lipoprotein staining material in the migration region occupied by albumin. This of course

is due to the albumin contained in the lipid poor plasma added to these fractions.

The electropherogram resulting from electrophoresis of fraction #1 is not shown in either fig. 3 or 4 because after staining nothing was visible. This is due mainly to the lipoprotein not entering the gel and being washed out of the application reservoir during processing. However, if sufficient of these large lipoproteins are present, they visibly distort the anodal edge of the application reservoir during electrophoresis.

Because of the renewal of interest in paper electrophoresis (18,21) it was decided to examine the paper electrophoretic migration characteristics of these fractions. The following migration characteristics were obtained:

(a) The $S_f0 - 10$ lipoprotein fraction formed a well-defined zone with a migration rate corresponding to the beta-lipoprotein of whole plasma.

(b) The $S_f10 - 30$ lipoprotein fraction had similar migration characteristics.

(c) The $S_f20 - 100$ lipoprotein fraction had a predominantly pre-beta mobility with a "smear" back toward the origin.

(d) The $S_f50 - 400$ lipoprotein fraction was mainly deposited at the origin with some forward "smearing" into the beta-lipoprotein area.

(e) The $S_f > 400$ lipoprotein fraction remained at the point of application.

The above observations are in agreement with those of

Smith (16) and compatible with those of Oncley (43) who used a slightly different fractionation procedure. However Lees and Fredrickson (42) indicate lipoproteins of $S_f > 400$ may have pre-beta mobility. This disparity may be due to an incorrect identification of the lipoprotein species involved. The nomogram of Dole and Hamlin (44) was used by Lees and Fredrickson to predict the minimum S_f value and thus identify the fractions isolated in an angle head rotor. This nomogram could likely be used to predict minimum S_f values with some accuracy using a swing-bucket type rotor. However, despite intimations by the authors, the ability to predict minimum particle sizes in a fractionation carried out in an angle head rotor is doubtful. This is because in addition to the obvious difficulty in allowance for convection, one must also take into consideration the depth of the serum layer and the depth of the layer removed (45), both of which affect the effective radial path length. When the Dole nomogram was used here to predict fraction ranges isolated with an angle head rotor, a gross overestimation of the minimum S_f value was observed. This could account for the different statements as to the constitution of the pre-beta fraction obtained by paper electrophoresis which vary from $S_f20 - 40$ (46) up to S_f400 and greater (42).

In summary, agarose gel electrophoresis of lipoprotein fractions separated by agarose gel electrophoresis, produces well defined bands. This is a marked improvement

over the "trailing" observed on paper electrophoresis of S_f20 - 100 and S_f50 - 400 lipoprotein fractions. It should also be noted that there is a distinct separation of S_f0 - 30 and S_f20 - 400 lipoprotein fractions following agarose electrophoresis of normal plasma. In abnormal plasma definition between these groups becomes more difficult probably due to a significant increase in the level of lipoproteins in the S_f10 - 100 range.

Lastly, it should not be stated categorically that the electrophoretic mobility of a lipoprotein in agarose gel is directly proportional to the S_f value (for S_f0 - 400 lipoproteins), however a general trend in this direction is apparent.

C. Chemical Analysis of Lipoprotein Fractions

Fractions 2, 3, 4 and 5 of low and very low density lipoproteins, isolated by preparative ultracentrifugation as described in the previous section, were further characterized by chemical analysis. These analyses include cholesterol, triglyceride, phospholipid and protein.

1. Methodology

(a) Cholesterol

Total cholesterol determinations on fractions 2, 3 and 4, as well as the whole plasma were carried out as described by Zak (47). The proteins are first removed by means of precipitation with a strongly acid, ferric chloride-acetic acid reagent, followed by centrifugation. The color reaction is then effected by addition of concentrated

sulfuric acid to an aliquot of the supernatant. Since fraction 5 contains bromide, which is known to interfere with Zak's procedure (48), a technique (49) based on that of Pearson, Stern and McGavack (50) utilizing paratoluene sulphuric acid, was used for cholesterol analysis of this fraction.

(b) Triglyceride

Triglyceride was determined by the thin layer chromatographic (TLC) method of Pinter, Hamilton, and Miller (51). A total lipid extract of serum is made with ethanol-ether (2:1). The extract is evaporated under nitrogen, redissolved in hexane, applied to a TLC plate of silica gel H, and chromatographed in a solvent system of petroleum ether, diethyl ether, and acetic acid. After thirty minutes running, the plate is removed from the solvent system, allowed to air dry and then sprayed with 7-dichlorofluorescein dissolved in ethanol. This allows visualization of the lipid components, with cholesterol esters appearing near the solvent front, followed by triglycerides, free cholerterol, diglycerides, monoglycerides and phospholipids in descending order. The triglycerides, along with the surrounding silica gel are scraped off and reacted with alkaline hydroxylamine. The resulting hydroxamate formed with the fatty acid is reacted with ferric perchlorate and quantitated by photometry, by comparison with a similarly treated standard of tripalmitin.

The protein precipitated by ethanol-ether during the initial extraction step was saved for quantitation,

and the phospholipid along with the surrounding silica gel was scraped off the TLC plate for quantitation.

(c) Phospholipid

A wet digestion (52) was carried out on the phospholipid isolated by thin-layer chromatography. The phosphorus was then quantitated by the Fiske and Subbarow method (53) and the phospholipid estimated by multiplying the quantity of phosphorus by twenty-five (54).

(d) Protein

The precipitated protein remaining from the ethanol-ether extraction step of the thin-layer chromatography isolation procedure was dissolved in 3% sodium hydroxide by heating in a 70° C water bath for 15 minutes. The resulting solution was cooled, biuret added and then allowed to stand for one-half hour. This solution had a slight degree of opacity to it, which was cleared with an ether extraction step, prior to spectrophotometric quantitation at 550 nanometers (nm). The protein value obtained includes protein other than lipoproteins. To determine the amount of non-lipid protein and thereby correct this value, the following was done. A 30 microlitre portion of fractions 2, 3 and 4 obtained by preparative ultracentrifugation was electrophoresed as described in II. A. 1. The electrophoretic strip was cut down the middle so as to produce two matching electropherograms. One half was stained with Ponceau 3R solution which stains protein but not lipid. The other half was stained with Sudan Black B to determine

TABLE 5

Chemical Composition of the Fractions Isolated by Preparative Ultracentrifugation. Protein, Cholesterol, Triglyceride and Phospholipid have been Calculated as being 100% of the Lipoprotein Complex.

Subject	Protein	Cholesterol	Triglyceride	Phospholipid
		Fraction #2 (S_f50 - 400)		
1	5%	8%	52%	35%
2	7	14	69	11
3	2	7	76	15
4	7	16	66	11
5	8	29	51	12
6	8	12	58	21
Mean	6	14	62	18
		Fraction #3 (S_f20 - 100)		
1	8	14	65	14
2	11	21	56	12
3	7	14	48	31
4	12	26	46	16
5	9	35	40	15
6	12	15	54	20
Mean	10	21	52	18
		Fraction #4 (S_f10 - 30)		
1	10	25	25	40
2	14	43	19	24
3	12	28	25	35
4	17	47	18	19
5	21	52	13	14
6	19	23	32	25
Mean	16	36	22	26
		Fraction #5 (S_f0 - 10)		
1	34	49	7	9
2	30	47	6	17
3	30	44	8	18
4	27	42	19	21
5	40	47	10	3
6	22	44	13	21
Mean	31	46	9	15

which areas contained lipoprotein. By comparing the Sudan Black B stained electropherogram to the Ponceau 3R stained electropherogram, non-lipid protein and lipid associated protein areas were delineated. The percent protein associated with lipoprotein was then determined by densitometry of the Ponceau 3R stained strip. The appropriate correction was applied to the total protein value (previously obtained by the biuret method) to obtain the value for lipid-protein.

2. Discussion

The fractions studied chemically were obtained from the following subjects:

Subject #1. Normal male age 34.

#2. Normal male age 44.

#3. Normal male age 24.

#4. Abnormal male type II.

#5. Abnormal male type III.

#6. Abnormal male - diabetic.

All subjects had fasted a minimum of 12 hours prior to procuring the blood specimen.

The lipid composition of the fractions separated by the preparative ultracentrifuge is summarized in table 5. For the purpose of summarization, mean values have been calculated utilizing figures obtained for both normal and abnormal subjects. One notes that the percentage protein content decreases as the S_f value increases, which is to be expected, since the density of the lipoprotein is proportional

to the protein content and the less dense the particle, the greater the flotation rate. This means there must be a net increase in the lipid components with increasing S_f. The decreasing cholesterol content with increasing S_f is more than compensated for by the increasing triglyceride content. Of interest is that in contrast to the other components which either increase or decrease steadily, the percentage phospholipid content appears to reach a peak in the S_f 10-30 group.

There are a number of assumptions made in correcting the lipid associated protein content of fractions, 2, 3 and 4 for non-lipid protein. It is assumed that the protein present as lipoprotein has the same dye uptake characteristics as non-lipid protein. It is further assumed that the dye uptake is accurately assessed, and that there was not any non-lipid protein located in the same area occupied by lipid associated protein.

The protein percent composition figures of fraction 5 are undoubtedly high in that they were not corrected for non-lipid protein. Assuming the same amount of non-lipid protein is present as found in fraction 4, the values are 10% high.

Due to the rather small number of samples and the inclusion of data from both normal and abnormal subjects, it was important to validate the above data obtained in this study by comparison with literature values. However, the bulk of literature data is presented in terms of only

TABLE 6

Comparative Chemical Composition Data for

$S_f0 - 20 \pm 10$ and $S_f20 - 400$ Lipoproteins.

Data Source	Protein	Cholesterol	Triglyceride	Phospholipid
		$S_f0 - 20 \pm 10$		
Present Study	27	44	12	17
Ref: 55	25	45	11	19
56	21	47	9	23
57	21	45	11	22
		$S_f20 - 400$		
Present Study	9	18	56	18
Ref: 55	10	13	55	20
56	7	22	52	18
57	8	19	50	18

two broad groups of lipoprotein, $S_f0 - 20 \pm 10$ and $S_f20 - 400$. Therefore to facilitate comparison, the chemical data used in this study was reassembled to conform to these two lipoprotein classes. This data along with that of Lindgren (55), Bragdon (56) and Oncley (57) is presented in table 6. Despite the differences in isolation procedures and assay methods, and the small number of specimens analyzed, the results are in remarkably good agreement. The apparent indifference as to whether one is comparing $S_f0 - 10$, $S_f0 - 20$, or $S_f0 - 30$ lipoprotein is due to the fact that $S_f0 - 10$ lipoprotein is present in relatively large amounts compared to the other species.

In summary, the chemical composition data presented compares favorably with available literature and augments the preparative and analytical ultracentrifuge characterization studies on the lipoprotein groups, fractionated for agarose gel electrophoretic studies.

D. Electron Microscopy

Electron microscopy was used to further characterize the lipoprotein fractions isolated for agarose gel electrophoretic studies.

Despite the fact that electron microscopy enables one to directly visualize individual lipoprotein particles, its use in this field has been slow in developing. An early attempt to photograph lipoproteins using the technique of electron microscopy was made by Macheboeuf (59) in 1949. Visualization of individual molecules however was

difficult.

Some improvement was made by Beischer (60) who used osmic acid to stain preparations of certain lipoproteins. The staining procedure was carried out after the lipoprotein had been allowed to dry on a collodion membrane. This technique produced uneven and incomplete staining, and the preparations were so concentrated that visualization of the individual particles were again difficult.

Hayes and Hewitt (61) obtained excellent results using osmic acid fixation prior to application of the specimen to the paralodion membrane. The preparation was then shadowed with a platinum-palladium-gold alloy, prior to viewing with the electron microscope. This procedure, with slight modifications, was used in the present study.

1. Methodology

Lipoprotein fractions were isolated by preparative ultracentrifugation as previously described, from a non-fasting plasma sample taken from a 34 year old normal male. The lipoprotein fractions were fixed with a buffered (pH 7.4) osmium tetroxide solution (58). Approximately 0.02 ml. of each lipoprotein fraction was pipetted into one ml. of the buffered one percent osmium tetroxide solution. After fixation for twenty-four hours at room temperature, 0.2 ml. of a latex suspension in distilled water was added to serve as an "internal standard". A small drop of this mixture was placed on a Formvar-coated 200-mesh copper grid previously prepared, and the

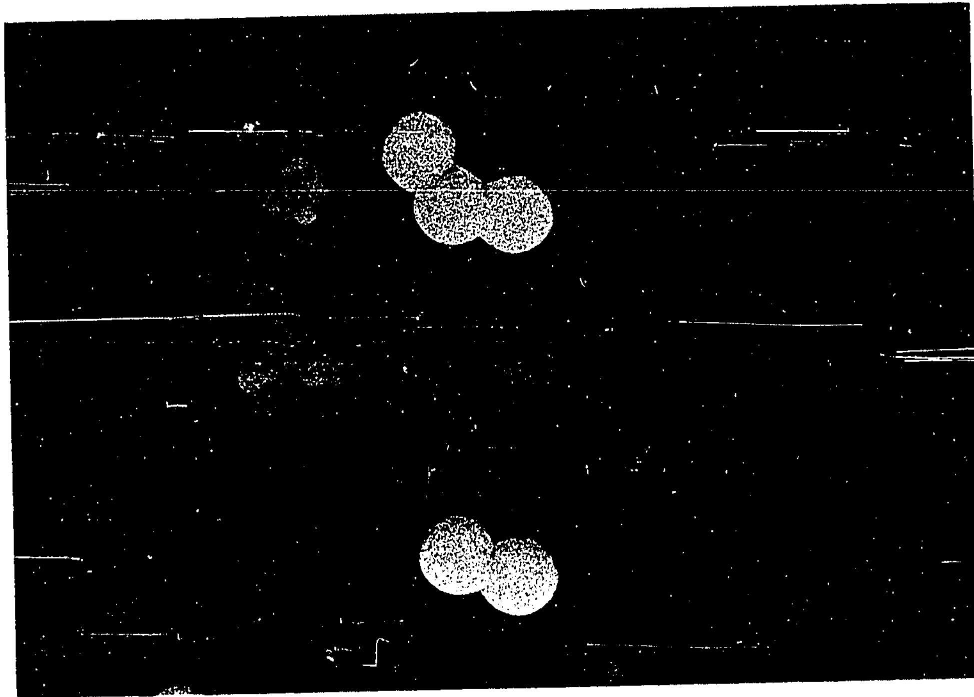

FIGURE 5: Electron micrograph of $S_f > 400$ lipoprotein particles separated by preparative ultracentrifugation. The large particles are latex markers of 1880 A° diameter.

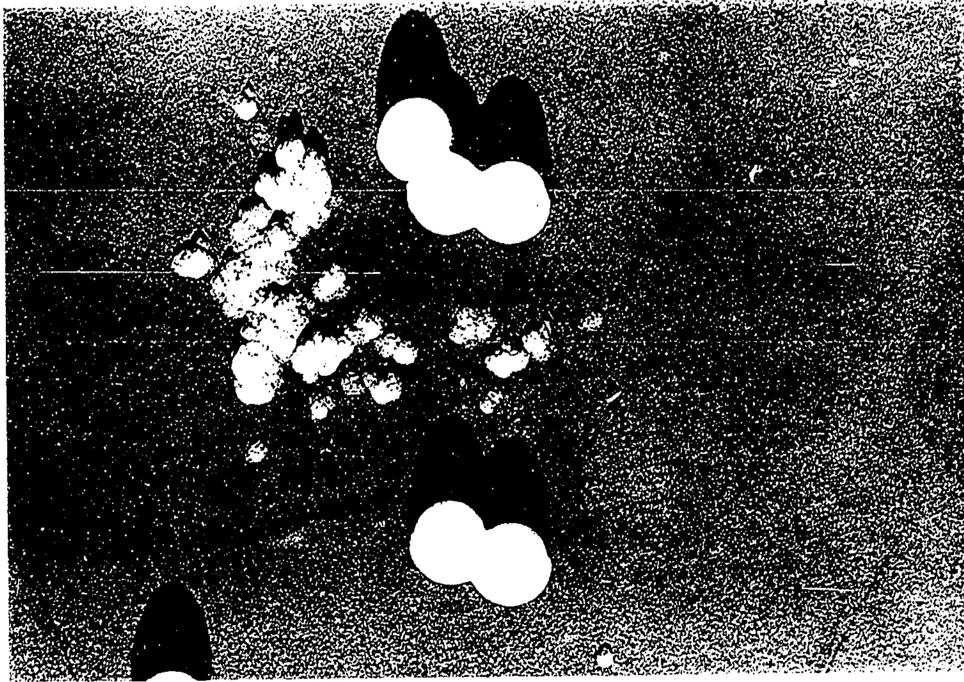

FIGURE 5: Electron micrograph of $S_f > 400$ lipoprotein particles separated by preparative ultracentrifugation. The large particles are latex markers of 1880 A° diameter.

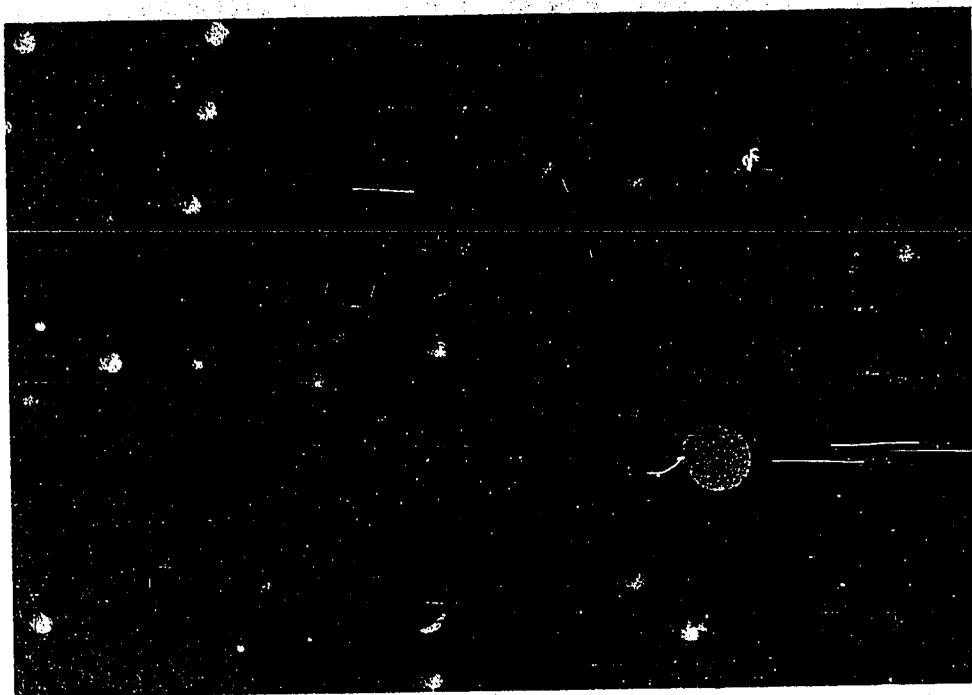

FIGURE 6: Electron micrograph of S_f50 - 400 lipoprotein particles separated by preparative ultracentrifugation. The large particle is a latex marker of 1880 A° diameter.

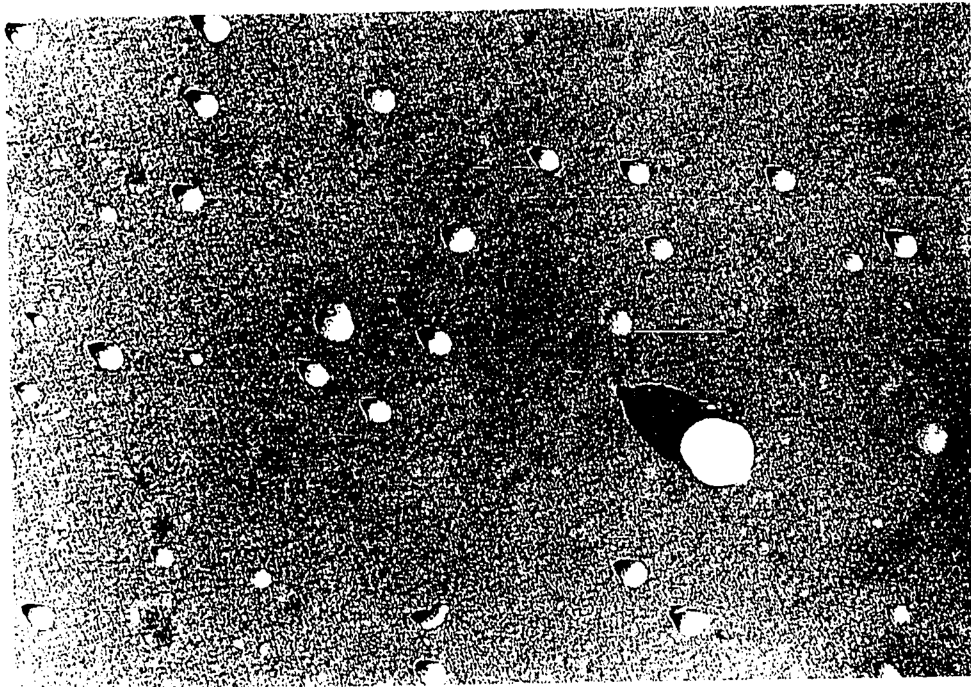

FIGURE 6: Electron micrograph of $S_f50 - 400$ lipoprotein particles separated by preparative ultracentrifugation. The large particle is a latex marker of 1880 A° diameter.

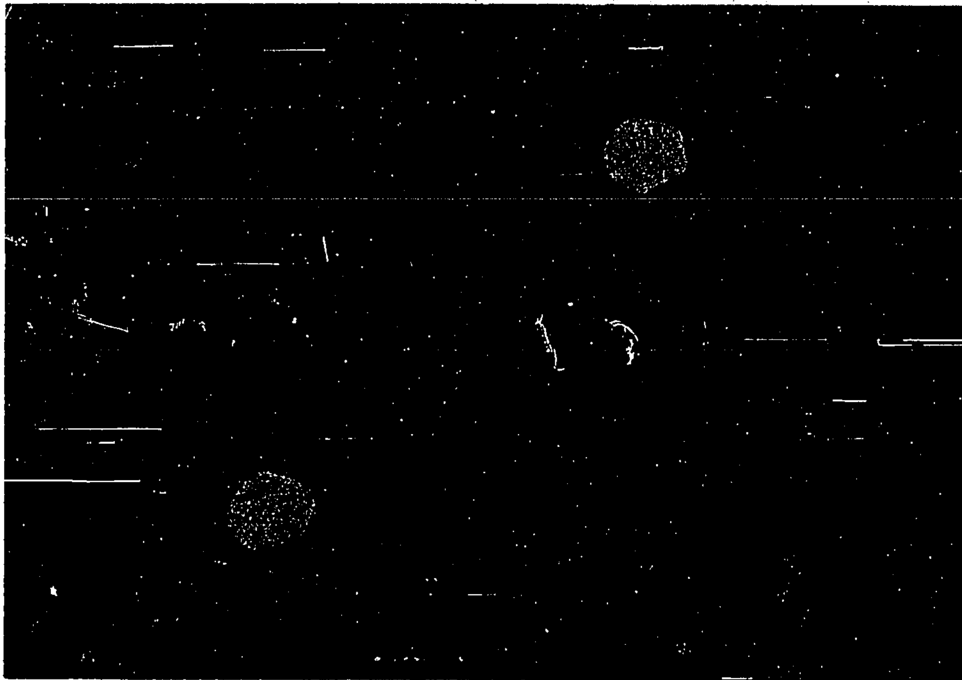

FIGURE 7: Electron micrograph of $S_f20 - 100$ lipoprotein particles separated by preparative ultracentrifugation. The two large particles are latex markers of 1880 A° diameter.

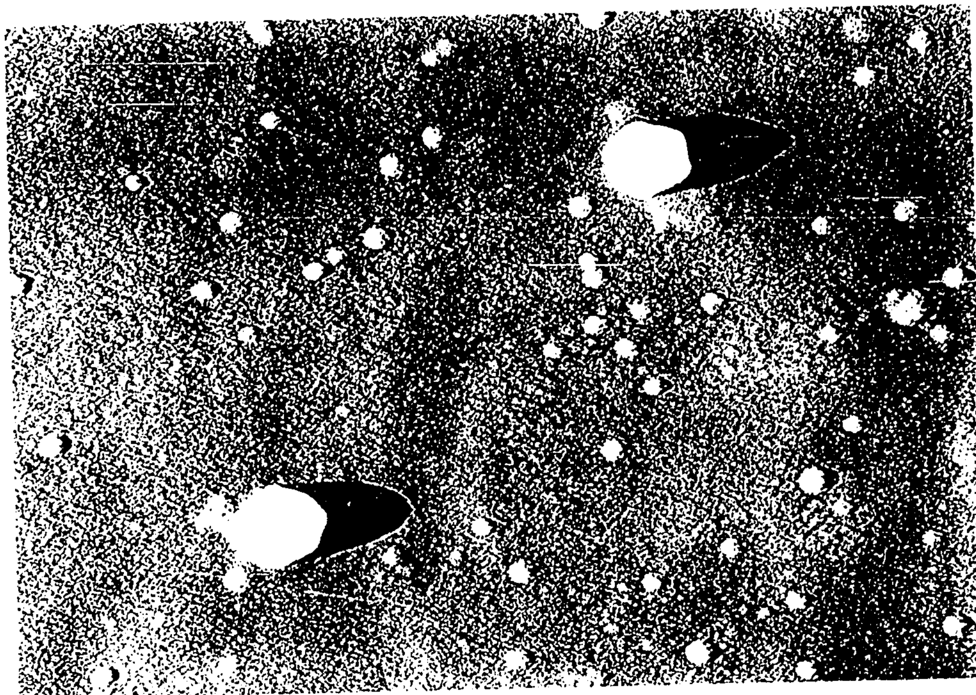

FIGURE 7: Electron micrograph of $S_f20 - 100$ lipoprotein particles separated by preparative ultracentrifugation. The two large particles are latex markers of 1880 A° diameter.

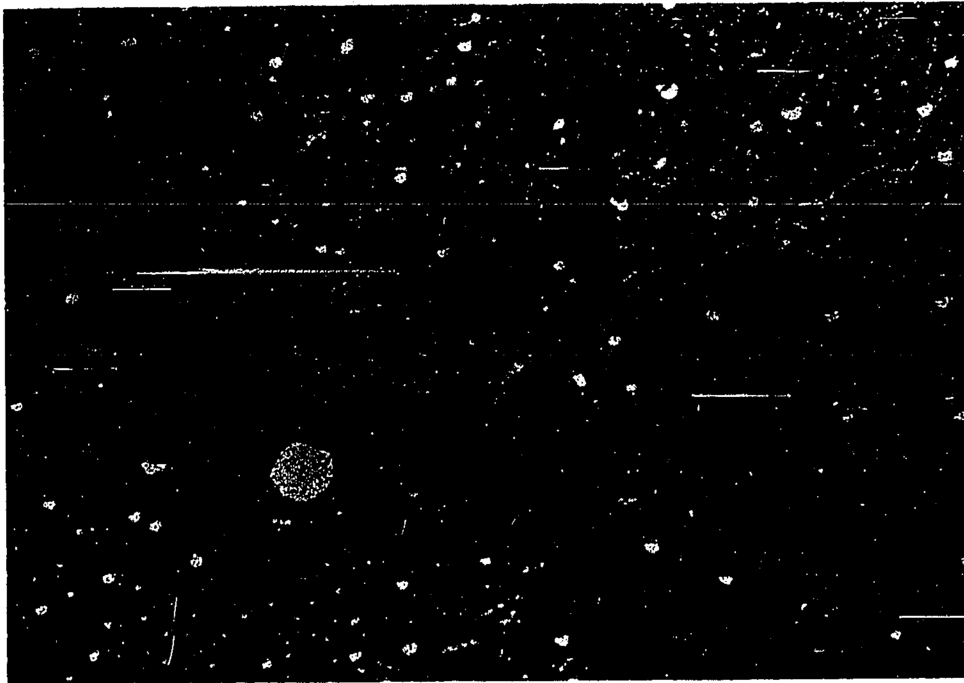

FIGURE 8: Electron micrograph of S_f10 - 30 lipoprotein particles separated by preparative ultracentrifugation. The large particle is a latex marker of 1880 A° diameter.

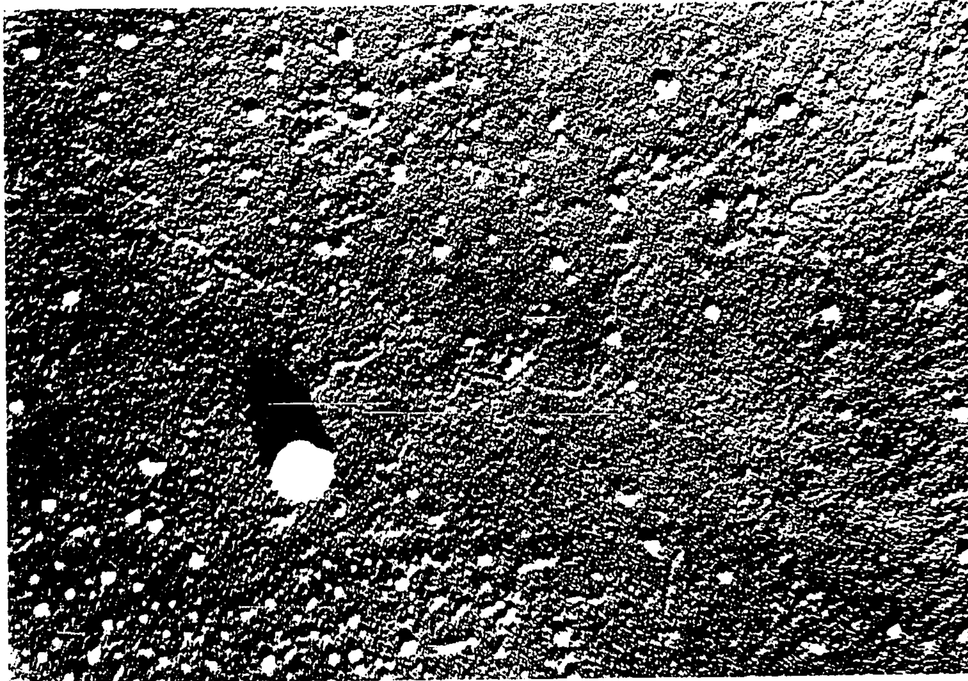

FIGURE 8: Electron micrograph of S_f10 - 30 lipoprotein particles separated by preparative ultracentrifugation. The large particle is a latex marker of 1880 A° diameter.

FIGURE 9: Electron micrograph of S_f0 - 10 lipoprotein particles separated by preparative ultracentrifugation. The large particle is a latex marker of 1880 A° diameter.

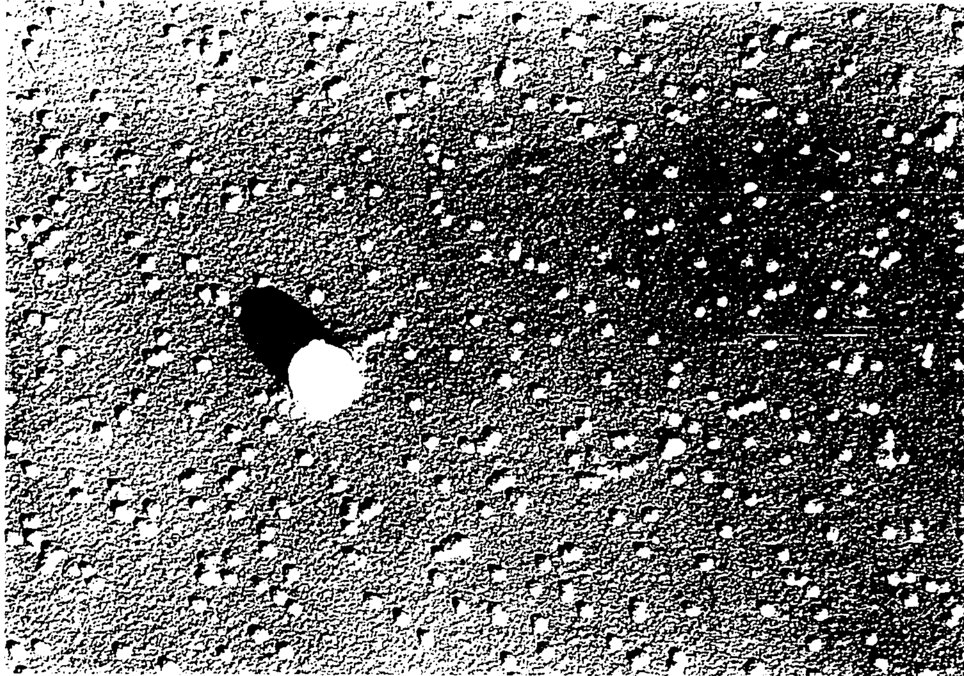

FIGURE 9: Electron micrograph of S_f0 - 10 lipoprotein particles separated by preparative ultracentrifugation. The large particle is a latex marker of 1880 A° diameter.

TABLE 7

Approximate Diameters of Lipoprotein Within each Fraction. These are Estimated from Electron Micrographs (figs. 6-9).

Fraction Number	Diameters in A° Mode	Range	Comments
#2 (S_f50 - 400)	680	540-970	This range excludes two particles of 320 A° diameter
#3 (S_f20 - 100)	530	410-610	
#4 (S_f10 - 30)		210-610	Over 50% of the particles in the figure shown are between 200 and 300 A°. There is a fairly even distribution of the remaining particles between 300 and 610 A°.
#5 (S_f0 - 10)	360		An estimated 99% of the particles are within ± 30 A° of the mode

drop was then immediately taken up with a corner of an absorbent tissue. The mounted specimen was then shadowed in an Edwards Model 12E6/930 vacuum evaporator using carbon-platinum electrodes. Electron micrographs were taken on a Siemens Elmiskop I electron microscope, on Ilford N-40 plates.

The electron micrographs obtained are shown in figures 5-9. The large white particles seen in each figure are latex markers of 1880 A° diameter. Approximate diameters for fractions 2 - 5 are given in table 7. No allowance has been made for the depth of the shadowing alloy deposited on the particles. The mode has not been derived statistically but is a visual estimation of the most common particle diameter.

2. Discussion

The appearance of the electron micrographs of fractions 2,3 and 5 (figures 6, 7 and 9) are consistent with those obtained by others (61,63,64). Fraction 1 (figure 5) lipoproteins are not well visualized, due to inadequate fixation. This might be expected as the majority of the lipid ingested prior to obtaining this non-fasting plasma specimen originated from milk products. The lipid fixative action of osmium tetroxide is due to its reaction at ethylenic double bonds of unsaturated fatty acid. Milk lipids consist mainly of triglyceride containing saturated fatty acids, thus osmium tetroxide does not react well with them. A similar phenomenon was noted by Hayes (62) who was not

able to visualize lipoprotein of $S_f > 400$ in the plasma produced by butterfat feeding. However good micrographs were obtained when corn oil was fed.

Electron micrographs of fraction 4 ($S_f 10 - 30$), (figure 8), show a rather wide range of particle size. In particular the small particles seen here are not present in the electron micrographs made by Lindgren and Nichols (41). The particles in figure 8 are smaller than those of the $S_f 0 - 10$ species, yet they must have a higher percent fat content or they would not have been isolated with the $S_f 10 - 30$ fraction. Therefore these particles are not consistent with a continuous conversion (with glyceride removal) of lipoproteins of $S_f 2 - 10^5$ as postulated by Lindgren and Nichols (41).

It is interesting to further consider the diameter of 360 A° for the $S_f 0 - 10$ particle obtained from the micrograph (figure 9). Hayes, Murchio, and Lindgren (64), on examination of earlier electron micrographs of Hayes and Hewitt (61), concluded this component had dimensions of 350 x 175 A° and the appearance of an oblate ellipsoid. From volume and density measurements the molecular weight was estimated to be about 6×10^6. However light scattering data (65) within the narrow $S_f 6 - 8$ lipoprotein band had yielded molecular weights in the range of 2.8 to 3.1×10^6 which was approximately 10 percent higher than those obtained from ultracentrifuge data assuming spheres (41). Further, dissymmetry data (65) indicated

axial ratios of from 2.5 to 3.1 suggesting an ellipsoidal shape (150 x 350 A° if prolate). Hayes (64) using a special spray technique for application of the lipoprotein suspension to the paraloidion membrane, but using the same shadowing technique as previous (61) obtained a molecular weight of 6.9 x 10^6. Considering the above facts they concluded that the particles seen in electron micrographs must be dimers which are individually, prolate ellipsoid in shape.

The result of 360 A° obtained for S_f0 - 10 lipoprotein checks well with Hayes figure of 350 $\pm$ 25 A° (61,64). However, closer examination of this micrograph of the S_f0 - 10 class reveals a few lipoprotein particles "shaded" by the latex particle. Because of this "shading" the particles have not received a "coat" of carbon-platinum used in the shadowing procedure. Measurement of these uncoated particles and with comparative adjustments to the dimensions of the latex marker particle results in a diameter of 260 A° rather than the 360 A° for the shadowed particles. This means that there is 50 A° of alloy on either side of the shadowed lipoprotein particles. If one assumes there is an equivalent depth of alloy on the top of the particle, it means it would have a depth of 125 A° rather than 175 A° (64). Thus the actual dimensions of the particle are about 260 x 125 A°. Assuming that these lipoproteins are oblate ellipsoids, which is what they appear to be in electron micrographs, one can recalculate the molecular weight

as follows:

The volume of an oblate ellipsoid is equal to $4/3\pi$ a^2b, where a is the radius of the long axis and b the radius of the short axis.

260A° = 26 millimicrons = 26×10^{-9} meters = 26×10^{-7} centimeters = 2.6×10^{-6} centimeters.

Similarly, 125 A° = 1.25×10^{-6} centimeters.

The volume of each particle is therefore = $4/3\pi$ $(1.3 \times 10^{-6})^2$ (0.62×10^{-6}) = 4.4×10^{-18} c.c. Lipoproteins in the peak area of $S_f0 - 10$ are known to have a density of 1.03 g./ml., therefore each particle would weigh $1.03 \times 4.4 \times 10^{-18}$ g. = 4.5×10^{-18} g. Since one mole contains 6.02×10^{23} particles, the G.M.W. is therefore (4.5×10^{-18}) (6.02×10^{23}) = 27×10^5 or 2.7×10^6. This figure is in excellent agreement with light scattering and analytical ultracentrifuge data. Thus these lipoproteins are probably oblate ellipsoids with dimensions of approximately 260 x 125 A°, rather than prolate ellipsoids with the longest dimension in the neighborhood of 350 A°.

In summary, the electron micrographs add further information in respect to the physical dimensions of the lipoprotein groups fractionated for agarose gel electrophoretic mobility studies. The results are mainly as expected in light of the previous preparative and analytical ultracentrifuge and chemical studies in that particles with a high percentage lipid content tend to be of greater size than those of lesser lipid content. There appears to be an

exception however in the S_f10 - 30 lipoproteins, some of which appear smaller than counterparts (S_f0 - 10 lipoproteins) having lesser percentage lipid content and conversely there are others that are physically large enough that one would expect them to be in a higher S_f category.

E. Immunoelectrophoretic Characterization of Lipoprotein Fractions

The low density and very low density lipoproteins have been studied in detail in the previous sections. The study will be extended to include high density lipoproteins in this section. This necessitates different preparative ultracentrifuge conditions. Since the low and very low density lipoproteins behave as a single antigen, as will be discussed later, they have been isolated by preparative ultracentrifugation as a single fraction. The high density lipoproteins were separated into three fractions by preparative ultracentrifugation. The fractions were then studied by immunoelectrophoresis. They were also subjected to agarose gel electrophoresis to relate the immunoelectrophoretic data to the electrophoretic fractionation. Details of the procedures used follow.

1. Methodology

(a) Preparative Ultracentrifuge Fractionation

Density < 1.063: Adjustment of the density of the plasma to 1.063 g./ml. was carried out as described by DeLalla and Gofman (66). This solution was then centrifuged in a #50 rotor at 36,500 r.p.m. (approximately 80,000 G), for 21 hours at 10°C. Approximately 2.5 ml. of the

supernatant was removed, adjusted to a volume of 3 ml. with density = 1.063 NaCl solution and labelled "D < 1.063". The infranatant was then adjusted to a volume of 8 ml. with D = 1.063 NaCl solution.

Density > 1.063 and < 1.125: Adjustment of the density of the infranatant was carried out according to Havel, Eder, and Bragdon (36), using a stock solution of NaBr, D = 1.388 g./ml. to increase the density to 1.125 g./ml. This solution was then centrifuged in a #50 rotor at 44,000 r.p.m. (approximately 117,000 G) for 22 hours at 10°C. Approximately 2.4 ml. of the supernatant was removed and the volume adjusted to 3 ml. with D = 1.125 g./ml. solution, and labelled "D > 1.063 and < 1.125". The infranatant was then adjusted to a volume of 8 ml. with D = 1.125 g./ml. solution.

Density > 1.125 and < 1.20: Adjustment of the density of the above infranatant was carried out according to Havel, Eder, and Bragdon (36), using a stock solution of NaBr D = 1.388 g./ml. to increase the density to 1.20 g./ml. This solution was then centrifuged in a #50 rotor at 44,000 r.p.m. for 26 hours at 10°C. Approximately 2.5 ml. of the supernatant was removed and the volume was adjusted to 3 ml. and labelled "D > 1.125 and < 1.20". The infranatant was labelled "D > 1.20".

(b) Immunoelectrophoresis

Aliquots of the above four fractions were dialyzed against 0.9% NaCl containing 0.001 molar E.D.T.A. (di-

sodium salt) for approximately 18 hours. Immunoelectrophoresis was then carried out as described in the LKB-Produkter-6800A Immunoelectrophoresis Operating Manual. Anti-β-lipoprotein serum (rabbit) and anti-α_1-lipoprotein serum (rabbit) obtained from Hoechst Pharmaceuticals Inc.* were used for this study. A pooled serum specimen was run with each fraction as a control. After electrophoresis, diffusion and removal of unreacted protein, the immunoelectrophoretic slides were stained with an Amido Schwarz 10 B solution.

(c) Electrophoresis

Agarose gel electrophoresis and staining of each of the dialysed fractions was carried out as described in Sections II. A. 1, and II. A. 2(b).

2. Discussion

It is generally agreed that the $S_f0 - 400$ lipoproteins ($D < 1.063$ and > 0.96 g./ml.) are antigenically distinct from the high density lipoproteins (67,68,69). There is some divergence of opinion concerning the antigenic homogeneity of the $S_f0 - 400$ lipoproteins as a group, i.e., concerning the relations among ultracentrifugally separated subfractions of these lipoproteins. For example Aladjem (70) obtained multiple zones of precipitation among the $S_f0 - 400$ lipoproteins, and although admitting to cross reaction between subfractions and their antisera

*Hoechst Pharmaceuticals Inc., Cincinnati, Ohio, 45229, U. S. A.

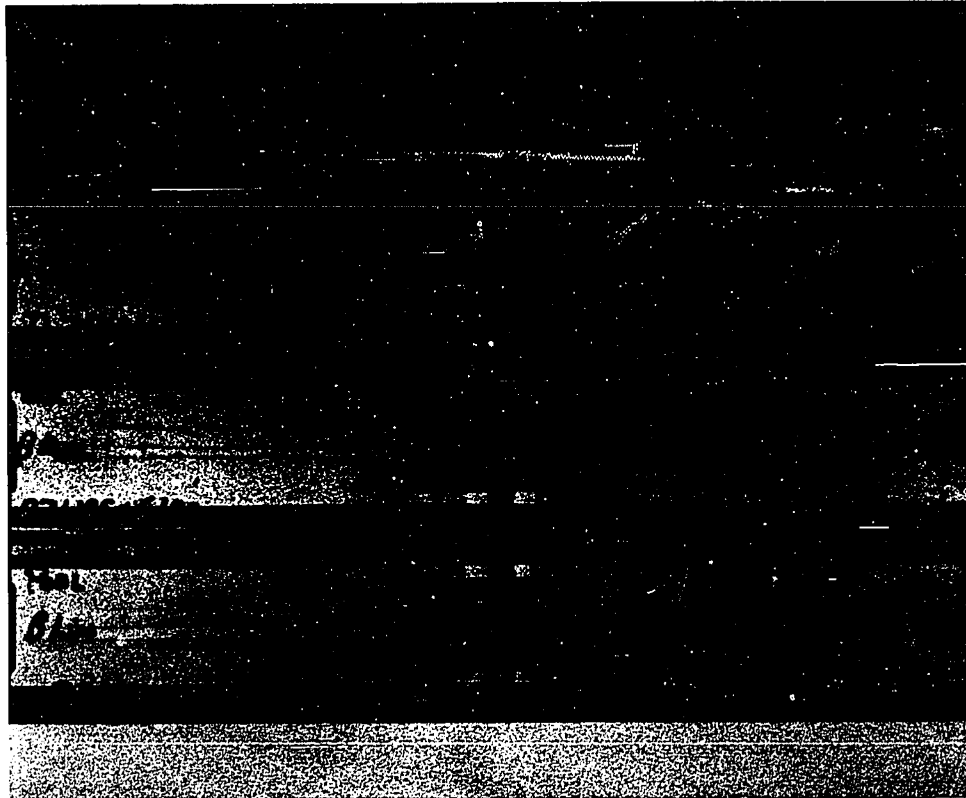

FIGURE 10: Immunoelectropherograms of lipoprotein fractions. The upper electropherogram in each slide is that of a pooled serum specimen and the lower immunoelectropherogram is one of the lipoprotein fractions as indicated. Beta-lipoprotein antisera was used for the immunoelectropherograms on the left and $alpha_1$-lipoprotein antisera for those on the right.

FIGURE 10: Immunoelectropherograms of lipoprotein fractions. The upper electropherogram in each slide is that of a pooled serum specimen and the lower immunoelectropherogram is one of the lipoprotein fractions as indicated. Beta-lipoprotein antisera was used for the immunoelectropherograms on the left and $alpha_1$-lipoprotein antisera for those on the right.

states that they are not immunochemically identical. Heide, Schmidtberger and Schwick (71) have distinguished two lipoproteins corresponding to different density classes of low density lipoproteins. In view of the wide range of particle size in this group of lipoproteins, this type of heterogeneity is not unexpected. Levine et al. (67), Scanu, Lewis and Page (69), Korngold and Lipari (72), Tracy, Merchant and Kao (73), and Walton and Darke (74), all agree that the various subfractions of the S_f0 - 400 lipoproteins behave as a single antigen.

The immunoelectropherograms obtained in this study are shown in figure 10. The upper immunoelectropherogram in each slide is that of a pooled serum specimen and the lower immunoelectropherogram is one of the fractions as indicated. Beta-lipoprotein antisera was used for the immunoelectropherograms on the left and alpha$_1$-lipoprotein antisera for those on the right.

The density $<$ 1.063 g./ml. lipoprotein fraction, on agarose gel electrophoresis showed two lipoprotein bands corresponding to mobilities of S_f0 - 20 and S_f20 - 400 lipoproteins. Immunoelectrophoretically this fraction, using β-lipoprotein antisera, produced a single, relatively straight precipitin line. This fraction did not produce a precipitin line against α_1-lipoprotein antisera.

The fraction of density $>$ 1.063 and $<$ 1.125 g./ml. on agarose gel electrophoresis produced a single lipoprotein band located in the α_1-albumin region. Immuno-

electrophoretically this fraction produced a single arced precipitin line with α_1-lipoprotein antisera. It did not react with β-lipoprotein antisera.

The fraction of density $>$ 1.125 and $<$ 1.20 g./ml. on agarose gel electrophoresis, produced a single lipoprotein band located in the α_1-albumin region. Immunoelectrophoretically this fraction produced two precipitin arcs with α_1-lipoprotein antisera. Only one of these precipitin lines is visible in figure 10 as one was too faint to reproduce photographically. This is consistent with results found by Scanu and Granda (75), and Levy and Fredrickson (76). This lipoprotein fraction did not react with β-lipoprotein antisera.

The fraction of density $>$ 1.20 g./ml. on agarose gel electrophoresis produced a single lipoprotein band located in the α_1-albumin region. It did not react with either α_1-lipoprotein antisera or β-lipoprotein antisera. Since only two proteins have this migration rate, i.e., α_1 lipoprotein and albumin, it is postulated that the lipoprotein band in this fraction is due to lipids associated with albumin.

In summary, the immunoelectrophoretic results indicate that the lipoproteins of density $<$ 1.063 g./ml., migrating in the β-α_2 protein region are, antigenically β-lipoproteins. The lipoproteins having a density greater than 1.063 g./ml., found in the α_1-albumin region in agarose gel electrophoresis, antigenically are composed of

at least two components, one which reacts with α_1-lipoprotein antisera and another which is, antigenically, neither α_1 or β lipoprotein. It is postulated that the latter component consists of lipid associated with albumin.

F. Factors Affecting Migration Rates

The main purpose of the work described in this section was to study the reproducibility of lipoprotein migration using the outlined electrophoretic procedure (II. A). Further to obtain reproducible results it is necessary to recognize the many factors that affect this. Among these, storage of lipoproteins at 4° C, effect of cooling gel during electrophoresis, and lipoprotein concentration have been explored in this study. Other factors will be discussed on a theoretical basis only. This information is essential if agarose gel electrophoresis is to be used to follow changes in a patients' lipoproteins during diet and/or drug therapy.

1. Methodology

(a) Reproducibility of migration rates within a single run.

To determine this, one and the same normal fasting plasma specimen was applied five times to each of three agarose gel electrophoretic strips. Electrophoresis and staining were carried out as described in II. A. 1 and II. A. 2(a). Distances migrated in the allotted time of 30 minutes were determined by measuring from the

midpoint of the application slot to the anodal front of each fraction (β, pre-β and $alpha_1$-albumin lipoprotein), and were estimated to the closest 0.5 mm. The intense demarcation band of the S_f0 - 10 lipoprotein was used for the above β-lipoprotein calculations. The term pre-β lipoprotein is used synonymously with S_f20 - 400 lipoproteins.

(b) Effect of storage at 4°C on lipoprotein migration rate.

As comprehensive study of patient's lipoproteins may require several weeks work, it was mandatory to determine the stability of plasma lipoproteins under usual storage conditions. Frederickson (21) has reported that freezing irreversibly alters the electrophoretic behavior of some of the lipoprotein fractions. Thus the following study was performed with plasma stored at 4°C. Seven ml. of fasting blood was taken from a normal donor, centrifuged and the plasma removed and stored at 4°C. At intervals over a 28 day period a portion was removed for electrophoretic study using lipoprotein staining and measurement procedures as described in F. 1(a) above.

(c) Effect of cooling during electrophoresis on lipoprotein migration rates.

Electrophoresis, lipoprotein staining, and calculations were carried out as per F. 1(a), except one electrophoretic run was carried out without cold water circulating through the electrophoretic cell.

TABLE 8

Reproducibility of Migration Distance of Lipoprotein Fractions Separated in a Single Electrophoretic Run.

Test Number	Migration Distance in mm. β Lipoprotein	Pre-β Lipoprotein	Albumin Bound Lipid
1	7.0	14.0	27.5
2	7.0	14.5	28.5
3	7.0	15.0	28.5
4	7.0	14.5	28.0
5	7.0	14.5	28.5
6	7.0	15.0	29.0
7	8.0	15.5	30.0
8	7.5	15.0	29.0
9	7.0	14.0	27.0
10	8.0	15.5	30.0
11	8.0	15.0	29.0
12	7.5	15.0	28.5
13	7.0	15.0	29.0
14	8.0	15.5	30.0
15	8.0	16.0	30.0
Mean	7.4	14.9	28.8
Standard deviation	0.5	0.6	0.9
Coefficient of variation	6.4%	3.8%	3.2%

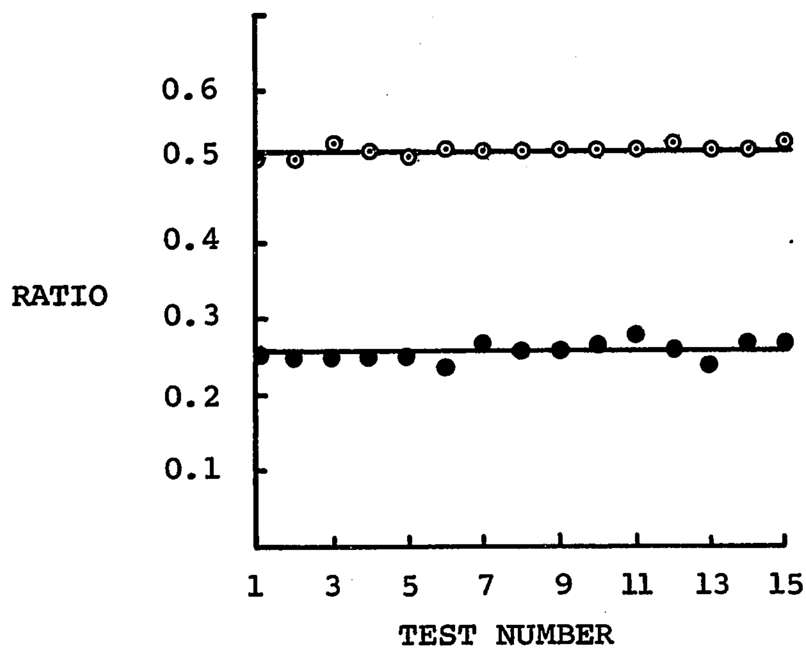

FIGURE 11: Reproducibility of mobility within a single run. Migration rate expressed as the ratio of β (●) and pre-β (o) lipoprotein to albumin bound lipid.

(d) Reproducibility of migration rates of serial dilutions of a single sample.

Serial dilutions, of a normal fasting plasma specimen were made up to a dilution of 1/32. The diluent used was 0.9% NaCl containing 0.001 molar disodium ethylenediaminetetra-acetic acid. Electrophoresis, lipoprotein staining and calculations were carried out as described in the previous section [F. 1(a)].

2. Discussion

Within run reproducibility of plasma lipoprotein migration rates during agarose gel electrophoresis is excellent. The coefficient of variation expressed as percentage is 6.4 for β lipoproteins, 3.8 for pre-β lipoproteins and 3.2 for the $alpha_1$-albumin lipid fraction. In table 8, the actual migration distance of the lipoprotein fractions following 30 minutes electrophoresis is given in centimeters (absolute migration rate). As may be seen the migration values are quite reproducible within a single run. However if the migration position of an individual fraction changes while others remain constant, this would have considerable bearing on the degree of separation of the different fractions. A means of expressing this relative relationship is shown in figure 11 where the ratio of β or pre-β migration distance to the migration distance of albumin lipid is given in graphical form. In this instance, the relative relationships show that essentially no fraction changed in mobility more than any other.

TABLE 9

Effect of Storage of Plasma Lipoproteins at 4°C on Electrophoretic Migration Distance.

Day	Migration distance in mm.		
	β Lipoprotein	Pre-β Lipoprotein	Albumin Bound Lipid
1	7.0	17.0	28.5
2	7.5	16.0	28.0
3	8.0	16.5	31.0
4	7.5	15.5	28.5
5	7.0	14.5	27.5
8	7.0	15.0	29.0
11	7.5	15.0	29.0
15	8.0	15.5	31.0
21	8.0	15.5	30.5
28	8.0	14.0	28.0

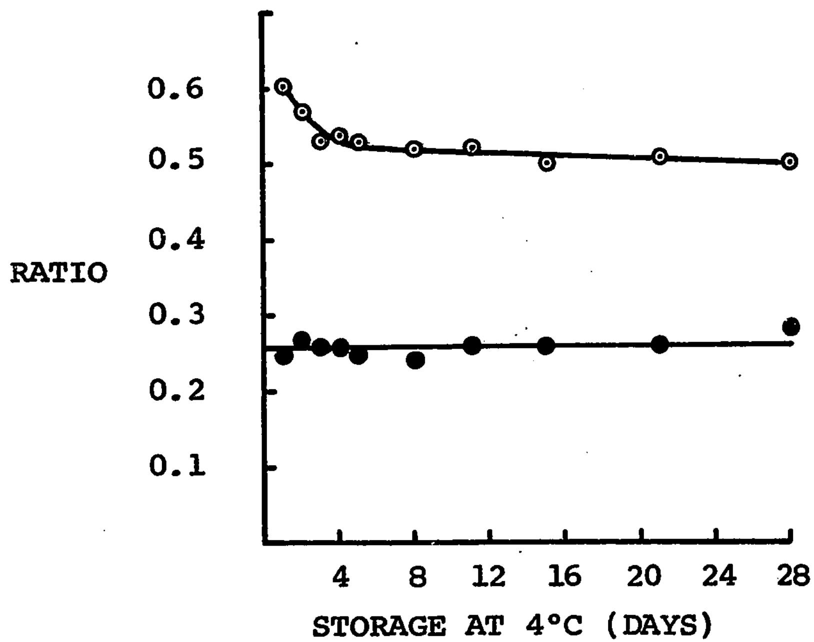

FIGURE 12: Effect of sample storage at 4°C on β and pre-β lipoprotein migration rates. Migration rate expressed as the ratio of β (●) and pre-β (o) lipoprotein to albumin bound lipid.

TABLE 10

Effect of Cooling During Electrophoresis on Migration Distance.

Electrophoresis condition	Migration distance in mm.		
	β Lipoprotein	Pre-β Lipoprotein	Albumin Bound Lipid
Cooled (cold tap water)	8.3	15.0	29.5
Room temperature	10.5	16.1	34.2

The mobility of β lipoprotein and $alpha_1$-albumin lipoprotein remains relatively constant over a period of at least 28 days when stored at 4°C (table 9). However there is a marked decrease in mobility of the pre-β lipoproteins. The change is most rapid in the first five days (17.0 - 14.5 cm.). The relative mobilities graphed in figure 12 most readily demonstrates this phenomenon.

A decrease in pre-beta mobility is the general pattern seen with stored normal plasma. However, one needs to be aware that occasionally the lipoproteins of some abnormal plasmas (elevated plasma lipids) have been observed to increase in mobility on storage. This may be due to oxidation (89), however Walton (88) found this could be prevented with the addition of 0.01% sodium azide.

Data from the above study also provides information regarding day to day reproducibility of the migration rates of plasma lipoproteins separated by agarose gel electrophoresis. Examination of table 9 and figure 12 with this in mind shows that the migration value of 2 of the 3 fractions is quite constant.

Cooling the gel during electrophoresis slows the migration rate of all of the lipoprotein fractions (table 10). It is well known that increasing temperature increases the mobility of all ion species.

Also of interest, is the effect of absolute concentration of lipoprotein on migration rate. Walton (88) studied the effect of dilution of lipoprotein fractions

TABLE 11

Effect of Serial Dilution of Plasma on Migration Distance of Beta and Pre-beta Lipoproteins.

	Migration distance in mm.		
Dilution Factor	β Lipoprotein	Pre-β Lipoprotein	Albumin Bound Lipid
1	7.5	14.5	28.5
1/2	8.5	14.0	28.0
1/4	9.5	14.0	29.0
1/8	10.0	14.0	27.5
1/16	10.5	14.5	27.0
1/32	11.5	- *	26.0

*The pre-beta band was not visible at this dilution.

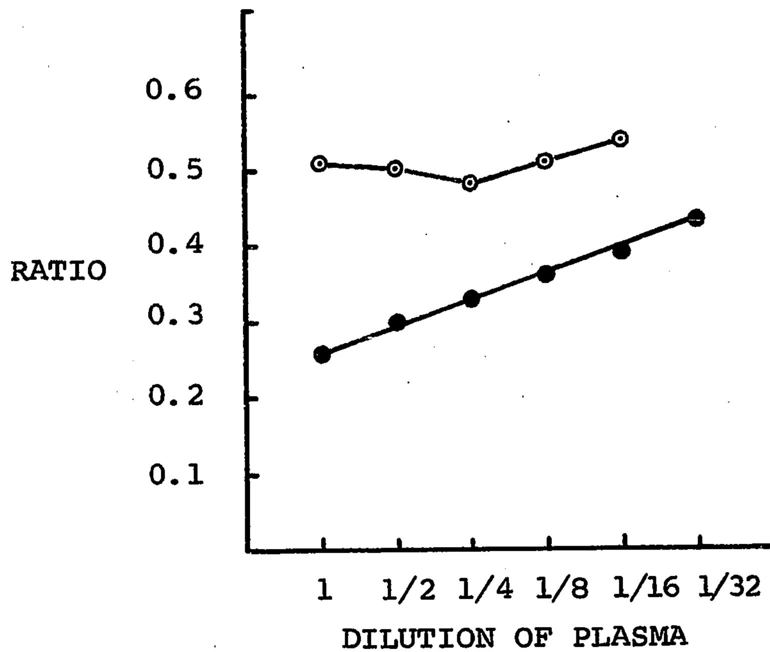

FIGURE 13: Reproducibility of migration rates of β (●) and pre-β (o) lipoproteins following serial dilutions of plasma. Migration rate expressed as the ratio of β (●) and pre-β (o) lipoprotein to albumin bound lipid.

on migration rate, and found increased mobility of $S_f3 - 9$ lipoproteins with increased dilution. Similar but less marked changes occurred in the $S_f20 - 400$ groups. Dilutions of plasma electrophoresed under the conditions of this study (II. A. 1), show similar results (table 11 and figure 13). From the above it is obvious that migration rate of β lipoproteins ($S_f0 - 10$) is partially affected by their concentration. The significance of this finding depends a great deal upon what the user of agarose gel is attempting to do with this technique.

A considerable amount has been written about the theory of electrophoretic separation. The following appears to be pertinent to agarose gel electrophoresis and needs to be considered when attempting to optimize reproducibility of migration rates.

Some common factors which one standardizes for electrophoretic procedures are:

(a) Buffer composition, ionic strength and pH.

(b) Voltage per unit distance.

(c) Electrophoresis time.

Buffer composition and pH present little difficulty, however the ionic strength of the supporting medium is not easy to control. The gel must be allowed to set and during this time some water will be lost due to evaporation, which will result in an increase in the ionic strength of the buffer in the gel. To obtain reproducible migration rates one should therefore control the gelling steps in

terms of time and atmospheric conditions. There may also be slight changes in ionic strength during electrophoresis.

All other factors being constant, the force F, exerted on an ion in an electrical field is related to the voltage V, as shown in the following formula (77):

$$F = \frac{V}{d} Q$$

where d is the distance between the electrodes and Q is the net charge on the ion. The "effective" net charge on an ion will be affected by the ionic strength of the buffer. This is due to the grouping of electrolyte ions of the opposite charge around the solute ions. The effect of the background electrolyte concentration on the mobility of an ion can be shown to be inversely related to the square root of the ionic strength of the background electrolyte (78).

The driving force can also be calculated at any point p in terms of amperes (I), cross-sectional area (Ap), and the specific conductance (ohm^{-1} $cm.^{-1}$), kp.

$$F = \frac{I}{ApKp} Q$$

Thus the necessity of having an even distribution of gel throughout the electrophoretic strip. All other factors being constant, the amperage will be governed by the smallest cross-sectional area. A "thin" area of gel will cause a reduction in the current for the whole electrophoretic strip. It is also important to prepare the application reservoir in the same manner each time, as this will probably, at least towards the end of the electrophoresis, be the zone having the smallest cross-sectional area.

Electroendosmosis occurs when ionized groups are not permitted to migrate in an electrical field, which causes the surrounding liquid to move to obtain electro-dynamically the same conditions as in electrophoresis. An example of ionized groups which are not able to migrate are the sulfate and carboxyl groups which are structural components of agaropectin. Agarose contains very little if any charged groups and therefore electroendosmosis is minimal in this medium. This has been demonstrated by Rapp (33).

The mobilities of ions in a stabilizing media of filter paper were shown by Kunkel and Tiselius (79) to be lower than those measured in free boundary electrophoresis. They proposed a tortuous path theory which describes the lowered mobility in solid state stabilizing media as being due to an increased path length around the support particles and due to a decreased electric field strength acting along this increased pathway. A second theory termed the barrier theory (80) was proposed in which the support is treated as being composed of randomly distributed obstructions which slow the migrating particles by collision or by mechanical restriction of the free pathways. The effective electrical field strength is taken as being equal to the measured one. The electrophoretic process is considered to be composed of two steps (a) a step in which the migrating ion proceeds normally in a straight path toward the electrode of the opposite charge, and (b) a second step in which the ion has run into a particle of the supporting medium and must

diffuse laterally around the obstruction before it can again move normally in a forward direction.

According to the latter theory, the obstructive factor should depend upon the size of the ion from the smallest to the largest. There is evidence that obstructions do influence the migration rates in stabilizing media such as starch gel, polyacrylamide, and Sephadex gels, where the capillary spaces approach the molecular dimensions of the molecules undergoing electrophoresis (81,82,83,84,85).

One would expect this phenomena to be evident in the agarose gel electrophoresis of plasma lipoproteins. However results indicate the larger S_f0 - 400 lipoproteins have the greatest mobility. What is evident however is the fact that there is an exclusion limit, that is, a specific particle size above which, lipoproteins cannot enter the gel matrix.

The exclusion limit for a 4% and 2% agarose gel (Sepharose*) prepared specifically for gel filtration techniques has been estimated by gel filtration studies to be 3-4 $x\ 10^6$ and 20-30 $x\ 10^6$ (expressed as $\overline{Mw}$ of dextran) respectively (86). The 3-4 $x\ 10^6$ M.W. would correspond approximately to S_f12 lipoproteins (45) and the 20-30 $x\ 10^6$ M.W. to S_f100 lipoproteins.

Lipoproteins of $S_f > 400$ are known to possess a net negative charge at alkaline pH (87). Since they do not enter the gel one can postulate an exclusion limit for

*Pharmacia Fine Chemicals, Uppsala, Sweden.

1/2% agarose gel of approximately 100 x 10^6, the M.W. of S_f400 lipoprotein (45). This appears to be a reasonable figure when compared to the results obtained by gel filtration experiments for 4% and 2% agarose gel.

Fatty acid levels elevated beyond the binding capacity of albumin to which they are normally bound can also cause an increase in the mobility of the lipoproteins. This was demonstrated by Gordon (90) who studied the interaction of oleate with albumin and β lipoprotein and the effect of this binding on electrophoretic mobility. Up to a mole ratio (oleate to albumin) of three there was no effect on the electrophoretic mobility of β lipoprotein, however the mobility of the albumin increased with increasing concentrations of oleate. At a mole ratio of four the rate of increase in electrophoretic mobility of the albumin fraction was falling off and the mobility of the β lipoprotein was increased. Between a mole ratio of 5 and 7 the mobility of albumin was more or less constant, but there was a considerable increase in the mobility of the β lipoproteins. He postulated that the increase in mobility of lipoproteins on administration of heparin, observed by Nikkila (91), Rosenberg (92), Herbst and Hurley (93), and Bolinger, Grady and Slinker (94), was due to the liberation of fatty acids which are evolved in the course of metabolism initiated by the heparin. Gordon observed that the increase in mobility of β lipoprotein due to the binding

of fatty acids could be returned to normal by the addition of dialyzed bovine albumin. Further evidence of the ability of albumin to restore abnormal mobilities to normal is presented by Shafrir (95).

In summary, reproducibility of the migration rate of plasma lipoprotein on agarose gel electrophoresis is affected by a number of factors common to all electrophoretic procedures. Examples are buffer composition, ionic strength and pH, voltage per unit distance, electrophoresis time, and temperature. There are other factors that must be controlled due to the use of a gel supporting medium, such as the gel setting conditions, distribution of the gel on the electrophoretic strip, and uniformity of size of the application reservoir. However if the procedure is rigidly adhered to the reproducibility of the migration rate of plasma lipoproteins in agarose gel electrophoresis is excellent. Storage of normal plasma at 4°C results in a decrease in the migration rate of the pre-β lipoproteins. It is therefore recommended that plasma is electrophoresed immediately after collection.

G. Quantitation of Lipoproteins by Densitometry

The qualitative information derived from agarose gel electrophoresis of plasma lipoprotein is valuable, and in many cases sufficient for sequential clinical studies. However it is difficult to interrelate results obtained in different laboratories, in terms of a subjective evaluation. Also certain clinical studies would be aided by

quantitative measurement. It is therefore desirable to "quantitate" the fractionation of plasma lipoproteins obtained by agarose gel electrophoresis. This necessitates a study of dye uptake and densitometry of the separated fractions.

In respect to visualization and/or "quantitation" of electrophoretically separated plasma lipoprotein, Oil Red O is the most commonly used stain, because it stains only the lipoprotein and not the gel (less background). It has been used either alone or in combination with Fat Red 7B for staining plasma lipoproteins separated by cellulose acetate (96), agarose (97), polyacrylamide (98) and of course, paper electrophoresis. Sudan Black B was chosen for this study primarily because preparation is easier, the dye is more stable, and it produces a more intense stain than Oil Red O (99). However of major importance, only two hours staining time is necessary vs. the 6 to 16 hours required when Oil Red O is used.

A number of factors may affect the "dye uptake"* by lipoprotein fractions separated on agarose gel. These factors include the following, and the methods of studying them are detailed below.

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*In this section the term "dye uptake" is not used in a literal sense but is the apparent amount of dye absorbed by lipoprotein as determined by densitometry. Variation in "dye uptake" as a result may be any one or a combination of: (a) an actual variation in the dye uptake; (b) variation in dye eluted during the rinsing procedure; (c) variation due to inadequacy of densitometry.

(a) Lipoprotein concentration

Increasing amounts of plasma were added to gel as follows. Sufficient 0.9% sodium chloride was added to 0.01, 0.025, 0.050, 0.10, 0.20, 0.30, 0.40, and 0.60 ml. of plasma in 10 ml. volumetric flasks, to give a total volume of 1.0 ml. Then sufficient 0.5% agarose solution (II. A. 1) which had been cooled to 40°C, was added to bring the volume to 10 ml. The resulting solution was mixed well, then poured and spread evenly onto a 15 cm. length of 35 mm. motion picture film leader. The gel was allowed to set, then dried, stained with Sudan Black B, and rinsed using concentrations of rinse and stain as previously described under "Quantitative Staining", [II. A. 2(b)]. A blank consisting of 1.0 ml. of sodium chloride and 9.0 ml. of the 0.5% agarose solution was included in the run. The strips were then scanned with a recording, automatically integrating reflectance densitometer (Chromoscan, Joyce, Loebl and Co. Ltd.). A Chromoscan filter #5021 was used in conjunction with a Beckman 594 millimicron interference filter, to limit the width of the band pass and a 0.5 x 10 mm. slit width was used. The scanning rate to recording rate ratio was 1:3. In each case the count was made over a 5 cm. length of the recording.

(b) Volume of plasma applied to gel for electrophoresis

Quantities of 5, 10, 15, 20 and 25 microlitres of plasma were electrophoresed, stained and scanned with the

Chromoscan integrating reflectance densitometer. For purposes of evaluation of the effect on densitometry, the effect on dye uptake by the following individual fractions was studied.

(i) S_f0 - 400 lipoproteins

(ii) $alpha_1$ plus albumin lipoprotein

(iii) application area

(c) Time of staining electropherogram

All other conditions being constant, the staining time was varied from one-half to six hours. Densitometry is reported on three fractions as before.

(d) Time of methanol denaturation of separated lipoprotein

All other conditions being constant, the electrophoretic strips were treated with methanol for 15, 30, 45 and 60 minutes, prior to drying the gel in preparation for staining.

(e) Ethanol concentration of the gel rinse solution

All other conditions being constant, the electrophoretic strips, after staining, were rinsed in 0, 10, 20, 30, 40, 50 and 60 percent ethanol. Each electrophoretic strip was rinsed three times for a duration of approximately 3 minutes per rinse (total rinse time of ten minutes). This was followed by a rinse in distilled water. Results for dye uptake are averages of duplicate determinations.

(f) Rinsing time of stained electropherograms

All other conditions being constant, the electrophoretic strips, after staining, were rinsed for 30, 60, 120 and 180 seconds in 50 percent ethanol, followed by a rinse in distilled water. Results for dye uptake are the average of five determinations.

(g) Age of stain

A Sudan Black B solution which had been used on five occasions over a period of four weeks and which was stored in a tightly closed opaque container when not in use, was compared with a freshly prepared Sudan Black B solution. Five electrophoretic strips of one and the same plasma were stained in each of the solutions.

(h) Effect of densitometer scanning rate to recording rate ratio on dye uptake by lipoprotein fractions

The same electrophoretic strip was scanned at the three different gear ratio settings available on the Chromoscan. A single scan only was made at each gear ratio.

(i) Densitometer slit width

All other conditions being constant one and the same electrophoretic strip was scanned using slit widths of 1/2 and 1 mm. The percent figures are the result of a single scan at each slit width.

(j) Check of linearity of chromoscan integrator mechanism

The recording pen was advanced manually in increments

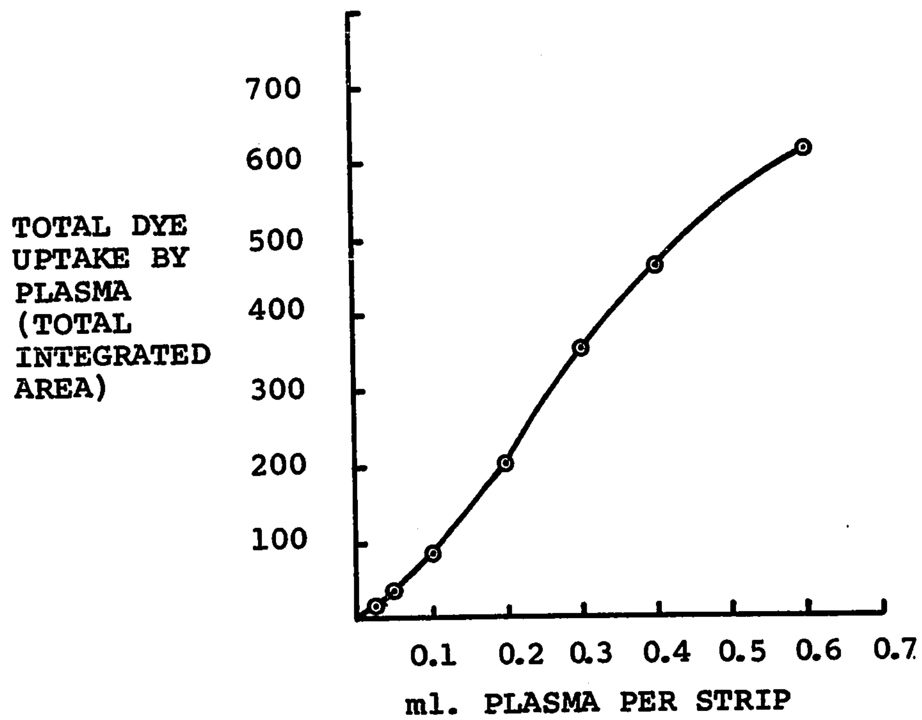


FIGURE 14: Whole plasma in agarose, stained with Sudan Black B, and quantitated by reflectance densitometry.

of approximately 4 mm. Integration was carried out over a 5 cm. distance at each increment and the results plotted.

(k) Within run reproducibility

Ten electropherograms were run on one and the same plasma using the quantitative procedures outlined in sections II. A. 1 and II. A. 2(b). The standard deviation of the dye uptake of each fraction (s.d.) was calculated under the formula: $s.d. = \sqrt{\frac{\Sigma x^2}{N - 1}}$, where x is the difference of the individual test from the mean and N is the number of tests. The coefficient of variation (V) expressed as a percentage was calculated using the formula: $V = \frac{s.d.}{M} \times 100$, where M is the mean.

(l) Stability of Stained Electropherogram

Six electropherograms which had been stained and scanned on July 6, 1967 were rescanned April 26, 1968. During this nine month period they were stored in the dark at room temperature.

Discussion

As shown in figure 14, densitometric quantitation of varying aliquots of whole plasma, dispersed over a unit area of agarose gel, and stained with Sudan Black B produces a sigmoidal curve, when the dye uptake is plotted against plasma concentration. The sigmoidal curve observed, as opposed to a straight line relationship, is a composite of at least two operating factors. (a) The tendency for the dye uptake to depart from linearity at higher concentrations

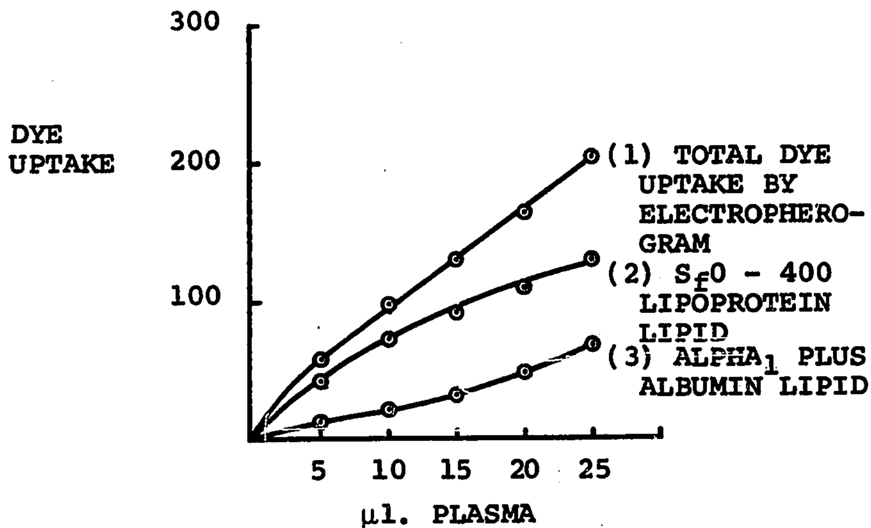


__FIGURE 15:__ Total integrated area of each electropherogram plus the integrated area of the major lipoprotein fractions vs. quantitity of plasma applied.

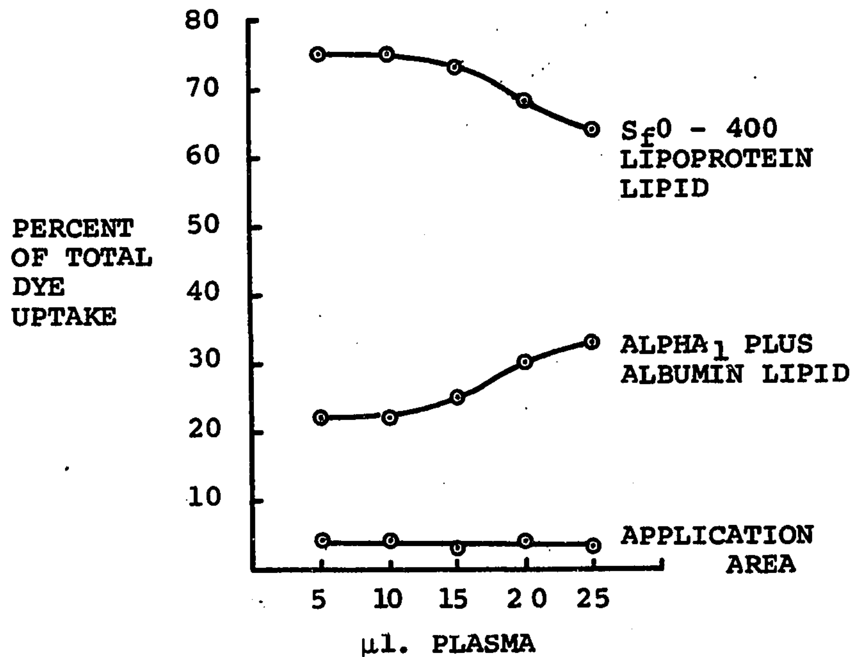


__FIGURE 16:__ Change in percentage dye uptake among lipoprotein fractions with increasing quantities of applied plasma.

due to the broad band pass of the uncompensated filter densitometer; (b) the non-linearity of the lower portion of this curve which probably is not related to the optics of the densitometer. The reason for the lack of a linear response between lipid concentration and dye at low concentrations is not clear.

The previous study of dye uptake vs. amount of plasma was done on whole plasma added to agarose gel prior to gelling and stained after drying of the gel but without electrophoresis. It was of interest to determine if this lack of linearity displayed by the dye uptake of whole plasma would be reflected in the dye uptake values obtained for the individual fractions, separated by agarose gel electrophoresis. Increasing amounts of plasma were electrophoresed and the dye uptake by the fractions $S_f0 - 400$ and alpha$_1$ plus albumin lipid studied (figure 15). The stained $S_f0 - 400$ lipoprotein fraction has a relatively high absorption. This absorption is in the upper portion of the dye uptake curve shown in figure 14. Increasing concentrations therefore, will not produce a linear increase in the integrated area representing this concentration. The integrated area underestimates the actual lipid content by larger amounts as lipid concentration is increased. In contrast, the stained alpha$_1$-albumin lipid fraction has a relatively low absorption. This absorption is in the lower portion of the dye uptake curve shown in

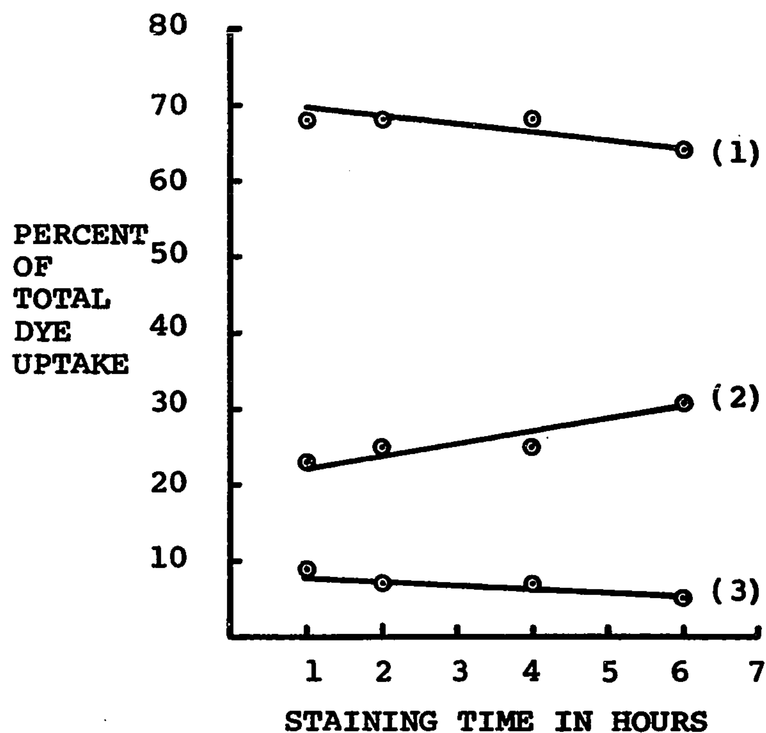


FIGURE 17: Effect of length of staining time on percentage dye uptake of separated lipoprotein fractions.

1. S_f0 - 400 LIPOPROTEIN LIPID
2. $ALPHA_1$ PLUS ALBUMIN LIPID
3. APPLICATION AREA

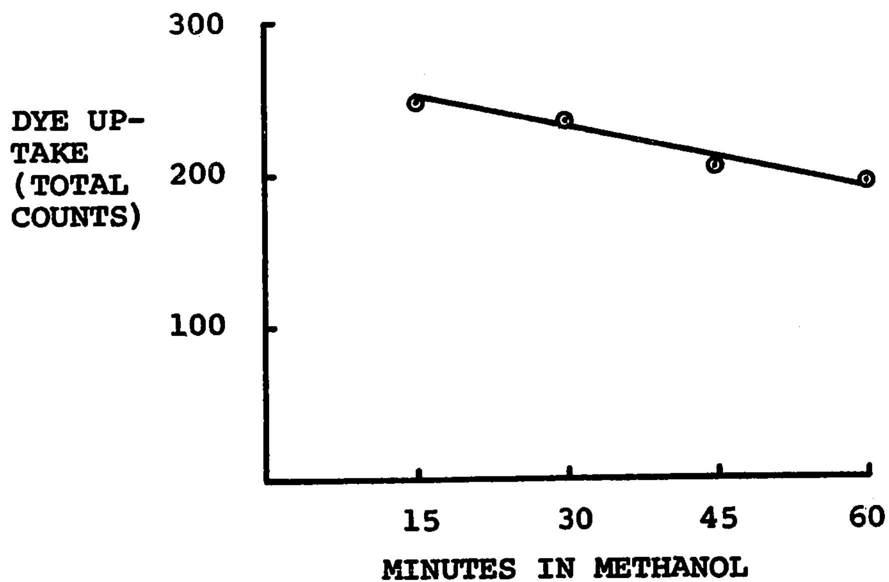


FIGURE 18: Effect of exposure to methanol on the total dye uptake by electrophoresed lipoprotein fractions.

figure 14. Accordingly there is an initial relative underestimation of lipid content. However, as the total lipid concentration is increased, the relative estimation of lipid in this fraction increases. Thus the percent dye uptake by the S_f0 - 400 lipid will decrease and that of the $alpha_1$-albumin lipid increases with increased quantities of applied plasma (figure 16). Decreasing percent dye uptake values for S_f0 - 400 lipid and increasing percent dye uptake values for $alpha_1$-albumin lipid can be expected if for any reason the total dye uptake is increased. This problem is also encountered when studying the relationship between staining time and uptake of dye (figure 17). As before, it was useful to examine the behavior of the individual lipoprotein fractions. It may be noted that the percent dye uptake by S_f0 - 400 decreased somewhat with time, and the $alpha_1$-albumin increased with time.

A brief 15 minute exposure to methanol after electrophoresis and prior to drying is necessary to ensure transparency of the electropherogram. Although methanol will precipitate the protein, it is also likely to elute some of the lipid from the lipoprotein complexes. On the evidence of decreased dye uptake as shown in figure 18, there is some lipid lost by increasing time of exposure to methanol.

It is also possible that some lipid is lost during staining, since the dye mixture contains approximately

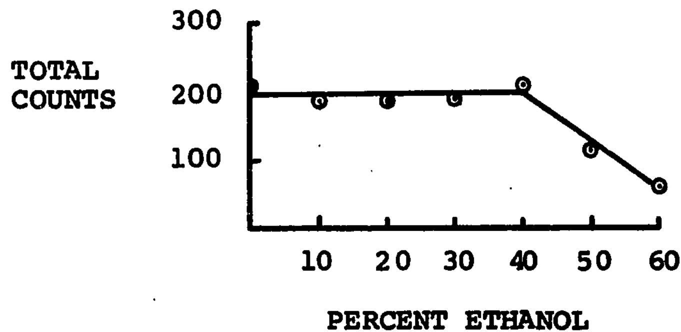


FIGURE 19: Effect of ethanol concentration in the rinse solution on total dye uptake of all lipoprotein fractions.

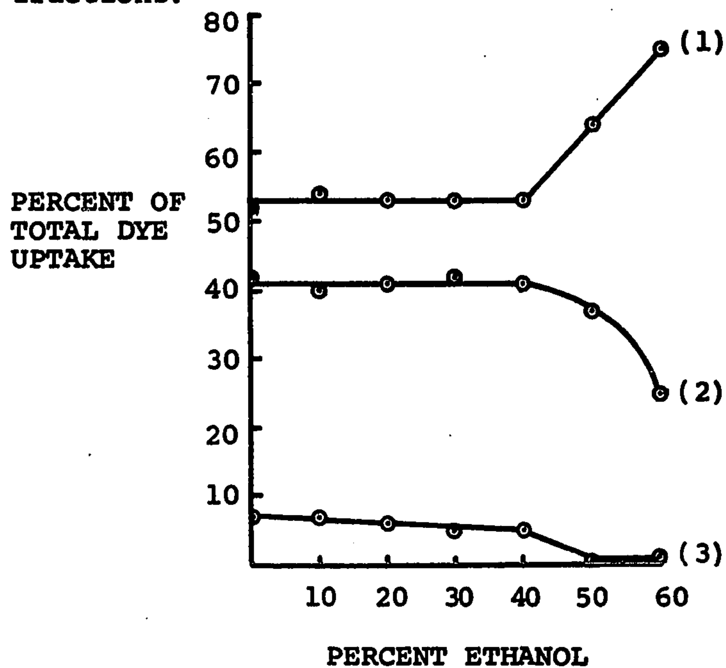


FIGURE 20: Effect of ethanol concentration in the rinse solution on percentage dye uptake of specific lipoprotein fractions.

(1) S_f0 - 400 LIPOPROTEIN LIPID

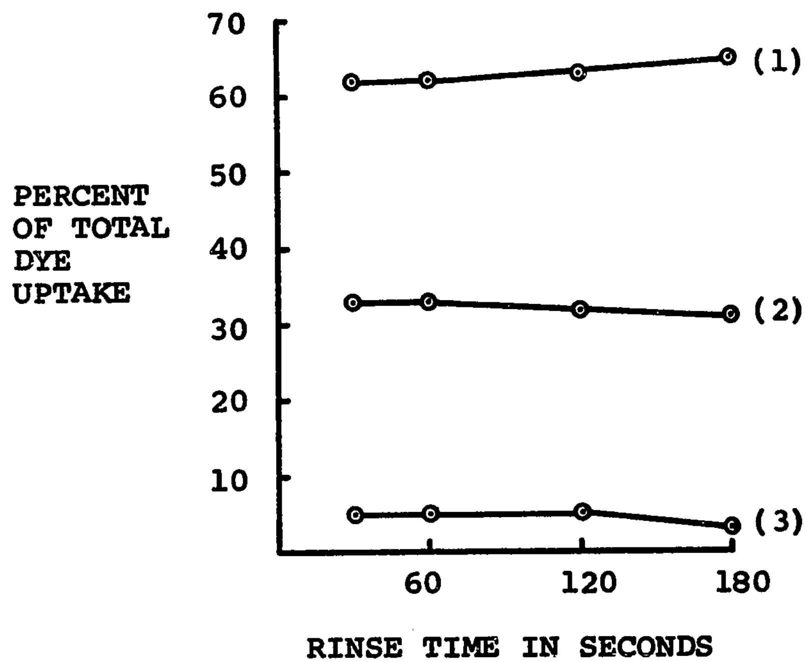
(2) $ALPHA_1$ PLUS ALBUMIN LIPID

(3) APPLICATION AREA

55% V/V ethanol. This particular aspect was not studied. Jencks, Durrum and Jetton (101) found dye uptake, by lipid on paper electrophoretic strips given a preliminary soaking in 60% ethanol at 30°C for 18 hours, to be reduced by approximately 30%. The two-hour staining time at room temperature is therefore not likely to remove an appreciable quantity of lipid.

After staining it is necessary to rinse the strips in ethanol to remove unbound dye. As can be seen in figure 19 increasing the concentration of ethanol up to 40% V/V in the gel rinse solution does not have any effect on the dye uptake by all of the lipoproteins, on the agarose strip. This is due to Sudan Black B being practically insoluble in solutions less than 40% ethanol. However at 50 and 60 percent ethanol concentration in the rinse solution, increasing amounts of the dye are removed from the stained lipoprotein by rinsing.

The increased removal of dye at 50 and 60% ethanol rinse concentrations causes a change in the percent dye uptake for the individual lipoprotein fractions (figure 20). The logical conclusion to draw from the information in figure 20 is to use a 40 percent ethanol rinse solution. However at this concentration, the electropherogram retains sufficient unbound Sudan Black B to interfere with densitometry. A 10-minute rinse time was used in this experiment. It was therefore decided to try rinsing



<u>FIGURE 21</u>: Effect on dye uptake of length of rinse in 50 percent ethanol.

(1) S_f0 - 400 LIPOPROTEIN LIPID

(2) $ALPHA_1$ PLUS ALBUMIN LIPID

(3) APPLICATION AREA

TABLE 12

Effect of Stain Age on the Dye Uptake by Plasma Lipoprotein Fractions.

	Percent dye uptake Fresh Stain	Percent dye uptake "Old" Stain
Application area	4	5
$S_f0 - 20$ lipid	34	40
$S_f20 - 400$ lipid	23	23
$Alpha_1$ plus albumin lipid	39	32

TABLE 13

Effect of Densitometer Scanning Rate to Recording Rate Ratio on the Apparent Dye Uptake of Separated Lipoprotein Fractions.

	Scanning rate to recording rate ratio.		
	1:1	1:3	1:9
Application area	10	10	9
S_f0 - 20 lipid	34	34	33
S_f20 - 400 lipid	25	27	27
$Alpha_1$ plus albumin lipid	31	30	31

the strips in 50% ethanol for varying short periods of time (30-180 seconds). As seen in figure 21 the percent dye uptake values were constant up to 60 seconds. The 30-second rinse with 50% ethanol was adopted as it removed sufficient unbound dye to make the strips suitable for densitometry.

Fresh Sudan Black B dye solution provides a higher uptake of dye by lipoproteins than "old" solutions. The resulting change in dye uptake when a Sudan Black B solution, used at five-day intervals over a period of four weeks, was compared with a freshly prepared stain, is shown in table 12. Comparing the "aged" stain values with the fresh stain, there is a decreased percent dye uptake value for the $alpha_1$-albumin lipid and an increased percent dye uptake value for the S_f0 - 10 lipoprotein lipid. The total dye uptake by all fractions decreased from an area densitometry count of 375 to 295 over the four-week period.

A number of checks were made on the reflectance densitometer to ensure that the results were not being affected by some of the simple mechanics of the unit being used. The scanning rate does not appreciably affect the percent dye uptake as is shown in table 13. Also, slit width does not affect densitometry measurements as the same percent distribution of dye was obtained at 1/2 and 1 mm. slit widths. The integrator was checked and found to be integrating correctly as evidence by the linear increase

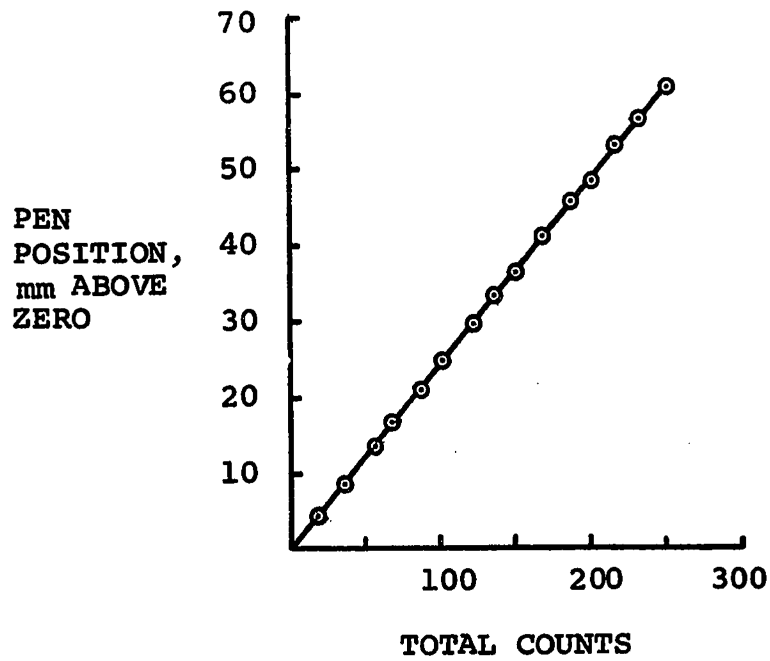


FIGURE 22: Check for linearity of Chromoscan integrator mechanism.

TABLE 14

Within Run Reproducibility of the Dye Uptake by Lipoprotein Fractions. Percent Distribution of the Total Dye Uptake was Determined by Reflectance Densitometry Using the Chromoscan Densitometer.

	Percent distribution of dye between fractions.			
Electrophero-gram number	Application Area	$S_f0 - 20$ Lipid	$S_f20 - 400$ Lipid	$Alpha_1$ plus Albumin lipid
1	5	31	31	34
2	6	32	29	33
3	6	32	30	33
4	4	33	31	33
5	5	32	29	34
6	5	31	30	34
7	5	34	30	31
8	5	30	31	33
9	5	32	30	32
10	5	31	31	33
Standard deviation	0.6	1.3	0.8	0.9
Coefficient of variation	12%	4%	3%	3%

TABLE 15

Stability of the Stained Lipoprotein Electropherograms.

Electropherogram Number	Percent uptake of dye					
	S_f0 - 20 Lipid		S_f20 - 400 Lipid		Alpha$_1$ Plus Albumin Lipid	
	A*	B**	A	B	A	B
1	51	49	10	13	38	38
2	48	50	14	13	38	37
3	56	57	11	11	32	32
4	50	50	17	15	33	34
5	46	44	9	11	46	45
6	64	62	9	10	27	28

*Dye uptake of the lipoproteins determined by densitometric scanning of the electropherograms the day following staining.

**Dye uptake of the lipoproteins determined by densitometric scanning of the same electropherograms approximately nine months later.

of integration with recording pen advancement as shown in figure 22.

The within run coefficients of variation for percent dye uptake of the lipoprotein fractions are shown in table 14. The average coefficient of variation for the major lipoprotein fractions was about 4 percent. The instability of plasma lipoproteins made it difficult to study day to day variation. However once the strips are stained they are stable for at least nine months when stored in the dark, as indicated by the results shown in table 15.

In summary then, the densitometric quantitative estimate of dye uptake by varying aliquots of plasma, as outlined, is not linear. When one considers the effect of this non-linearity on the distribution of dye between the two major lipoprotein fractions (percent dye uptake), analysis is somewhat complex. For example, the $S_f0 - 20$ lipoprotein lipid has a high absorbance when stained; thus any factor which causes an increase of stain concentration in this fraction will result in a relative decrease in the percent dye uptake figure. Further, heavier staining will have the opposite effect on $alpha_1$-albumin lipid, that is the percent dye uptake figure for this fraction will increase. The reason for this can be due to either or both of: (a) a relative decrease in the recorded density of the $S_f0 - 20$ fraction as explained above; (b) the

reflectance densitometry value for $alpha_1$-albumin lipid is in the lower portion of the uptake curve (figure 14) where there is initially a relative underestimation of the lipid content. Any factor causing a shift to a higher absorbancy will therefore cause an increase in the percent dye uptake value for this fraction.

In summary, reproducibile separation and estimation of lipoproteins separated by agarose gel electrophoresis is possible. From the detailed studies just described, the following emerge as the most critical aspects of the procedure. These are the variables that require priority attention:

1. The Sudan Black B stain must be prepared in a constant manner. Also the stain must be freshly prepared or at least stain of relative age must be used to compare results between different lipoprotein samples.

2. The volume of the lipoprotein applied (plasma sample size) must be constant.

3. The time for staining shall be 2 hours $\pm$ 5 minutes. Rinse time must be controlled to 30 seconds $\pm$ 5 seconds.

4. The composition of the rinse solution must be constant.

5. Electrophoresis of plasma must be done within 24 hours after venipuncture. Lipoprotein patterns alter with specimen age and no feasible method of storage was

found that would prevent this.

Further work in the area of quantitative densitometry should include construction of a compensating cam similar to that which has been used for densitometry of plasma proteins (26). Such a cam could produce a linear relationship between densitometric quantitation and lipid concentration. However, in the interim, densitometry for quantitation is useful, as clinically significant lipoprotein abnormalities are of a much greater magnitude than the analytic deviations observed in this study.

III. SUMMARY AND CONCLUSIONS

Lipoprotein terminology in the literature is not consistent. This is due primarily to different separations obtained using different analytical systems, and the subsequent use of "operational" definitions. For example, pre-beta lipoproteins as fractionated by paper electrophoresis refers to lipoproteins of approximately S_f20 - 100 (16), while present work reveals that by agarose gel electrophoresis, the operational definition would mean lipoprotein of S_f20 - 400.

It is necessary then to define operational terminology for each fractionating system in terms of some common factor such as density, and/or flotation rates. However this does not always completely clarify the issue, as is illustrated by the following example. According to Lindgren and Nichols (41) and Jones (102), all lipoproteins of S_f >20 should float at the small particle density of plasma (1.006 g./ml.), i.e., if plasma is centrifuged for sufficient G-minutes the supernatant will contain lipoprotein of S_f> 20 and the infranatant lipoprotein of S_f <20. However, Smith (16) found lipoproteins up to S_f27 in the infranatant. For this reason when the terms S_f0 - 20 and S_f0 - 30 are used in this text, they must be considered equivalent species.

Lipoproteins with densities > 1.006 g./ml. may be fractionated by use of specific density adjustments and

centrifugation. However to accomplish fractionation at densities < 1.006 g./ml. (S_f's > 20), one must rely on differential flotation rate procedures, which result in considerable overlapping of the S_f values of the fractions separated. Flotation rates may be assigned with fair precision, but as one approaches S_f400, a region is reached where technical difficulties prevent accurate evaluation of S_f values in the analytical ultracentrifuge (103). Thus the term S_f400, frequently used in the text, must at best be considered somewhat an approximation.

Keeping the above qualifying statements in mind, the following conclusions are made with respect to agarose gel electrophoresis [II. A. 1 and II. A. 2(a)] of human plasma lipoprotein fractions, separated by preparative ultracentrifugation.

1. Those lipoproteins of $S_f > 400$ do not enter the gel. They are partially washed out of the reservoir and are not visualized to any extent, as they are particularly vulnerable to the rather high ethanol concentration of the rinse solution (80% ethanol) of the qualitative procedure. When whole plasma is electrophoresed and stained as outlined in the quantitative procedure, lipoproteins of $S_f > 400$ if present, are seen only as a faint stain on the anode edge of the sample slot wall.

2. The S_f0 - 20 lipoproteins are the slowest

migrating group that show distinctly after staining. S_f0 - 10 and 10 - 20 lipoproteins, isolated from one another by preparative ultracentrifugation and individually electrophoresed on agarose gel, have slightly different migration rates. The difference is not enough to provide a clear cut division when both are present, as in plasma. However, the majority of the S_f10 - 20 lipoproteins will be found on the leading edge of the S_f0 - 20 group.

3. Lipoproteins of S_f20 - 400 migrate further and are located in the alpha$_2$-globulin region. There is evidence that lipoproteins of S_f approaching 400 are located at the anode side of the alpha$_2$ band. Lipoproteins with a lower S_f have a lesser migration rate and when elevated levels are encountered in abnormal plasma, may spread back to fuse with the S_f0 - 20 group.

Thus there appears to be a definite and continuous correspondence between S_f values and agarose gel electrophoretic mobility for lipoproteins of S_f0 - 400, i.e., the higher the flotation value the farther the migration distance.

4. Protein, cholesterol, triglyceride, and phospholipid content of S_f0 - 20 and S_f20 - 400 lipoprotein groups as determined here compared favorably with values found in the literature. It is interesting to note that the percent contribution of protein, cholesterol and triglyceride either increases (triglyceride) or decreases

(protein and cholesterol) in a continuous manner between S_f0 and 400. However the phospholipid reaches a peak concentration in the S_f10 - 30 lipoprotein group.

5. Electron micrographs of the various lipoprotein fractions had particle sizes and appearances mainly as expected. The exception was the S_f10 - 30 group which had a much greater particle size distribution (210 - 610 A°) than reported by others. Many of the lipoprotein particles in this group were smaller than the S_f0 - 10 lipoproteins which have a lower percentage lipid content, while others were sufficiently large that, providing the percent lipid content was higher, they should have appeared in a higher S_f fraction when separated. This is evidence that the S_f10 - 30 lipoproteins do not fit directly into the proposed orderly spectrum of low density and very low density lipoproteins commonly hypothesized (41).

6. Immunoelectrophoretically, $alpha_1$ lipoprotein was identified as comprising part of the lipid migrating in the $alpha_1$-globulin albumin region. In addition a fraction of the lipid migrating in the $alpha_1$-albumin region did not react to either $alpha_1$ or β-lipoprotein antisera.

Quantitative determination of the lipoprotein fractionated by agarose gel is a highly desirable objective. As has been found for protein electrophoresis, quantitation is affected by many variables. These include amount of

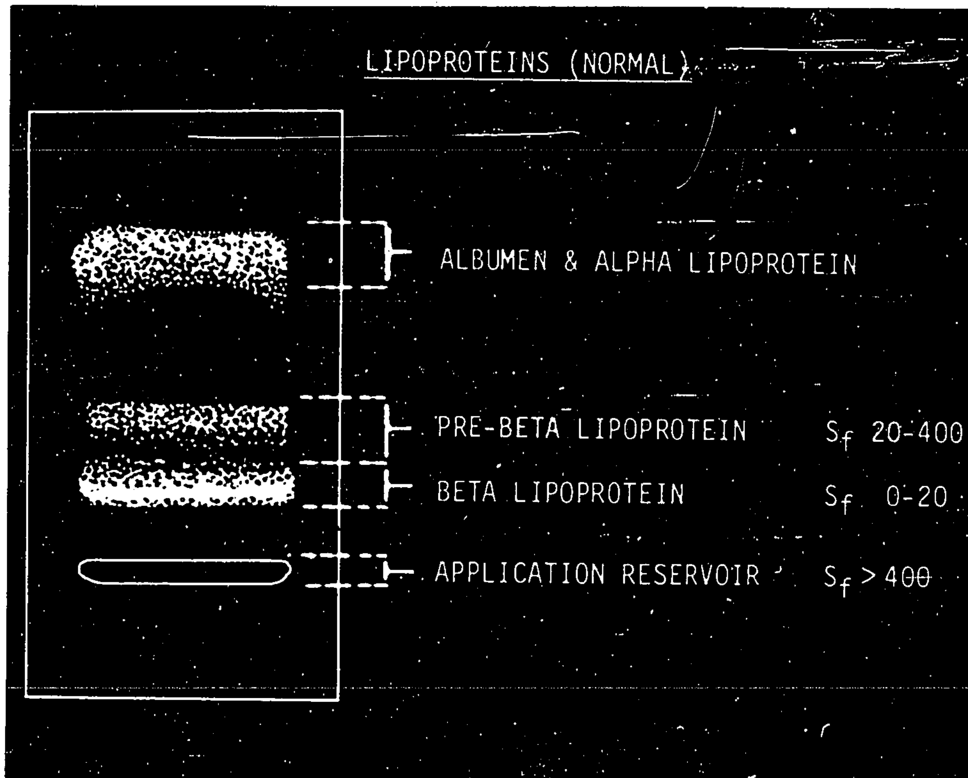


FIGURE 23: Diagramatic illustration of the fractionation of plasma lipoproteins obtained by agarose gel electrophoresis as outlined in the text.

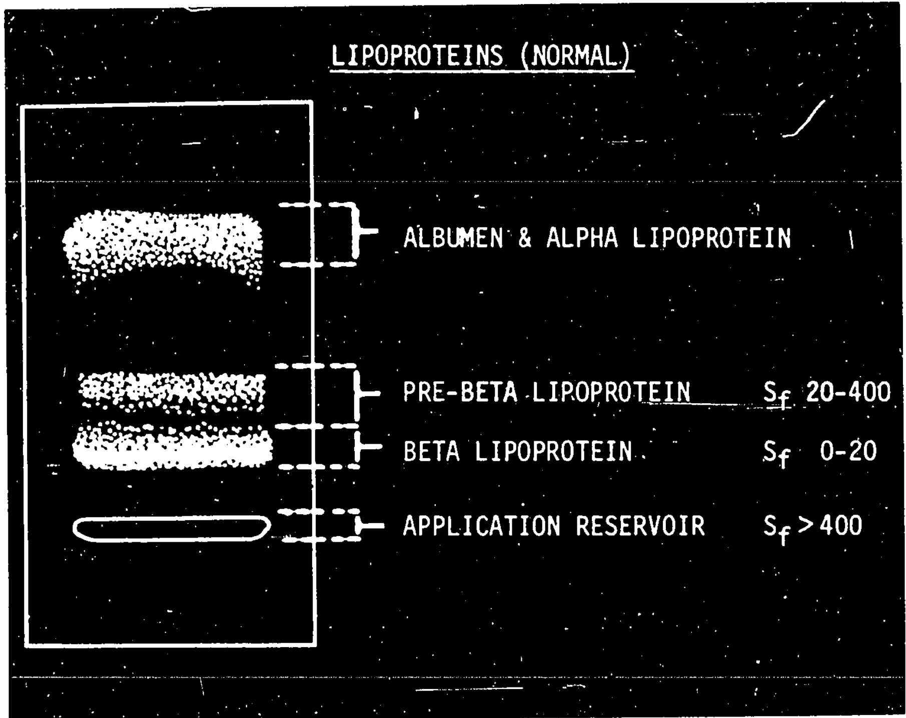


FIGURE 23: Diagramatic illustration of the fractionation of plasma lipoproteins obtained by agarose gel electrophoresis as outlined in the text.

plasma applied, variation of staining or rinsing time, and age of staining solution, all of which affect the apparent dye uptake of the lipoprotein fractions separated by agarose gel electrophoresis. The within run coefficient of variation for quantitative fractionation of the plasma lipoproteins using the proceudre outlined is about 4% for the major lipoprotein fractions. Thus it may be seen that if procedural conditions are rigidly adhered to the relative amounts of lipoprotein fractions can be estimated with precision adequate for noting changes of clinical significance.

The information obtained, regarding the nature of lipoprotein fractionation by the developed agarose gel technique is best summarized in a diagram (figure 23). The "operational" terminology (methodology related) is shown and is related to the measured flotation values. With this information, any laboratory using agarose gel separation of lipoprotein under the described conditions, should now be aware of the physical identity of the separated fractions.

The growing clinical interest in hyperlipidemias requires a deeper understanding of the nature of the lipoprotein fractions being altered by drug and/or diet therapy. Agarose gel electrophoresis, as now characterized, would appear to make available this type of information.

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APPENDIX I

Analytical Ultracentrifugation

Flotation rates of the plasma lipoprotein fractions, separated by preparative ultracentrifugation, were obtained using a Model E Spinco ultracentrifuge. A standard 12 mm, 4° sector, Kel-F cell was used. The temperature of the aluminum alloy rotor was maintained at $20 \pm 1^{\circ}C$. A bar angle of 65° was used and the Schlieren patterns were recorded on Kodak metalographic plates. Rotor speeds and recording time intervals are listed on page 24. Peak distances from the axis of rotation were measured using a Gaertner two dimensional micro comparator. Flotation values were then calculated as indicated on page 24.

It should be emphasized that the analytical ultracentrifuge studies were carried out at $20^{\circ}C$, rather than the conventional $26^{\circ}C$ traditionally used for lipoprotein studies. Thus the S_{f20} values will be slightly lower than S_{f26} values.

APPENDIX II

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis has been used to fractionate human serum lipoproteins (98). Similar lipoprotein fractions might be expected from polyacrylamide and agarose gel electrophoretic fractionation, providing the gels were of equal pore size and that lipoprotein-gel interactions were similar. However the pore size of polyacrylamide gel in the concentrations commonly used tend to be smaller. At a polyacrylamide gel concentration of 7.5 per cent, the low density lipoproteins remain at the top of the main gel. High density lipids are resolved into three to five components, but the nature of these components is not known. Interpretation of polyacrylamide gel electropherograms of serum lipoproteins in general has not been achieved.