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The Paper Chromatography of Human Serum Lipoproteins

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THE PAPER CHROMATOGRAPHY OF HUMAN
SERUM LIPOPROTEINS

by

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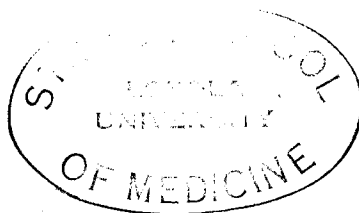
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LIFE

Leonard Jerome Banaszak was born in Milwaukee, Wisconsin, on February 1, 1933. He graduated from Custer High School in Milwaukee in June, 1951. From September, 1951, to June, 1955, he attended the University of Wisconsin, and received the Bachelor of Science degree in chemistry in June, 1955.

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He is co-author of the following publications: "Centrifugal Force in Paper Chromatography and Electrophoresis", Anal.

Chem. 31, 825 (1959), and "Tupor Chromatographic Separation of Human Serum Lipoproteins", Clin. Chem. 5, 270 (1959).

PREFACE

The presence of lipoproteins in human serum is now a well accepted fact. The involvement of lipid material in several pathological conditions of man, particularly atherosclerosis, has drawn much attention to the lipoproteins of serum. Aside from the clinical significance of lipoproteins, there is also considerable interest in their molecular nature.

The recent development of a paper chromatographic technique for serum lipoprotein separation raised the question as to what ultracentrifugal density classes correspond to the chromatographic lipoprotein fractions. One of the objectives of this thesis is to develop a correlation between the lipoprotein fractions obtained from the ultracentrifuge and those obtained by paper chromatography.

The observation that certain lipoprotein fractions on chromatograms stained with Sudan Black B faded faster than others, led to further investigation of these reactions.

Very special thanks are extended to Dr. Hugh J. McDonald for his guidance and encouragement throughout this investigation.

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CHAPTER I
CHEMISTRY OF LIPOPROTEINS

Lipids are a heterogeneous group of biochemicals usually characterized by their solubility in organic (non-polar) solvents and by their insolubility in water (polar) solvent. The term lipid includes such materials as fatty acids and their conjugates, triglycerides, waxes, certain hydrocarbons (i.e. carotenoids), tocopherols and various sterols and their derivatives. The state of existence of non-polar material such as lipids in a relatively polar environment raises a problem which is only partially understood. The evidence of lipid-protein complexes explains the solubility and stability of lipid material in the aqueous fluids of animals. However, the nature of lipid-protein interaction and factors influencing lipoprotein formation remain ill-defined. Early investigators felt that a lipoprotein was an unstoichiometrical combination of lipid and protein. From the work of the past ten years, however, there seems to be little doubt that the lipoproteins of blood serum are definite biochemical entities.

Most of the studies on the chemistry of lipoproteins have been performed with human serum lipoproteins. Only a few investigators have employed the serum of animals (2). To date, the same general classes of lipoproteins have been found in

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both animal and human serum. Lipoproteins are also present in animals and plants as probable structural components of many types of cells. The knowledge of this structural role is limited by the lack of techniques for lysing cells and subsequent methods of isolation (15). Present methods of breaking cells result in the breakdown of components which are interlinked in the intact cell.

In regard to the chemistry and interrelationship of serum lipoproteins, there is a question as to the number of distinctly different lipoprotein components present in serum. At the present time, it is impossible to resolve this problem. Some insight, however, regarding the number of lipoprotein components present in serum may be obtained by examining the chemistry of lipoproteins and the methods available for their analysis. The latter will be described in Chapter II.

The existence of emulsified fat, sometimes referred to as chylomicra, in serum is a well-established fact. These fat globules are of microscopic size, 0.5 to 1.0 micron in diameter and were first recognized by Boyle in 1665 (27). Emulsified fat particles are found in serum normally only in transient periods, post-prandially (53). More recently investigators use the term chylomicron in describing a series of low density lipoproteins. These chylomicrons are not visible microscopically. "Chylomicrons" as used in this thesis, will refer to the large molecular weight lipoproteins having a density less

than 1.019.

The first lipoprotein of a homogenous nature was isolated by Macheboeuf in 1929 (41). It was prepared by adjusting the pH of horse serum to 3.9 and utilizing the technique of ammonium sulfate precipitation. During the next ten years comparatively little work was done in the field of lipoprotein research because of the lack of experimental techniques for their isolation and characterization. The more recent development of ultracentrifugal and electrophoretic procedures made possible great strides in the understanding of serum proteins and lipoproteins.

In general, two classes of lipoproteins have been isolated by the ultracentrifugal methods. They are referred to as "low density" and "high density" lipoproteins. The microscopic "emulsified fat" is not considered since it is only found in lipemic serum. The low density lipoproteins have the same electrophoretic mobility as the beta globulins and are called beta lipoproteins. The high density lipoproteins have the same electrophoretic mobility as an alpha globulin and are often referred to as alpha lipoproteins. Macheboeuf's lipoprotein was shown to be an alpha lipoprotein (42).

Adair and Adair (1) described the two classes of lipoproteins as follows: The alpha lipoproteins are estimated to have molecular weights of 200,000. They are composed of approximately sixty-five per cent protein and thirty-five per cent

lipid, and have the solubility characteristics of a pseudo-globulin. Beta lipoproteins are thought to be spherical in shape and have diameters of about 180 A. Solubility data indicates that beta lipoproteins are euglobulins. They are composed, approximately, of twenty-five per cent protein and seventy-five per cent lipid. Euglobulins are true globulins in that they are insoluble in salt-free water; pseudoglobulins, while possessing the general properties of the globulins, are soluble in salt-free water. The distinction between these types of globulins, while useful, is by no means well-defined.

An interesting sidelight on the physico-chemical properties of beta lipoproteins was proposed by Surgenor (72). He pointed out that because of the lack of lipid characteristics, the outermost aspects of the beta lipoproteins might possibly be proteins. From the approximate dimensions of protein chains and assuming the protein to be spread out in a monolayer, Surgenor calculated that there is only enough protein to cover about one-half of the lipid surface. He then postulates that the polar groups of phospholipid compose the remaining outer-most "shell". The alpha lipoproteins because of the greater protein to lipid ratio, can be imagined as lipid material completely surrounded by protein.

COMPOSITION

Kritchevsky (33) has tabulated the data of Havel et al

(14,26) regarding the composition of human serum lipoproteins.
This is given in table I. The density classes referred to are
those obtained from ultracentrifugal analysis. These ultra-
centrifugal density classes are one way of describing the lipo-
protein fractions of serum. The density class 1.019 to 1.063
are beta lipoproteins; the density class 1.063 to 1.210 are alpha
lipoproteins. These two fractions represent the major portion
of lipoproteins present in serum. The most obvious difference
in composition is in the cholesterol and protein content. In
this case the beta lipoproteins contain approximately fifty per
cent cholesterol and twenty-one per cent protein. The alpha lipo-
proteins contain forty-seven per cent protein and only twenty
per cent cholesterol. This fact may be important because chol-
esterol is most probably involved in the etiology of atheroscler-
osis. Therefore beta lipoproteins or their serum concentration
may be a secondary factor in the onset of atherosclerosis. This

TABLE I

COMPOSITION OF HUMAN SERUM LIPOPROTEINS (PER CENT)

Density class	Cholesterol	Phospholipid	Triglyceride	Protein
less than 1.019	22	18	52	7
1.019 to 1.063	49	24	5	21
1.063 to 1.21	20	27	6	47
greater than 1.21	2	27		

will be discussed under the clinical aspects of lipoproteins in Chapter II. The ultracentrifugal density class labeled "greater than 1.21" represents the serum with all presently known lipoproteins removed.

BINDING STUDIES ON LIPOPROTEINS

Avigan (4) experimented on the solubilizing characteristics of human serum toward C^{14} and H^3 labeled cholesterol. The cholesterol was adsorbed on Celite 545 by evaporation of a pentane solution of ths sterol. The cholesterol-Celite particles were then incubated with serum and the serum lipoproteins isolated ultracentrifugally. The distribution of cholesterol in the various fractions was then determined by counting the radioactivity. The results are shown in table II.

TABLE II

INCORPORATION OF 4-C14-CHOLESTEROL IN LIPOPROTEIN FRACTIONS

Density class	Cholesterol incorporated ug./ml.
LESS THAN 1.019	40
1.019 to 1.063	85
1.063 to 1.21	17
GREATER THAN 1.21	5

The density class 1.019 to 1.063, beta lipoproteins, incorporates most of the cholesterol. Serum with the lipopro-

teins removed, density greater than 1.21, can take up very little
cholesterol. The incorporated cholesterol differed in two res-
pects from particles of cholesterol injected into the blood stream
in an alcoholic solution: (1) Lipoprotein incorporated choles-
terol exchanged with the cholesterol in the red blood cells.
(2) The alcoholic suspension of cholesterol was taken up rapid-
ly by the tissues; the lipoprotein incorporated cholesterol was
not. Most of the natural steroids studied behaved as cholesterol.
This included sitosterol, progesterone, testosterone, deoxycorti-
costerone and corticosterone.

Other lipid material, such as fatty acids, demonstrates
a different distribution between the serum proteins. Unesteri-
fied fatty acids have been shown to be of considerable metabolic
significance. They represent a transport form of lipid material
readily available as a substrate for oxidation. Goodman and
Shafrir (22) have studied the binding of oleic, palmitic, stearic
and linoleic acids to plasma proteins by using C^{14} labeled com-
pounds. Their results indicated that while these compounds
represent eighty to eighty-five per cent of all fatty acids
present in serum, only three-tenths per cent are bound to the
lipoproteins of density less than 1.019 and of density 1.019 to
1.063 (chylomicrons and beta lipoproteins respectively). It
was also demonstrated that the alpha lipoprotein, density 1.063
to 1.21, binds even less of the unesterified fatty acids.
Albumin binding is responsible for practically all of the trans-

port form of unesterified fatty acids. Bermes (8) obtained similar results employing ionography.

Gordon (23) in an earlier investigation had demonstrated the binding of oleate to both albumin and beta lipoproteins. He had shown, by determining the ionographic mobilities of albumin and beta lipoproteins in the presence of increasing amounts of oleate, that: (1) The oleate was bound first to the albumin. (2) As the concentration of oleate was increased, greater amounts of this fatty acid were bound to the beta lipoproteins. Gordon did not consider the concentrations of the fatty acids, albumin and beta lipoproteins in comparison with their relative proportions in serum. The binding of fatty acids to the beta lipoproteins probably has no physiological significance.

Certain other non-polar material has been associated with the beta lipoprotein fraction (58). This includes estriol, vitamin A and vitamin E, and some of their metabolic homologues. It is these components which probably account for the oxidative changes occuring in isolated lipoproteins (8,20,59,60). One oxidative change is demonstrated by alterations in the visible spectra of the beta lipoproteins. It is catalyzed by very low concentrations of cupric ion and has been attributed to the oxidation of beta carotene (13).

Beta lipoproteins undergo another alteration which must be considered in their laboratory preparation. Oncley (59) points out that water is very important to the maintenance of

their structure. Freeze drying destroys the well-defined solubility characteristics of beta lipoproteins (74). This is in contrast to other serum proteins like albumin and gamma globulin which are best preserved in the dry state.

Lovern (40) summarized extraction studies on lyophylized lipoproteins and postulated that there are at least two general types of linkages involved in lipoproteins. (1) A link or bond so weak that it can be ruptured by ether when an outer barrier (water?) has been destroyed. (2) The other bond requiring more drastic treatment such as boiling with alcohol to break it. Lovern postulates that the weaker bond may be a lipid-lipid linkage and the stronger bond a lipid-protein linkage.

Dervichian (19) in an earlier review had postulated essentially the same linkages as Lovern but was more specific. Dervichian said that non-ionic lipids cannot associate by themselves with proteins while they can associate with other ionic lipids. Unfortunately he presented no evidence for this statement. Dervichian's lipoprotein consisted then of the following: protein--ionic lipids (phospholipids, fatty acids)--non-ionic lipids (esters of cholesterol, cholesterol, triglycerides). In contrast, Palmer and Chargaff (66) proposed that lipoprotein molecules are formed by the interaction between the alkyl-side chains of certain amino acids in the protein and the non-polar portion of lipid molecules.

Goodman (22) studying the binding of fatty acids to

beta lipoproteins indicated that two classes of binding sites existed. The large difference between the numbers of these two binding sites permitted speculation on the mechanism of the fatty acid interaction. His reasoning was that in one class, the large number of sites available indicated relatively little specificity. The main factor may be that of the "solubility" of the fatty acid hydrocarbon chain in the lipid portion of the lipoprotein. Goodman also proposed that in a small number of instances, the carboxylate end of the fatty acid ion may interact with one or more side groups of the protein portion of the beta lipoproteins. In summary, the accumulated data is still insufficient to make any definite conclusions regarding the types of bonds involved in the structure of lipoproteins.

TRANSPORT AND METABOLISM

Further evidence regarding the definition of lipoprotein molecules may be obtained by examining their biological function. Most of the work regarding the biological function of lipoproteins, so far, has been done _in vitro_ and therefore, the possibility of structural changes resulting from isolation procedures may exist. Another thing to consider is that various investigators have utilized lipoprotein fractions of different density classes in their investigations. This fact makes it difficult to compare the work of one investigator with that of another.

One approach to the metabolic interrelationships of the

lipoproteins is the determination of N- and C-terminal amino
acids and amino acid composition. An N-terminal amino acid has
a free alpha amino group; a C-terminal amino acid has a free
carboxyl group alpha to an amino group. Table III lists the amino
acid composition of alpha and beta lipoproteins and albumin (61).
The high concentration of leucine may be of considerable signi-
ficance. This amino acid might be expected to have attraction
for the aliphatic moiety of lipid material.

Oncley (61) describing N- and C-terminals has reported
that the two alpha lipoprotein fractions of density 1.09 and 1.14
have similar amounts of both N- and C-terminal amino acids.
These alpha lipoproteins contain one mole of N-terminal aspartic
acid and one mole of C-terminal threonine per 100,000 g. of pep-
tide. It is then suggested that the lipoprotein of density 1.09
has two peptide chains which are thought to be the same as those
of the density 1.14 lipoprotein. This was done to account for
the differences in molecular weights since the lipoproteins of
density 1.09 have a molecular weight about twice that of the
lipoproteins of density 1.14.

The protein portion of the combined alpha lipoproteins
has a molecular weight of about 75,000 (71). This protein moiety
was shown to be similar to an alpha-1 serum globulin by analyti-
cal ultracentrifugation and free solution and paper electrophor-
esis. Grundy (25) applied the same technique to low density
(beta) lipoproteins. The protein moiety of the beta lipoproteins

TABLE III

AMINO ACID COMPOSITION OF THE PLASMA LIPOPROTEINS AND ALBUMIN

Amino acid residues per 100,000 g. of
peptide moiety

	beta lipo- proteins	alpha lipo- proteins	albumin
glycine	33.4	36	21.4
alanine	49.3	66	
valine	49.5	55	66.0
leucine	131.7	135	84
isoleucine	ca 0	ca 0	13
phenylalanine	45.0	38	47.3
proline	28.6	(28)	44.4
tryptophan	?	?	1.0
serine	51.1	55	35.3
threonine	49.5	45	42
half-cystine	41.1	26	52.2
methionine	ca 0	ca 0	8.7
aspartic acid and asparagine	76.0	62	78.2
glutamic acid and glutamine	73.5	131	118.6
tyrosine		19	26.0
lysine		57	84.3
histidine	ca 6.5	17	22.6
arginine	20.0	42	35.7

See (61).

was different from the protein portion of the alpha lipoproteins. This was confirmed by the amino acid studies of Avigan (3). He showed that the beta lipoproteins, density 1.019 to 1.063, contained C-terminal serine and N-terminal glutamic acid. This is different from the C-terminal threonine and N-terminal aspartic acid of alpha lipoproteins.

Prior to the studies of the protein portions of the lipoproteins, some investigators thought that low density lipoproteins were converted to high density lipoproteins. This was suggested to occur *in vivo* by Graham (24) and *in vitro* by Boyle (12). They felt that the conversion occurred by the removal of lipid material from low density lipoproteins, thus resulting in high density lipoproteins. In view of the differences in the amino acid composition of the protein moieties of the low and high density lipoproteins, such a simple conversion of one class of lipoproteins to another, would appear to be impossible.

Rodbell (70) subsequently has shown that at least two different peptides or proteins are present in the density class less than 1.019. In this thesis this fraction is referred to as chylomicrons. One of the peptides of the lipoproteins of density less than 1.019 was probably the same as that of the high density, alpha lipoproteins. This was demonstrated by a modification of the "finger printing" technique of Ingram (28). Ingram's technique consists of comparing two dimensional chromatograms of a partial acid hydrolyzate of the peptide. Korn (32) had postulated

a mechanism in 1955 which would account for the results of Rodbell. Korn's idea was that triglycerides plus alpha lipoproteins yielded chylomicrons. The latter was broken down enzymatically by lipoprotein lipase to yield alpha lipoproteins and fatty acids.

Oncley (61) using iodinated (I^{131}) lipoproteins gets results that conflict with the work of Rodbell. Oncley labeled the following four lipoproteins with radioactive iodine: microscopic serum emulsified fat, lipoprotein of density 0.98 (chylomicrons), beta lipoprotein of density 1.03 and the combined alpha lipoproteins. A tracer amount of the iodinated lipoproteins was then injected into human subjects and blood samples were taken at various intervals. When the lipoprotein of density 0.98 was injected the label was quickly picked up by the beta lipoproteins of density 1.03. Only a small amount of the label is found in the other lipoprotein fractions. Other experiments indicated that after radio-iodinated alpha lipoproteins injection none of the radioactivity was found in the "emulsified fat" fraction, or in either the density 0.98 or 1.03 lipoproteins fraction. Extraction experiments had shown that the iodine was not associated with the lipid portion of the lipoproteins. This is in direct opposition to the previously mentioned work of Korn. It is also difficult to explain on the basis of Rodbell's work in which he showed that one of the peptides associated with the chylomicrons seems to be the same as the peptide portion of

alpha lipoproteins.

In conclusion, metabolic and transport studies offer some explanation as to the range of composition of different types of lipoproteins. The problem of the interrelationship amongst the lipoprotein fractions has not been solved. While it is true that knowledge of these relations depends on physical analytical procedures, clarification of the reactions which produce the various lipoprotein fractions will probably be necessary to determine which of the various analytical procedures results in fractions comparable to those contained in the organism.

CHAPTER II

ANALYTICAL PROCEDURES FOR LIPOPROTEIN STUDIES

As is generally true in the field of protein chemistry, the knowledge of lipoproteins is limited by the techniques available for separation, analysis and preparation. Investigators studying lipoproteins are further perplexed by the sensitivity of these compounds to different changes. This includes oxidation of certain lipid moieties and sensitivity to lyophilization or other conditions which tend to remove water. Certain alterations are readily detectable. For example, lyophilization or partial denaturation would result in noticeable changes in the solubility characteristics of the lipoproteins. Other changes may be slight and go undetected. The biochemist must always consider the possibility that his isolation or analytical procedure has altered the compound under examination. He must also keep in mind the possibility that a method is not sensitive enough to distinquish all the species of a given type of compound. Frequently, then, the results of an isolation or analytical procedure are not representative of a material as it exists in the organism.

CHEMICAL METHODS

Separation of plasma lipoproteins can be achieved by solubility methods. For the most part, the solubility character-

istics of the lipoproteins are similar to those of the other
serum proteins. The addition of small amounts of electrolytes
or relatively large amounts of glycine causes an increase in
their solubility. On the other hand, the addition of ethanol
causes a decrease in solubility (61). Lipoproteins have a
minimum solubility at pH values near their isoelectric point.

In 1946, during the course of fractionating plasma
proteins, Cohn and his colleagues (16) were able to isolate and
characterize two distinct lipoproteins. One was an alpha lipo-
protein; the other was a beta lipoprotein. The latter comprised
five per cent of the human plasma protein and contained seventy-
five per cent of the plasma lipid. The method, sometimes refer-
red to as the "alcohol fractionation procedure", is done at -5°C.
It involves fractional precipitation of the serum proteins by
altering ionic strength, pH and concentration of ethanol. The
same group in 1950 (17), introduced an improved method. The new
method used lower ethanol concentrations, so that the isolated
products might undergo less denaturation or isolation changes.

While such procedures, as devised by Cohn and coworkers,
are readily adapted to the fractionation of large quantities of
serum, they do not lend themselves to analysis of small amounts
of individual sera. Another drawback is the fact that the pro-
ducts are not of the highest purity. Usually the precipitates
obtained contain lipid-free protein to the extent of fifty to
seventy-five per cent of the total protein present (61). Further-

more, most investigators remain concerned about the effect of
the alcohol on the native components. For example, alcohol
may remove small amounts of lipid from the lipoproteins. Lipid
and protein analysis would then indicate a composition which
would be different from the comparable ultracentrifugally pre-
pared lipoproteins.

Another pure chemical method for the measurement of
serum lipoproteins was introduced by Bernfeld (5), in 1958.
He found that beta lipoproteins are the only proteins of human
serum to interact with macromolecular sulfate esters at pH 8.6.
At pH 7 and below the reaction becomes rather unspecific; other
serum proteins also react with the polyanion. The specificity
of the reaction for beta lipoproteins was determined by moving
boundary electrophoresis and chemical analysis of polyanion-
protein precipitate. Briefly the procedure is as follows: When
one to two milligrams of sulfated amylopectin is added to one
milliliter of serum, a precipitate forms which can easily be cen-
trifuged. Dilution of the mixture about 250 times results in
a precipitate which is highly dispersed and does not sediment
easily. The quantity of the precipitate can be estimated nephe-
lometrically and has been shown to be proportional to the concen-
tration of beta lipoprotein over a wide range of concentration.
The turbidity of the precipitating polyanion is negligible. Ex-
cess of the sulfated amylopectin will cause dissolution of the
precipitate.

Bernfeld failed to get a correlation between ultra-centrifugally isolated lipoprotein and his nephelometric index. This he attributed to the "arbitrarily" set conditions used with ultracentrifugal procedures. It is difficult to understand what Bernfeld meant by the word "arbitrarily". As will be pointed out subsequently, preparative ultracentrifugal lipoprotein methods were devised from analytical ultracentrifugal data. Also he was unable to correlate the cholesterol content of the serum with his index. Bernfeld attributed this to the varying amounts of cholesterol bound to alpha lipoproteins and consequently not measured. No mention was made of the effect of age of the serum, temperature or preparation of the polyanion, all of which might influence the results.

The simplicity of Bernfeld's method is very advanta-geous. It has the disadvantage of measuring only one class of lipoproteins, the beta lipoproteins. Since the procedure is relatively new, evaluation by other investigators has not appeared in the literature.

Some other chemical methods for studying lipoproteins are those concerned with the isolation and measurement of the lipid components. This includes the determination of free and esterified cholesterol, phospholipids and fatty acids. An excel-lent review of such procedures was made recently by Oncley (62).

ULTRACENTRIFUGAL METHODS

Because of the composite lipid and protein structure

of lipoproteins, they can be separated reasonably well in the
ultracentrifuge. The most important factor in ultracentrifugal
separation is the density of the substances to be separated. The
densities of some of the components of lipoproteins are as fol-
lows: triglycerides--0.92, cholesterol--1.06, peptide--1.35,
cholesterol esters--0.99 and phospholipid--0.97 (61). It is
obvious that a complex of any of the fore-mentioned substances
would have a density proportional to the relative amounts of
each component present. Serum has a density about 1.006.

In analytical ultracentrifugation, the rate of sed-
imentation, or flotation of the lipoproteins or proteins in a
centrifugal field is measured optically. Lipoproteins because
of their comparatively low densities are generally measured by
their flotation rates. These are conventionally described in
terms of S_f values. The S_f value is a measure of the rate of
flotation in a unit centrifugal field when suspended in a sodium
chloride solution of density 1.063 at $25^{\circ}C$. One Svedberg unit
has the units of centimeters/gram/second X 10^{-13} (62).

Preparative ultracentrifugation or differential flot-
ation is based on the same principles as analytical ultracentr-
ifugation. Rather than measuring the rate of flotation of vari-
ous substances, each particular lipoprotein is floated to the top
singularly and removed. This is done by adjusting the density
of the solution gradually, and each time ultracentrifuging for
an empirical time interval. The duration of ultracentrifugation

is a function of the S_f and is determined by the interval required to get the desired component reasonably concentrated at the top of the tube.

A certain amount of difficulty arises in comparing the results of the two types of ultracentrifugal methods. If the results are expressed in S_f values one may calculate the density of the particle if the diameter is known. On the other hand, by differential flotation it is not possible to calculate the S_f value. Theoretically the flotated substance represents a spectra of densities, the range of which will depend on the range of density adjustment.

Experimentally it is possible to measure the S_f of a differentially floated lipoprotein and hence correlate S_f with solvent and particle density ranges. This has been done by several workers (63,39), simply by measuring the S_f value in different solvent densities. The density of the lipoprotein will be equal to the density of the medium when S_f equals zero. It is felt that the physiological hydrated density is closely similar if not identical with that measured by this method (39). The physiological hydrated density is the density of the lipoprotein as it exists in serum. It was shown that S_f class 20--40 exhibited a hydrated density of 0.978 g./ml. and S_f class 0--20 had a density of 1.024 g./ml. The term "hydrated density" applies to the lipoprotein with its bound water.

The history of the ultracentrifuge as applied to the

analysis of serum could begin with the work of McFarlane (48-52).
He demonstrated two sedimenting peaks which were labeled A and
G. The slower sedimenting of the two peaks, A, contained mainly
the albumin fraction and G was thought to be the globulin.
McFarlane (49) noticed another "peak" which sedimented as a
shoulder on Fraction A. This phenomena disappeared on dilution.
Because it only appeared occasionally in his experiments, he lab-
eled it the X-protein. Pedersen (67) in 1945, was able to iso-
late quantities of the X-protein. He showed that ultracentrifug-
ation of the X-protein in the presence of salt resulted in two
peaks. One of these has a sedimentation constant the same as
albumin. He also found that the X-protein would float upon ultra-
centrifugation if the density was raised above 1.04. Because of
this low density, Pedersen postulated that the X-protein was a
complex of albumin, globulin and plasma lipid.

Gofman and co-workers (21) finally gave evidence that
the X-protein was a definite entity -- a lipoprotein. Lindgren
and his associates (38) in 1951, by means of preparative and ana-
lytical ultracentrifugation were able to isolate and characterize
a number of serum lipoproteins. Finally Havel, Eder and Bragdon
(26) modified the differential flotation technique by adjusting
to several different densities. Three different lipoprotein
fractions are prepared by this method. They are: density less
than 1.019, density 1.019 to 1.063 and density 1.063 to 1.210.
No mention is made as to how they arrived at these density

classes, but it is presumed that they used the analytical ultra-
centrifugal data of other investigators (18, 36).

The fraction of density greater than 1.210 still con-
tains ten to fifteen per cent of the serum lipid phosphorus but
practically no cholesterol. The associated phosphorus is non-
dialyzable and migrates with alpha-1--albumin fraction in starch
electrophoresis. The exact nature of this component is not
known (26).

ELECTROPHORETIC METHODS

When lipoproteins were linked with certain human dis-
eases, particularly atherosclerosis, great impetus was given to
finding a quick method of fractionation and measurement of serum
lipoproteins. Both paper and free solution electrophoresis were
already being used for the fractionation of serum proteins. Pap-
er electrophoresis or ionography was quickly adapted to the study
of lipoproteins. Ionography permits measurement of the lipopro-
teins in the presence of other serum proteins. Free solution
electrophoresis does not. In free solution electrophoresis,
staining is impossible. The alpha and beta lipoproteins cannot
be distinquished from the alpha and beta globulins.

Ionographic determinations of serum lipoproteins are
summarized by McDonald et al (44) and by Block et al (9). More
recent work describes careful comparison of ultracentrifugal
fractions with those obtained by electrophoresis. Pezold,

deLalla and Gofman (68) studied ionographically, the sedimenting
and the floating ultracentrifugal fractions at densities 1.063
and 1.210. Ionograms were stained for both lipid and protein.
Serum lipoproteins with density less than 1.210 contained both
alpha and beta lipoproteins. Those with density less than 1.063
represented ionographic beta lipoproteins and chylomicrons only.
Essentially the results were the same as the data of Kunkel and
Trautman (34).

Methods have also been devised to measure the lipid
components of lipoproteins following ionography. Nury et al (59)
and Bloomberg et al (11) have developed procedures for the meas-
urement of the cholesterol content of the lipoprotein fractions
obtained by ionography. Phospholipid may be determined following
ionography by various extraction procedures (14,56).

Most of the ionographic methods for determining lipo-
proteins, depend on the fact that the stains used to color the
lipoproteins are lipid specific. Sudan Black B had been used as
a lipid stain by histologists as early as 1933. In 1954, Swahn
(73) described the use of Sudan Black B in the ionographic deter-
mination of blood lipids. He showed that this stain does not
color other lipid-free serum proteins. Other workers have dem-
onstrated the lipid specificity of Oil Red O (29).

More recently, the commercial preparations of Sudan
Black B and Oil Red O have been shown to be impure. Bermes (6)
fractionated commercial Sudan Black B into ten components. All

of the fractions demonstrated lipid specificity. On the other hand, some components of commercial Oil Red O were shown to partially stain lipid-free proteins (35).

A method of staining serum lipoproteins prior to ionography was introduced by McDonald and Bermes (45). Elimination of staining following ionography resulted in patterns with essentially no background color. This facilitated densitometric measurement of the relative amounts of lipoproteins. The pre-staining technique was subsequently modified by Wilcox et al (76) and by McDonald and Ribeiro (46).

CLINICAL ASPECTS OF LIPOPROTEINS

Many diseases have been shown to cause aberrations in the normal serum lipid level. It is recognized now that practically all serum lipid material is associated with protein as biochemical entities, lipoproteins. With the relatively simple ionographic procedure for measuring lipoproteins, many studies have been made linking serum lipoprotein levels with various diseases. Similarly, ultracentrifugal studies have been made on many types of pathological serum. Also physiological factors have been reported to influence lipoprotein concentrations. Some of these factors are: (1) dietary intake of fat and cholesterol (2) thyroid function (3) experimental adrenal cortical hyperactivity (4) age (5) sex (6) pregnancy (24).

A continued effort is being made to correlate lipopro-

tein levels with atherosclerosis. Special attention is being given to the use of lipoprotein determinations in the diagnosis of atherosclerosis. There appears to be some correlation between high levels of low density lipoproteins and atherosclerosis. However, other physiological factors which influence the concentration of these components of serum make it very difficult to establish normal values. At best then, such determinations only indicate atherosclerotic tendencies but cannot be used as a primary diagnostic tool or as an indicator of the progress of such a condition.

CHAPTER III

MATERIALS AND METHODS

The methods and materials used in this investigation
are described in the following section. In a few instances, a
description of reagents and techniques will be made in the sec-
tion of the thesis denoted to results.*

SERUM

Pooled human serum was obtained from Cook County
Hospital through the courtesy of Dr. E.W. Bermes. It generally
represented samples from approximately fifty unselected patients.
In general the type of blood specimen was that required for det-
ermination of fasting blood sugar. For the preparation of ultra-
centrifugal fractions, the serum was used the same day it was
drawn. Serum for chromatography was used for several days after
it was taken. All samples including those obtained from the
ultracentrifuge and pre-stained samples were stored at $0-3^{\circ}C$.

Pre-stained serum was prepared according to the method
of McDonald and Ribeiro (46). To prepare the Sudan Black B solu-
tion, 100 ml. of ethylene glycol (Eastman Kodak, BP $195-197^{\circ}C$)
was heated to $100^{\circ}C$. One gram of Sudan Black B (Harleco,

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*Certain reagents and techniques were used in an attempt
to improve the chromatographic procedure. It was felt that these
slight changes could best be described in Chapter IV, so that a
comparison of the results could be attempted

lot no. 37) was added slowly with rapid stirring. After five minutes, the solution was filtered while still hot, through Whatman #1 filter paper. The filtrate was allowed to cool to room temperature and then re-filtered.

Pre-staining of the lipoprotein fractions and serum was accomplished by adding 0.10 ml. of the Sudan Black B solution to 0.50 ml. of the lipoprotein solution or serum. The mixture was allowed to stand for approximately one hour at room temperature with occasional mixing. This volume ratio was considered adequate since excess dye invariably appeared as a precipitate following centrifugation. The serum--dye mixture was centrifuged for ten minutes at full speed in a clinical centrifuge (International Clinical Centrifuge, Model CL). The precipitate was discarded. When the pre-stained serum was stored at 0-3°C., no further precipitation of the Sudan Black B occurred.

ULTRACENTRIFUGAL PREPARATION OF LIPOPROTEIN FRACTIONS

The ultracentrifugal method used in this thesis is fundamentally that of Havel (26). Some modifications in the preparation of the solutions of various densities in which the ultracentrifugaion was carried out, were introduced by Bermes (8). The modifications consist of the removal of heavy metal ions, especially copper, from the stock salt solution. Copper seems to be a normal contaminant of the distilled water supply.

The solutions of various densities were prepared as

follows: A stock salt solution of density 1.346 was prepared
by dissolving 153.0 g. of sodium chloride (Mallinckrodt, Reagent)
and 354.0 g. of potassium bromide (Mallinckrodt, USP) in one liter
of water. This solution was extracted with a saturated solu-
tion of dithizone (diphenylthiocarbazone, Eastman Kodak) in car-
bon tetrachloride. The stock salt solution was then extracted
five times with a total of 500 ml. of carbon tetrachloride.
Traces of the carbon tetrachloride were removed by boiling the
salt solution for thirty minutes. The various solvent densities
were prepared by dilution of the stock solution with double dis-
tilled water. The special distilled water was prepared by re-
distilling the standard distilled water in an all glass distil-
ling apparatus. Throughout the ultracentrifugal preparation, all
glassware used in the various manipulations were subjected to
scrupulous cleaning and were rinsed with the double distilled
water. For preparing solutions less dense than the stock sol-
ution the following formula was used:

$$Volume_{water} \text{ X density}_{water} + volume_{stock} \text{ X density}_{stock} =$$

$$density_{desired} (volume_{water} + volume_{stock})$$

The densities of the salt solvents were checked with either a
five or ten milliliter specific gravity bottle. Weighings were
performed on a Gramatic Analytical Balance or a Christian Becker
NA-1 balance.

The density of the serum or serum fraction was then adjusted, employing the formula indicated above. This was done by pipetting the desired amount of the serum and salt solutions directly into the Lusteroid ultracentrifuge tubes and filling the tube with a solution of the expected final density. Final filling and capping was performed using the technique of Bermes (8). All ultracentrifugations were carried out in a Spinco Model L Ultracentrifuge employing the number 40 rotor. The average centrifugal force was 105,400 times gravity. Temperature in the head chamber is maintained at 10-12°C. After centrifuging for about eighteen hours, the tubes are carefully removed from the ultracentrifuge head. The floated lipoproteins (supernatants) appear as a layer on the top of the salt solution. The tube is sliced directly below the supernatant lipoprotein concentrate by means of a tube slicer. The latter is a device for collecting the ultracentrifugally floated lipoproteins. The tube slicer consists of a series of plastic and rubber rings, which hold the ultracentrifuge tube firmly in place, and a horizontal knife blade. The blade is forced rapidly through the Lusteroid tube directly below the supernatant lipoproteins. The blade remains in place until the floated lipoproteins are removed by aspiration with a syringe. The bottom portion of the tube is removed and the contents (infranatant) collected.

Essentially two distinct isolation procedures were followed for preparing the alpha and beta lipoproteins.

Method I: To six tubes were added 2.0 ml. of the salt solution of density 1.346 and 10.0 ml. of serum. The tubes were then filled with a solution of density 1.063. After centrifuging for about eighteen hours at 40,000 RPM, the combined chylomicrons and beta lipoproteins fractions float to the top. The alpha lipoproteins and the other serum proteins remain in the bottom portion of the tube. The infranatant is adjusted to a density of 1.210 by adding 1.12 g. of potassium bromide (recrystallized three times, courtesy of Dr. E.W. Bermes) and 0.15 ml. of glass distilled water for every 4.50 ml. of infranatant. Repeating the ultracentrifugation once again for eighteen hours at 40,000 RPM, the alpha lipoproteins fraction floats to the top and is removed by slicing the tube.

Method II: This procedure was carried out simultaneously with Method I. To six tubes were added 2.0 ml. of 1.085 density salt solution and 10.0 ml. of serum. The tubes were capped and filled with a salt solution of density 1.019. These tubes were centrifuged along with the tubes of the first part of Method I. After removing the chylomicrons, supernatants, the solutions in the lower portions of the tubes were pooled. For part II, 9.0 ml. of the infranatant from part I were added to 3.0 ml. of a salt solution having a density of 1.196. The tubes were capped and filled with a salt solution of density 1.063. After again centrifuging for about eighteen hours at 40,000 RPM, the beta

lipoproteins are floated to the top and are removed by means of
the tube slicer.

The fractions obtained from both methods I and II are
listed in table IV. The fraction labeled <u>low</u> <u>density</u> consists
of both beta lipoproteins and chylomicrons.

TABLE IV

LIPOPROTEIN FRACTIONS OBTAINED FROM THE ULTRACENTRIFUGE

Fractions	Density range	Method
Low density, chylomicrons	less than 1.019	II
Low density, beta lipopro-teins	1.019 to 1.063	II
Low density	less than 1.063	I
High density, alpha lipo-proteins	1.063 to 1.21	I

METHODS AND MATERIALS OF PAPER CHROMATOGRAPHIC PROCEDURE

Whatman 3MM paper was used in all the chromatography
experiments. It was obtained in sheets, forty-six by fifty-seven
centimeters. Strips or sheets were cut from the stock paper in
a manner such that the direction of development was parallel
to the machine or "grain direction". For conventional descend-
ing development, the migrant was placed seven centimeters from
the top of the paper which was forty-three centimeters long. The
strips or sheets were developed in a large chromatography jar,
sixty centimeters in height and thirty centimeters in diameter.

In the bottom of the developing jar was placed a large dish which contained a sheet of Whatman 3MM paper. The bottom of this paper was immersed in the respective developing solution and extended above the liquid about three or four inches. It was felt that this paper facilitated equilibration of the atmosphere within the jar. Unless otherwise indicated all the chromatographic experiments were carried out at room temperature, approximately 25°C. The jar was protected from air currents by a card board box.

For the early experiments, the original Michalec buffer (55) was used. It contained 10.3 g. of sodium veronal (Mallinckrodt, USP), 0.04 g. of oxalic acid·$2H_2O$ (General Chemical Co., Reagent) and 0.61 g. of citric acid·H_2O (Baker Chemical Co., Reagent) in one liter of distilled water. The final pH was between 8.5 and 8.6. The ionic strength is 0.06. It was found later that a phosphate buffer gave equally good results. It was prepared by dissolving 7.08 g. of $Na_2HPO_4 12H_2O$ (Baker Chemical Co., Reagent) and 0.033 g. of $NaH_2PO_4·H_2O$ (Mallinckrodt, Reagent) in one liter of distilled water. This resulted in a pH between 8.5 and 8.6 and an ionic strength of 0.05. Unless otherwise indicated, the developing solution contained buffer/ isopropyl alcohol (Baker Chemical Co., Reagent), 60/40, volume/ volume; the Michalec developing solution as referred to in the subsequent text, consists of Michalec buffer/isopropyl alcohol in the same proportions.

Prior to the application of the migrant, the paper was pre-wetted with the buffer. In the early experiments, this was done by running the buffer onto the paper from a pipette or wash bottle. Even after blotting, however, streaks were seen in the paper; some areas seemed to have contained more buffer than others. This was remedied by submerging the paper completely in the buffer and then blotting between two sheets of Whatman 3MM paper.

Preliminary experiments had shown that about forty microliters of the ultracentrifuge fractions and/or serum were necessary to give reasonably detectable final Sudan Black B spots. The migrant was applied from a one-tenth or a two-tenths milliliter pipette to the chromatogram which was laid over two glass dishes. The origin of the chromatogram was suspended between the dishes so that the relatively large volumes of protein solution could not run out of the paper. If the protein solution was permitted to run on the pre-wetted paper without streaking, the initial spot was a little over one centimeter in diameter. Streaking of the solution across a strip one inch wide reduced the longitudinal length of the spot.

After development, the chromatograms were dried for thirty minutes at $105-110^{\circ}C$. They were stained for one hour at $40^{\circ}C$ in a saturated ethanol solution of Sudan Black B. The stain solution is prepared by heating one liter of sixty per cent

ethanol to boiling. One gram of Sudan Black B (Harleco, lot no. 66) is added slowly with stirring. The ethanol solution is cooled to room temperature and filtered twice through Whatman #1 filter paper. After the chromatograms are stained, excess Sudan Black B is removed by three rinses in fifty per cent ethanol. The chromatograms were dried at room temperature or dried in an oven for fifteen minutes at 105-110°C.

In order to improve the color development of the chromatograms, acetylated Sudan Black B was prepared according to the method of Lillie and Burtner (37). Two grams of Sudan Black B was dissolved in a mixture of sixty milliliters of acetic anhydride and forty milliliters of pyridine. The mixture was permitted to stand overnight and then poured into three liters of water. The acetylated Sudan Black B was removed by filtration. The precipitate, acetylated Sudan Black B, was dissolved in 200 ml. of acetone and re-precipitated by pouring again into three liters of water. The acetylated Sudan Black B was collected by filtration and air dried. Both pre-staining and post-staining solutions were prepared as with ordinary Sudan Black B.

A definite darkening of the stained color of the lipoproteins was observed visually with the acetylated dye. However, the additional color was not enough to warrant it's use, so that almost all chromatograms were developed with ordinary Sudan Black B.

Oil Red O was also tried in an attempt to improve the

staining of the finished chromatograms. One and one-half grams
of Oil Red O (Harleco, lot no. 14714) was dissolved in one liter
of boiling sixty per cent ethanol. After cooling to room temper-
ature, the solution was filtered through fluted Whatman #12 filter
paper. Staining of the lipoproteins required twelve hours in the
dye solution. The final color of the chromatograms was improved
particularly because of the reduced color of the background. It
was felt, however, that the long staining period limited use of
this stain to samples in which the time element is not critical.

The bromphenol blue solution chromatographed with the
lipoproteins was prepared by dissolving 0.10 g. of tetra brom-
sulfonphthalein (bromphenol blue, National Aniline, lot no.
13796) in 100 ml. of water. This will be referred to in the
remaining text as the "bromphenol blue marker solution".

The bromphenol blue protein staining solution was made
by dissolving 2.0 g. of bromphenol blue in 190 ml. of ninety-five
per cent ethanol saturated with mercuric chloride (Mallinckrodt,
Reagent) and 1.0 ml. of glacial acetic acid (Mallinckrodt, Rea-
gent). Chromatograms to be stained for protein were dipped in
the bromphenol blue stain solution and then rinsed three times
with a dilute acetic acid solution. The solution of dilute
acetic acid consisted of one milliliter of glacial acetic acid
in ninety-nine milliliters of water.

IONOGRAPHIC SEPARATION OF LIPOPROTEINS

Veronal buffer, pH 8.6, with an ionic strength of 0.05

was used throughout the ionographic experiments. A stock solution with an ionic strength of 0.10 was prepared by dissolving 20.6 g. of sodium veronal and 2.00 g. of veronal (Mallinckrodt, USP) in one liter of distilled water. The buffer was diluted with distilled water to give the buffer used in the ionographic experiments.

Ionographic separation of the lipoproteins was done with a Precision Scientific Company Ionograph using strips of Whatman 3MM paper, one inch in width. The strips were wetted with veronal buffer from a ten milliliter graduated pipette and allowed to equilibrate for one hour with an applied potential of 200 volts. The pre-stained serum or serum was streaked across the center of the paper strip and a potential of 5.0 volts/centimeter was applied. With a potential of this magnitude, good separation of alpha and beta lipoproteins was attained in three hours. The strips were then dried and developed as described in the section on paper chromatography.

CENTRIFUGALLY ACCELERATED PAPER CHROMATOGRAPHY OF LIPOPROTEINS

The apparatus and principles involved in centrifugally accelerated paper chromatography have been described in detail in a recent publication (47). The apparatus, Labline Model 5080 Chromatofuge, consists of a disk-shaped head rotating in a horizontal plane. The filter paper is contained within the head and is held in position at the center. During rotation, the

filter paper assumes a taut horizontal position. There is a circular opening, eight centimeters in diameter, in the center of the head. The developing solvent capillary has access to the chromatogram through this opening. Since the solvent capillary does not rotate, a non-rotating closure device is provided to seal the opening. The closure device has a Teflon ring which protrudes slightly from the other portions and rides on the rotating head. The closure device thus essentially seals the chromatogram chamber. The developing solvent is applied to the rotating paper in the form of a steady stream from a capillary tube. The tube is connected to the solvent reservoir by Tygon tubing. The reservoir must be maintained under a pressure of 8 to 15 lbs./in.^{2} of dry nitrogen (Airco) to maintain the steady stream of developing solvent onto the chromatogram. Located between the solvent reservoir and the capillary tube is a Teflon stopcock. This provides instantaneous starting and stopping of the solvent flow.

Circular sheets, eighteen inches in diameter, of Whatman 3MM paper was used in the centrifugally accelerated chromatographic experiments. The paper was pre-wetted from a wash bottle containing the phosphate buffer. This was the same buffer as that used in the conventional type of chromatographic development. After blotting the disk between two sheets of Whatman 3MM paper, it was rotated for five minutes at 200 to 800 RPM in an attempt to get an even distribution of the buffer on the paper

and to remove any excess buffer. After application of the migrant, the chromatofuge and solvent flow were started simultaneously. Chromatograms were developed for periods between five and thirty minutes with a solution of phosphate buffer/ isopropyl alcohol, 60/40, volume/volume. Drying and staining of the lipoprotein fractions were performed as previously described.

DENSITOMETRIC TECHNIQUES

The scanning device used to measure the color intensity of the Sudan Black B stained chromatograms consisted of a Bausch and Lomb Monochromator, a Welch Densichron with a blue probe, the Welch Log Converter and a Minneapolis Honeywell Brown Recorder. The theory and use of this scanning device has been described in detail by Urbin (75). The blue probe is a photocell with a maximum sensitivity at 400 mu. Chromatographic strips are drawn between the photocell and light source by an electric motor synchronized with the chart on the Brown recorder. The slit width regulating the band width of the light incident on the chromatograms, was kept as small as possible. Usually a slit width of one millimeter resulted in enough light to obtain a densitometric recording of the chromatogram. The scanning was performed at a wave length of 600 millimicrons. Sudan Black B has a maximum light absorption at 600 millimicrons. Because of the relative thickness of the Whatman 3MM paper and in order to use a slit width of one millimeter, it was necessary to use the

maximum sensitivity of the Densichron. This meant that the
instrument was set at zero optical density with the range switch
of the Densichron at position three and at a point on the chrom-
atogram which contained no stained lipoprotein. The zero opti-
cal density was generally re-set for each chromatogram.

Chromatograms and ionograms were scanned as soon as
possible after staining with Sudan Black B. When pre-stained
serum samples were used, the scanning was performed within two
hours following development. The area under the densitometric
recording was measured with a Keuffel and Esser compensating
planimeter (4236M, serial no. 803). At least two planimetric
measurements were made of each densitometric recording. The
measurements generally agreed within 0 to 0.3 centimeters^2.

BLEACHING TECHNIQUES

Experiments employing the chromatography of serum pre-
stained with Sudan Black B indicated that the color of the lipo-
protein fractions faded. It was also observed that the color
of the low density lipoproteins seemed to disappear faster than
the high density lipoproteins and that hydrogen peroxide accel-
erated the fading. An investigation was undertaken to determine
the fading properties of Sudan Black B bound to lipoproteins and
contained in aqueous solutions. The disappearance of Sudan Black
B color was then determined colorimetrically.

The ultracentrifugal lipoprotein fractions and serum

were pre-stained with Sudan Black B in the usual manner. Dilutions of the stained lipoprotein fractions were prepared in Klett colorimeter tubes graduated at five and ten milliliters. The Michalec and phosphate buffers used in these fading experiments were the same as the ones used for chromatography. Various concentrations of hydrogen peroxide were made up from a thirty per cent stock solution (Baker Chemical Co., Reagent). Dilute hydrogen peroxide solutions were prepared fresh for each experiment. The rate of fading of the Sudan Black B was determined in the Klett-Summerson colorimeter (Model 900.3) using the number sixty filter. The spectral changes resulting from the hydrogen peroxide treatment were determined with a Beckman DU spectrophotometer.

CHAPTER IV

THE PAPER CHROMATOGRAPHIC SEPARATION OF SERUM LIPOPROTEINS

Unlike many other biochemicals, the separation of proteins by paper chromatography has not, as yet, been reduced to a routine procedure. This could partially be attributed to the success of ionography which has, in a sense, overshadowed the use of paper chromatography in reference to proteins. There are also certain inherent hindrances in the use of paper chromatographic procedures for the separation of proteins. One difficulty often encountered is the irreversible adsorption of the protein to the paper. This adsorption is facilitated on dry paper and in the presence of organic solvents. In general, any factor which may be expected to cause denaturation of proteins, would increase this adsorption phenomena. Irreversible adsorption can be partially circumvented by pre-wetting the paper.

A number of systems have been successfully applied to the separation of proteins by paper chromatography. A review of these investigations has been published by Block, Durrum and Zweig (10).

In 1958, Michalec (54) reported the separation of serum lipoproteins by paper chromatography. He failed, however, to give any data regarding the nature of the fractions or the exact composition of his developing solvent. This chapter will detail the investigations undertaken to determine the nature of

the separable components and the application of the paper chromatographic method to pre-stained serum. This section will also include the densitometric measurement of the relative concentration of the lipoprotein fractions and the possibility of application of an altered procedure to centrifugally accelerated paper chromatography. Modifications will be introduced which will simplify the procedure. The applicability of the paper chromatographic method to a simple clinical procedure will be illustrated by comparison with the presently used ionographic determination of serum lipoproteins.

PAPER CHROMATOGRAPHIC LIPOPROTEIN FRACTIONS

If serum is subjected to paper chromatography using Michalec buffer/isopropyl alcohol, 60/40, volume/volume, two fractions each of which may be stained with Sudan Black B, are obtained. In order to determine the nature of these lipoprotein fractions, a comparison with the known fractions obtained by some other analytical technique must be made. The ultracentrifugal fractionation procedure was chosen because it yields lipoprotein fractions in comparatively large quantities. Furthermore, the lipoprotein fractions obtained by ultracentrifugation have been carefully studied by means of ionography (68) so that a comparison between paper chromatographic and ionographic lipoproteins can be made.

Figures 1, 2 and 3 illustrate the results of the paper

FIGURE 1

CHROMATOGRAPHIC DIAGRAM OF ULTRACENTRIFUGAL LIPOPROTEIN FRACTIONS

(1) Ten microliters of bromphenol blue (2) Serum (3) Chylo-
microns, density fraction less than 1.019 (4) Beta lipoproteins,
density fraction 1.019 to 1.063 (5) Chylomicrons and beta lipo-
proteins, density fraction less than 1.063 (6) Alpha lipopro-
teins, density fraction 1.063 to 1.21. Developed for two hours
with Michalec developer. Whatman 3MM paper pre-wetted with Micha-
lec buffer.

FIGURE 2

CHROMATOGRAPHIC DIAGRAM OF ULTRACENTRIFUGAL LIPOPROTEIN FRACTIONS

(7) Ten microliters of bromphenol blue (8) Serum pre-stained with Sudan Black B (9) Alpha lipoproteins stained with bromphenol blue. (10) Serum, stained with bromphenol blue (11) beta lipoproteins stained with bromphenol blue. Prepared as figure 1.
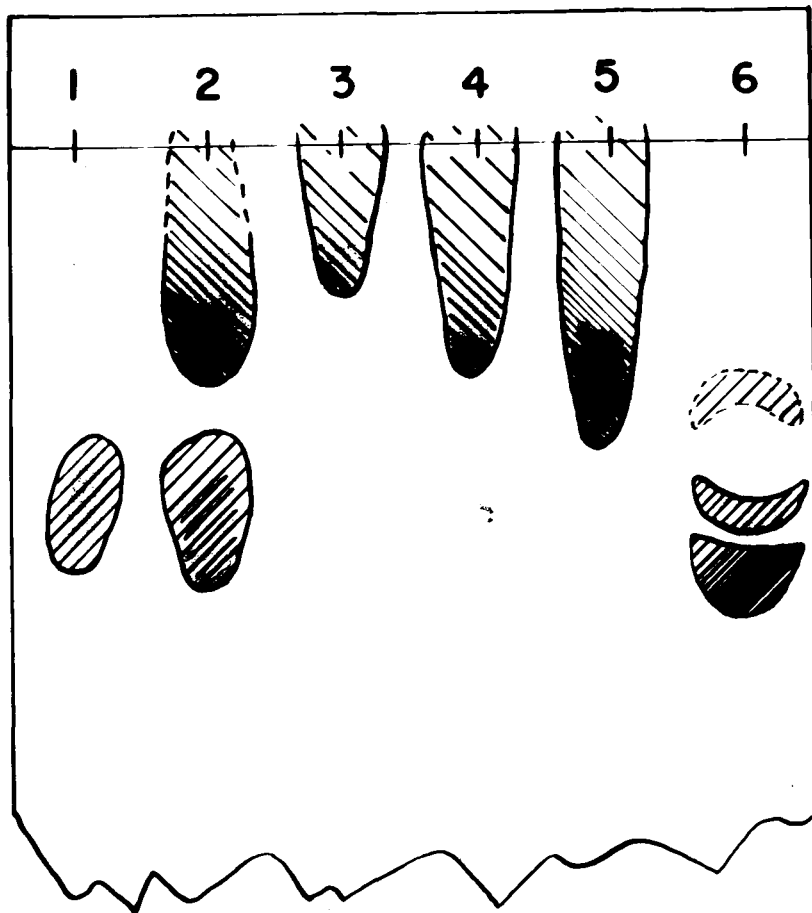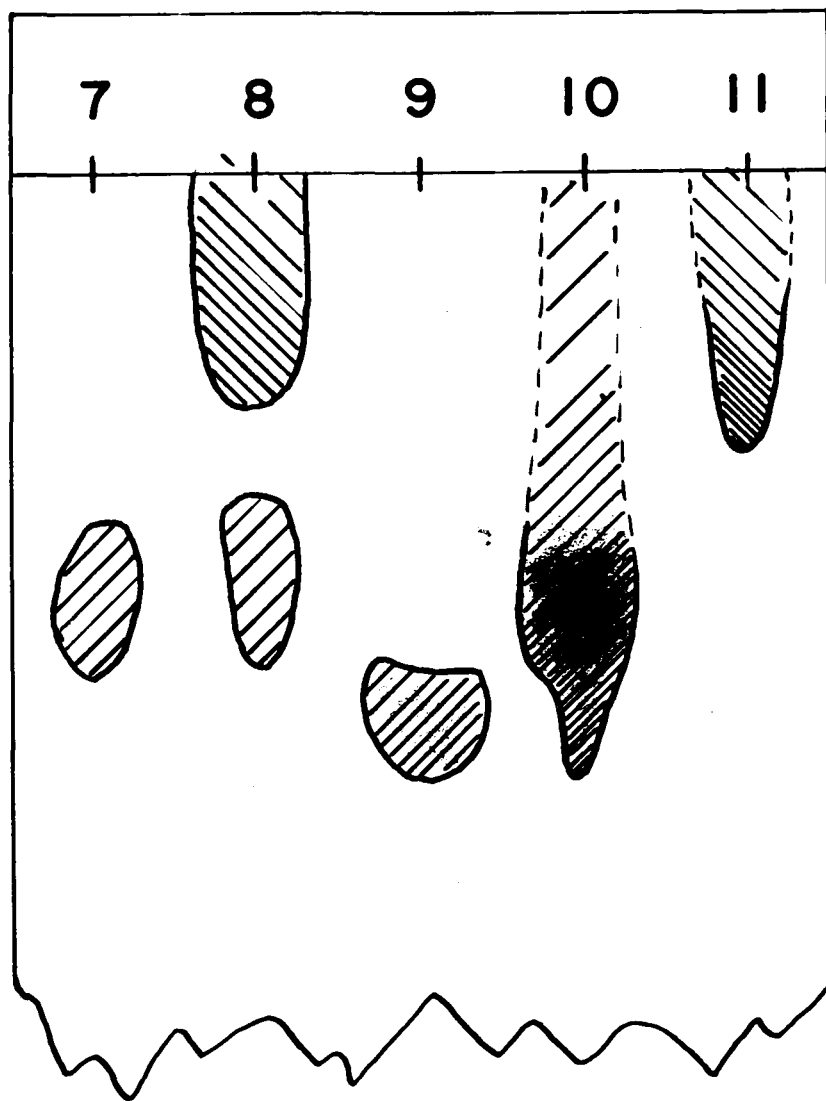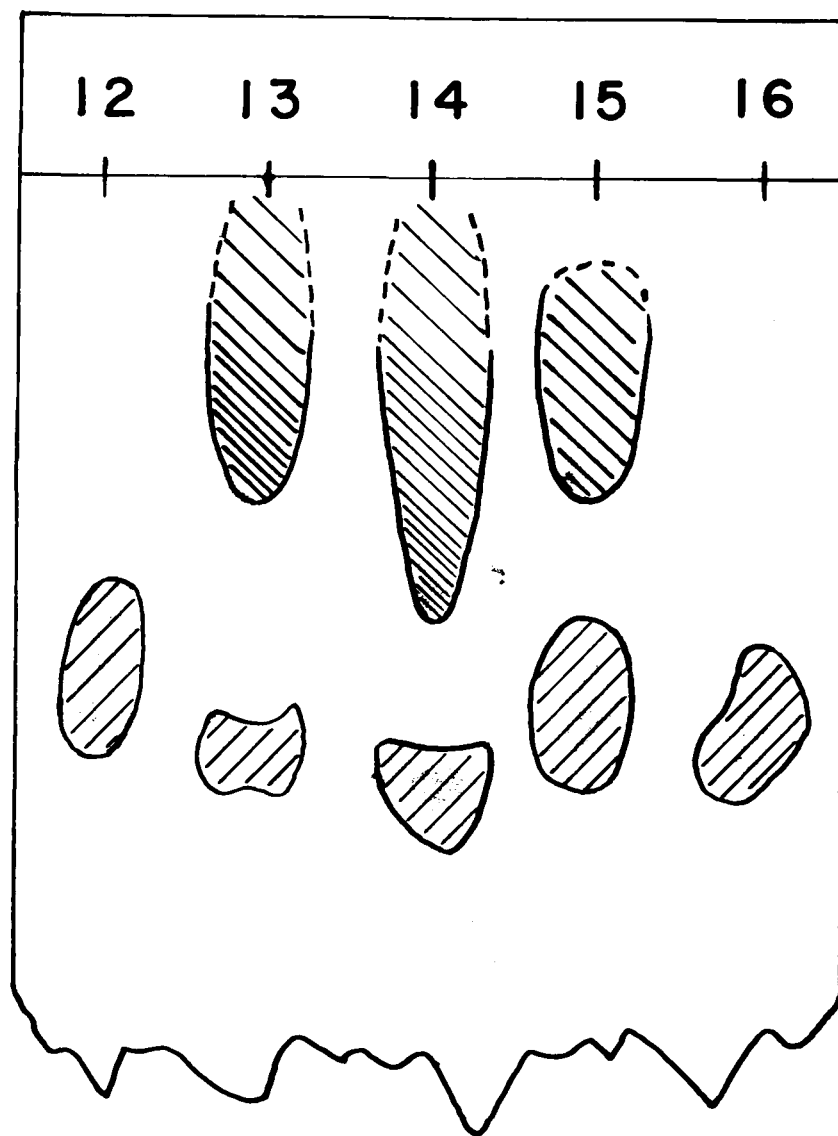
FIGURE 3

CHROMATOGRAPHIC DIAGRAM OF ULTRACENTRIFUGAL LIPOPROTEIN FRACTIONS

(12) Ten microliters of bromphenol blue (13) Reconstituted mix-
ture of alpha lipoproteins, beta lipoproteins and chylomicrons
(14) Alpha and beta lipoproteins, reconstituted mixture (15) Ser-
um minus chylomicrons (16) Serum minus chylomicrons and beta li-
poproteins. Prepared as figure 1.

chromatographic studies of ultracentrifugally prepared lipoproteins and serum. The descending solvent technique was used. The drawings for the photographs were prepared by tracing the spots directly from the chromatograms. Each figure represents the upper one-third of one chromatogram. While these chromatograms represent only one series of experiments, similar results were obtained by repeating both the ultracentrifugal and the subsequent paper chromatographic separation.

Numbers 1, 7 and 12 on figures 1, 2 and 3 respectively are developed spots of ten microliters of a 0.1% bromphenol blue solution. One spot of bromphenol blue was used on each chromatogram so that a comparison of lipoprotein spots could be made from different chromatograms. It was not found possible to calculate an R_f value because the developing solvent front could not be located on the pre-wet paper. The R_f value of a compound is the ratio of the distance moved by the migrant divided by the distance moved by the developing solvent. Both values are measured from the same reference point, usually the origin of the migrant. Since the experimental data required for R_f calculations could not be obtained, it was decided to compare spots from different chromatograms by comparing the ratio of the distance moved by the migrant divided by the distance moved by some reference substance, such as bromphenol blue. The R_b value of a particular lipoprotein is defined as the distance moved by the lipoprotein fraction divided by the distance moved by bromphenol

blue. The distances are measured from the origin to the leading edge of the final spots. Relatively large volumes, ten microliters, of the bromphenol blue was used to give an initial spot commensurate with the initial protein spots. The chromatograms for figures 1, 2 and 3 were developed under the same conditions so that a comparison between the figures might be made.

The portions numbered 2 and 8, represent the lipoprotein fractions obtained from serum by chromatography. Number 2 is serum which has been stained with Sudan Black B after development and number 8 is the pattern resulting from the chromatography of pre-stained serum. The two fractions obtained are the same in both cases. When pre-stained serum is used, the question arises as to whether or not Sudan Black B is being "washed" from the lipoprotein molecules. One spot on the pre-stained serum chromatogram might then be the Sudan Black B. This could not be the case because Sudan Black B, spotted and developed under the same conditions as the pre-stained serum, did not move from the origin.

Under the numbers 3, 4 and 5 are the lipoprotein fractions obtained from the ultracentrifuge which represent what many investigators refer to as low density lipoproteins. It is apparent that the slower moving lipoprotein component of serum represents the low density lipoproteins.

Chromatography of ultracentrifugal alpha lipoproteins, number 6, results in three spots. The very faint spot closest

to the origin may represent a trace of low density lipoprotein. The two other fractions which separate during the paper chromatography of the density class 1.063 to 1.21 from the ultracentrifuge, do not separate from serum under the same conditions. However, the farthest moving component of serum does correspond to the ultracentrifugal alpha lipoproteins. If serum with the low density lipoproteins removed is developed, number 16, only one spot is obtained for the alpha lipoproteins. Numbers 13 and 14 are reconstituted mixtures of ultracentrifugal lipoprotein preparations. A reconstituted mixture consists of mixing equal portions of the lipoprotein fractions isolated ultracentrifugally. These mixtures do not contain the same concentrations of the lipoproteins as is found in serum. Again the alpha lipoproteins appear as only one spot. If the chromatogram of the alpha lipoproteins is stained for protein with bromphenol blue, number 9, again only one fraction appears. This seems to indicate that the ultracentrifugal alpha lipoproteins preparation is contaminated with other serum proteins which are not resolved by this chromatographic procedure.

Protein staining with bromphenol blue of a serum chromatogram, number 10, indicates that practically all of the serum proteins move with the alpha lipoproteins.

R_b VALUES OF SERUM LIPOPROTEINS

In order to make a more accurate comparison between

lipoproteins contained in serum and the corresponding fractions
obtained from the ultracentrifuge, a series of experiments were
performed to determine the R_b values of the various lipoproteins.
Table V contains the results of these determinations. The
standard deviation (SD) was calculated from the formula:

$$SD = \sqrt{\frac{\Sigma(x-m)^2}{n}}$$

where $\underline{x}$ is an experimental R_b value, $\underline{m}$ is the arithmetic mean of
the corresponding series of $\underline{n}$ R_b determinations.

The beta lipoproteins listed in tables V and VI in
reference to serum chromatograms, represents all the low density
lipoproteins. It is the slower moving spot which appears on the
Sudan Black B stained chromatogram of serum; it has an average
R_b value of 0.55 for pre-stained serum and 0.62 for post-stained
serum. In contrast, the R_b of the ultracentrifugally isolated
low density lipoproteins is 0.74. The alpha lipoproteins on the
serum chromatogram have an R_b midway between the two alpha lipo-
proteins which appear on the ultracentrifugal alpha lipoproteins
chromatogram. Both fractions resulting from the chromatography
of serum pre-stained with Sudan Black B had an R_b value slightly
less than the corresponding fractions determined on chromatograms
of serum post-stained with Sudan Black B.

Since the R_b values are determined by measuring to the
leading edge of the lipoprotein spot, the initial volume of mig-

TABLE V

R_b VALUES OF LIPOPROTEINS

Lipoprotein		Average R_b	SD	Number of determinations
Pre-stained serum	alpha	1.03	0.03	6
	beta	0.55	0.03	6
Post-stained serum	alpha	1.13	0.05	6
	beta	0.62	0.05	6
Ultracentrifugal chylomicrons		0.39	0.03	3
Ultracentrifugal beta lipoproteins		0.57	0.04	3
Ultracentrifugal beta lipoproteins and chylomicrons		0.74	0.05	3
Ultracentrifugal alpha lipoproteins	alpha-1	0.97	0.04	3
	alpha-2	1.21	0.05	3

TABLE VI

SUDAN BLACK B CONCENTRATION OF THE LIPOPROTEIN FRACTIONS ON THE
CHROMATOGRAMS USED IN R_B DETERMINATIONS

Lipoprotein sample (forty microliters)	Area-centimeter^2
Chylomicrons	40.4
Beta lipoproteins	78.1
Beta lipoproteins and chylomicrons	87.2
Alpha lipoproteins	56.6
Post-stained serum beta lipoproteins	37.2
Post-stained serum alpha lipoproteins	14.9

rant applied to the paper is important. This was the same, forty microliters, for all lipoprotein solutions used in these experiments. However, the concentration of lipoprotein in the solutions may also be important. In table VI, the concentration of the lipoprotein solutions is presented. The concentration is represented by the area under the densitometric recording of the scanned chromatogram stained with Sudan Black B. The differences in the Sudan Black B staining properties, roughly proportional to the lipid material, of the low density lipoproteins may account for the differences in R_b values (R_b increases with increasing applied lipid material).

LOCATION OF SOLVENT FRONT ON PRE-WET PAPER

The use of R_b values or similar constants in describing the chromatographic properties of lipoproteins works well when the developing system and the buffer used for pre-wetting the paper are always the same. If however, the chromatographic developing conditions are altered, comparison of R_b values is ruled out. Any alterations in the developing solutions would be expected to change the migrating distance of the bromphenol blue as well as the distance travelled by the lipoproteins. In order to study the changes occurring in the chromatographic properties of the lipoproteins which are produced by alterations in the developing solution, a method for locating the solvent front would be very useful. This would permit calculation of R_f values.

Several attempts were made to devise a method for locating the solvent front on chromatograms prepared with pre-wet paper.

An investigation was made to see if a colored substance could be found which would move with the solvent front on a pre-wet chromatogram. The leading edge of the colored material would then mark the solvent front. Chromatographic strips were prepared and developed in the manner used for lipoprotein chromatography. The following materials were spotted on both pre-wet and dry paper: phenophthalein, methyl red, bromthymol blue, bromcresol green, bromcresol purple, phenol red, methyl orange, thymol blue and bromphenol blue. On dry paper developed with the Michalec buffer/ isopropyl alcohol solvent, bromphenol blue, thymol blue and bromcresol green moved with the solvent front. However, on pre-wet paper none of the dyes seemed to move with the solvent front. The distance of solvent movement was roughly determined by developing the chromatogram until the solvent dripped from the bottom of the paper.

Another fact was considered in an effort to locate the solvent front. In areas of the chromatogram where the developing solution has not passed, very little isopropyl alcohol should be present. Thus a substance which is insoluble in isopropyl alcohol would not diffuse as readily on the portion of the pre-wet chromatogram which has been developed. A metal ion was chosen since most metal ions are relatively insoluble in organic solvents. A saturated aqueous solution of ceric sulfate

(Merck, Reagent) was prepared. Using the ceric sulfate solution, it was possible to locate the solvent front on a chromatogram developed in an open jar. This was simply done by "painting" with a glass stirring rod, a streak of ceric sulfate along the edge of the chromatogram. The yellow colored ceric sulfate diffuses rapidly in the area containing no isopropyl alcohol (undeveloped region) and does not seem to "wet" the developed area. If the same procedure is followed but with the chromatogram developed in a sealed chromatography jar containing isopropyl alcohol, no difference in coloring çan be detected. Apparently enough isopropyl alcohol from the atmosphere is taken up by the undeveloped areas of the paper to negate the difference noted above.

Organic fluorescent materials dissolved in the developing solution were also investigated but they did not prove useful in locating the solvent front. In one experiment, the developer (Michalee buffer/isopropyl alcohol) contained 0.1 mg. % eosin. A descending chromatogram developed for two hours with the eosin solution resulted in the eosin not moving even to the origin of the lipoproteins, approximately three centimeters. Similar results were obtained with a 0.8 mg. % solution of fluorescein.

MODIFICATIONS OF THE PAPER CHROMATOGRAPHIC PROCEDURE

The paper chromatographic separation of serum lipopro-

teins results in two distinct fractions. The spot extending
from the origin outward (see figures 1,2,3) consists of at least
two corresponding ultracentrifugal fractions, beta lipoproteins
and chylomicrons. In an attempt to increase the resolution of
the paper chromatographic method, modifications of the develop-
ing solution were made.

Preliminary experiments had shown that Whatman 3MM
paper is superior to Whatman #1 for lipoprotein chromatography.
The application of forty microliters of protein samples to pre-
wetted Whatman #1 paper resulted in a very large, irregular,
initial spot. Also, Whatman 3MM paper is less subject to tearing
following the pre-wetting with the buffer and during the mech-
anical manipulations necessary in preparing the chromatogram.

Pre-wetting of the paper is a necessary step in the
chromatography of lipoproteins. Experiments performed on dry
paper using the ultracentrifugal lipoprotein fractions and serum
resulted in practically no movement of the Sudan Black B stain-
ing fractions. Staining for protein with bromphenol blue reveal-
ed a streak of protein extending from the origin to the solvent
front. In two experiments where the isopropyl alcohol content
of the developing solution was reduced, some movement of the
Sudan Black B staining fractions did occur on dry paper. However
the extreme irregularity of the spots made interpretation im-
possible.

Alterations in the isopropyl alcohol content of the

chromatographic developing solutions did not bring about any
improvement in the results which are obtained with the original
developer, buffer/isopropyl alcohol, 60/40, volume/volume. Fig-
ure 4 represents the chromatograms obtained with the different
developing systems. For each developing system, ultracentrifugal
lipoprotein fractions and pre-stained serum were chromatographed
with the serum. Forty microliters of the protein samples were
applied to one inch strips of Whatman 3MM paper. The strips
were forty-three centimeters long. All the chromatograms were
developed for two hours at room temperature.

Since no satisfactory way of telling how far the sol-
vent front had moved was found, evaluation of the results must
be given by the general appearance of the chromatograms. Table
VII indicates the different solvent front distances on dry paper
produced by variations of the isopropyl alcohol content. This
is given to illustrate the difficulty in comparing the chroma-
tograms on the pre-wet paper resulting from the different deve-
loping systems. The Sudan Black B staining fractions on chroma-
tograms 17 through 21 could conceivably have very similar R_f
values.

In general, on chromatogram number 17 only one spot is
distinct. On the original chromatogram definite streaking of
Sudan Black B could be found from the origin to the single spot.
On chromatogram number 18, two spots are present but there was
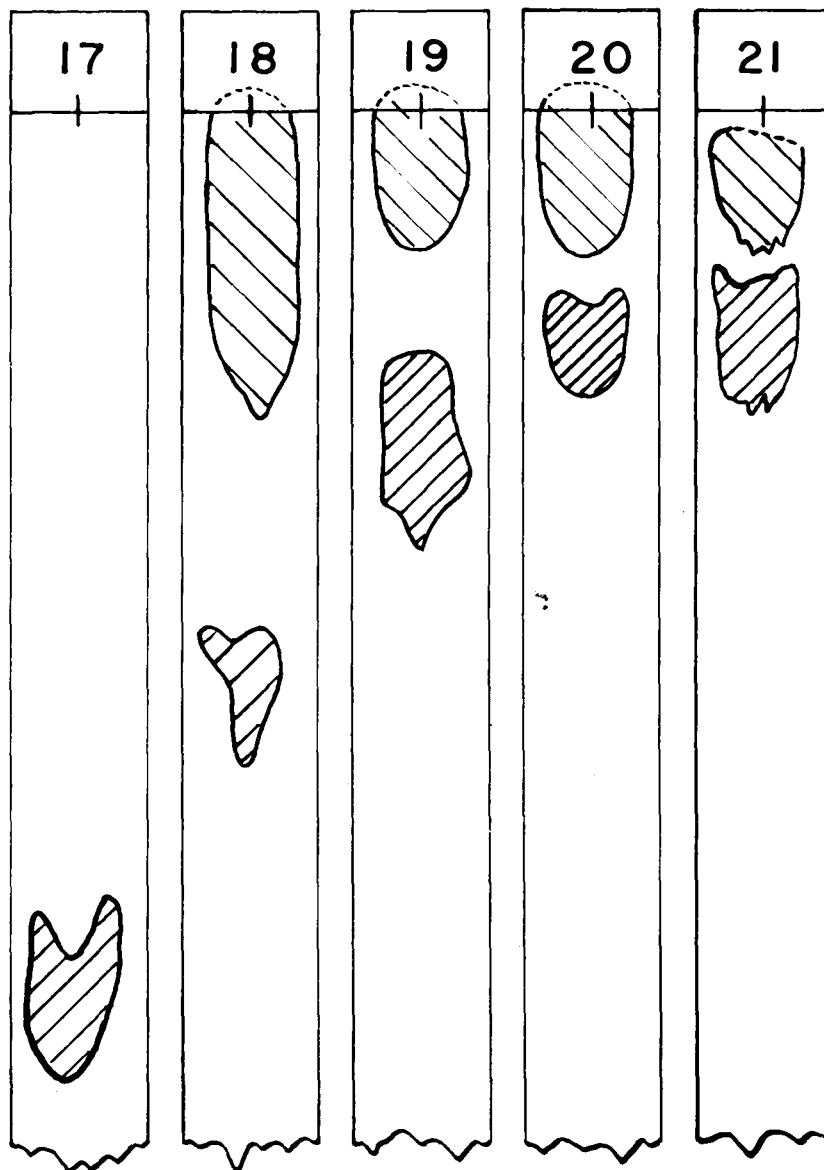
FIGURE 4

CHROMATOGRAPHIC DIAGRAMS OF SERUM LIPOPROTEINS

Whatman 3MM paper pre-wetted with Michalec buffer, developed for
two hours with isopropyl alcohol/Michalec buffer, volume/volume
(17) 0/100 (18) 20/80 (19) 40/60 (20) 60/40 (21) 80/20

TABLE VII

TWO HOUR SOLVENT FRONT DISTANCES OF DIFFERENT ISOPROPYL ALCOHOL/
BUFFER DEVELOPING SYSTEMS

Isopropyl alcohol/Michalec buffer v/v	Solvent front centimeters
0/100	23.0
20/80	14.0
40/60	10.0
60/40	7.3
80/20	5.0

a distinct color of Sudan Black B between the spots. When
the concentration of the isopropyl alcohol in the developing
solution reaches sixty per cent (v/v) it is difficult to dis-
tinquish the two fractions. At eighty per cent isopropyl alcohol
the spots are very irregular. A red color appears at the lead-
ing edge of the spots obtained from pre-stained serum when the
isopropyl alcohol content is above sixty per cent. This red
color is probably a fraction of the Sudan Black B which has
been extracted from the lipoproteins. The Sudan Black B stain
used in these experiments, has been shown to contain several
components (7).

If secondary butyl alcohol (Mathieson) is used
instead of isopropyl alcohol, a two phase system results with
the volume ratio 40/60, secondary butyl alcohol/Michalec buffer.
Serum chromatograms developed for two hours with the lower aq-
ueous phase resulted in no resolution of the lipoprotein compon-
ents.

Alterations in the ionic strength of the Michalec buffer resulted in no definite change in the lipoprotein patterns. Buffer solutions of ionic strength 0.03, 0.015 and 0.008 were prepared by diluting the Michalec buffer. The developing solutions contained the usual forty per cent by volume of isopropyl alcohol. On the two hour serum chromatograms, two lipoprotein spots are resolved. They move the same distance as when the buffer component of the developing solution has the usual ionic strength of 0.06. The alpha lipoproteins spot appeared to be irregular and poorly defined at the lower ionic strengths (0.015 and 0.008).

A series of chromatograms were developed at different pH values. Phosphate buffers with pH values of 5.8, 6.1, 6.4, 6.8, 7.3, 7.7 and 8.0 were prepared according to the directions of Kaplan (30). The chromatographic developing solutions contained forty per cent isopropyl alcohol and sixty per cent of the various buffers. The pre-wetted serum chromatograms were developed for two hours. In the pH range from 5.8 to 8.0, two lipoprotein fractions appeared on all the serum chromatograms. In general, the approximate distance traveled by both lipoprotein fractions was the same as that obtained for the Michalec buffer, pH 8.6. Chromatography with a developing solution of forty per cent isopropyl alcohol and sixty per cent citrate buffer, pH 3.8, resulted in no separation of the serum lipoproteins. All Sudan Black B staining material streaked outward from the origin about

six centimeters. The alpha lipoproteins spot as it appear-
ed on the chromatograms for pH 5.8 to 8.0 was increasingly more
uneven and indistinct in the more acid pH values. Increased
tailing and general unevenness has been reported at acid pH
values in the ionographic determination of serum proteins (44).
This has been attributed to greater protein-paper interaction
toward the acid pH values since paper has a negative charge.

The successful use of phosphate buffers in the chroma-
tography of lipoproteins led to the preparation of a phosphate
buffer, pH 8.6, ionic strength 0.05 (Chapter III). This is the
buffer which was used in the centrifugally accelerated chroma-
tography experiments and some of the densitometric studies.
Conventional chromatographic studies have shown it to give the
same results as the Michalec buffer.

DENSITOMETRY OF LIPOPROTEIN CHROMATOGRAMS

The relative concentrations of the lipoprotein compo-
nents of serum can be represented by the amount of lipid stain,
Sudan Black B, which is bound to these fractions. Densitometric
scanning may be used to determine the Sudan Black B associated
with the chromatographically resolved serum lipoproteins. The
chromatographic procedure for the determination of serum lipopro-
teins is easier to perform and requires less expensive equipment
than the presently used ionographic method. Scanning of the
chromatographic strips was chosen in preference to the ethyl

alcohol:acetic acid extraction of Sudan Black B bound to each fraction (54). Preliminary experiments had shown that complete extraction of the Sudan Black B associated with the lipoproteins was difficult. Furthermore, paper fragments remaining in the solution after extraction, often interfered with the colorimetric determination of the Sudan Black B.

Figure 5 represents the serum lipoprotein--Sudan Black B density distribution on a chromatogram and an ionogram prepared under similar conditions. The patterns are essentially the same. Two main peaks appear in both instances; the peak farthest from the origin represents the alpha lipoproteins while that closest to the origin consists of the low density lipoproteins, beta lipoproteins and chylomicrons. The gradual slope of the low density lipoproteins on the chromatogram is the result of tailing. Similarly on the ionogram, Sudan Black B color streaks from the origin out to the crest of the low density lipoprotein peak.

In order to demonstrate the usefulness of the chromatographic lipoprotein separation as a quantitative method, a standard curve was prepared. This is illustrated in figure 6 and the data recorded in table VIII. The area under the densitometric recording is proportional to the amount of pre-stained serum approximately in the range 10-50 microliters. If the chromatograms are post-stained with Sudan Black B, instead of using pre-stained serum, the results are erratic. They are

FIGURE 5
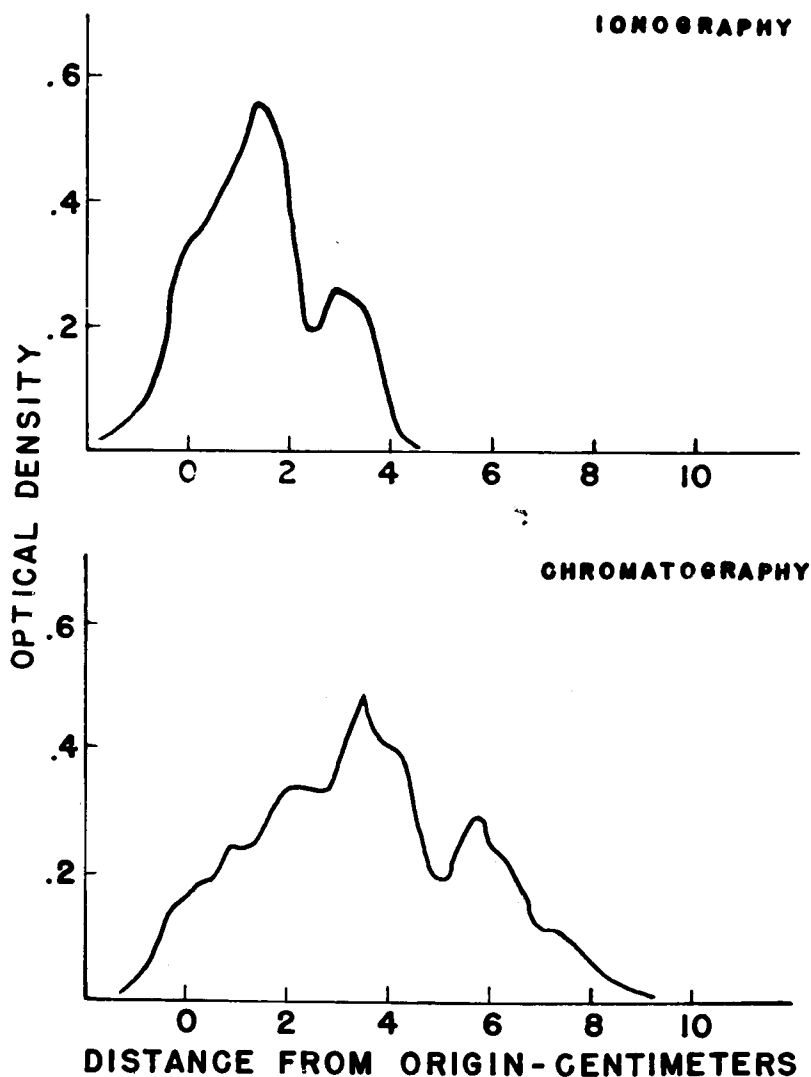
DENSITOMETRIC RECORDINGS OF SERUM LIPOPROTEIN SEPARATIONS

Forty microliters of pre-stained serum on Whatman 3MM paper.
Ionography--veronal buffer, pH 8.6, ionic strength 0.05, run for
three hours, 5.0 volts/centimeter. Chromatography--paper pre-
wetted with phosphate buffer, pH 8.6, ionic strength 0.05, deve-
loped for two hours with isopropyl alcohol/phosphate buffer,
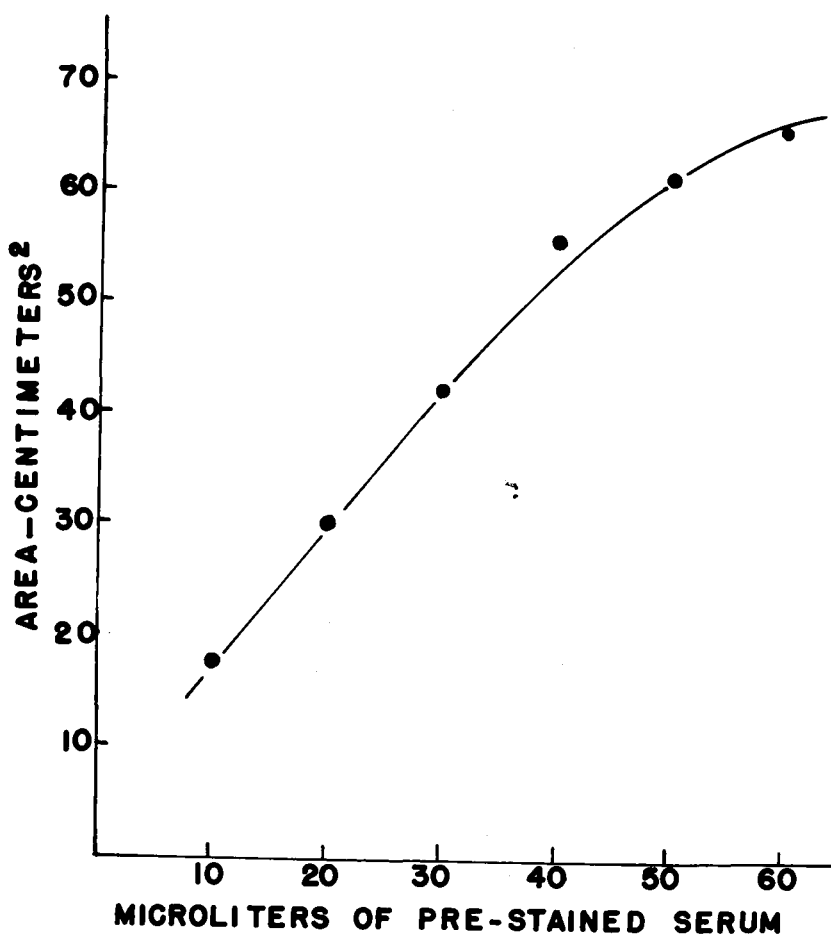40/60, volume/volume.

FIGURE 6

RELATIONSHIP BETWEEN THE TOTAL AREA UNDER THE DENSITOMETRIC RE-
CORDING AND THE APPLIED PRE-STAINED SERUM

Pre-stained serum applied to Whatman 3MM paper pre-wetted with
Michalec buffer. Developed for two hours with isopropyl alcohol/
Michalec buffer, 40/60, volume/volume. The densitometry was
performed within two hours after development.

listed in table IX, appendix. This can probably be attributed to the relatively high absorbance and uneven background of the post-stained chromatograms.

It was noted early in this investigation, that the color of the lipoprotein chromatograms stained with Sudan Black B appeared to fade. This seemed to be particularly true for chromatograms of pre-stained serum. In order to determine the color stability of the Sudan Black B stained lipoproteins on paper, an ionogram and a chromatogram were prepared and scanned over a period of approximately twenty hours. The results are tabulated in table X. Both the ionogram and the chromatogram are the ones described in figure 5. During the twenty hours after development no definite color loss was demonstrated. Since all chromatograms were scanned within two hours after development, no consideration need be given to color fading in the densitometric studies. Investigation of the fading reaction in solution will be given in Chapter VI.

Clinical investigators studying alterations in lipoprotein levels usually express their results in terms of lipoprotein ratios. One example is the ratio "beta lipoproteins" to "alpha lipoproteins". This is actually the ratio of the area under the low density peak, beta lipoproteins and chylomicrons, divided by the area under the peak representing the alpha lipoproteins (76). The separations necessary for these calculations, in the past, have been obtained by ionography. In table XI

TABLE X

DENSITOMETRIC STABILITY OF PRE-STAINED SERUM CHROMATOGRAMS

Time (minutes) after completion of separation	AREA UNDER THE CURVE-CENTIMETERS^2	
	alpha lipoproteins	chylomicrons and beta lipoproteins
Chromatography		
37	6.2	19.5
138	5.9	20.7
170	6.9	20.9
200	6.5	20.0
1315	6.5	19.5
Ionography		
33	4.0	16.4
85	3.6	16.5
98	4.2	15.5
123	4.0	15.1
1175	4.0	15.0

are tabulated the results of a series of experiments to determine the ratio of "beta lipoproteins" to "alpha lipoproteins" by the chromatographic procedure. Chromatograms of serums I and II, both pooled samples, were developed with isopropyl alcohol/Michalec buffer, 40/60, volume/volume. For the chromatograms developed of serum III, also a pooled sample, phosphate buffer was substituted for the Michalec buffer. For comparison with the ionographic ratios obtained by other investigators, Wilcox et al (59) determined beta/alpha lipoproteins ratios ranging from 1.6 to 6.6. Their determinations were made on individual serum samples which accounts for the wide range of values-sex, age and state of health variations. The values given in table XI should not be construed to be indicative of an approximate "normal" lipoproteins ratio. These results are presented merely to indicate the usefulness of chromatography as a clinical procedure for the determination of serum lipoproteins.

CENTRIFUGALLY ACCELERATED PAPER CHROMATOGRAPHY

Separation of serum lipoproteins can be accomplished by centrifugally accelerated paper chromatography using the same developing solutions as those employed for conventional techniques. Certain difficulties encountered in the use of centrifugally accelerated methods which prevent the densitometric scanning of the chromatograms decrease the value of the technique as a quantitative procedure.

TABLE XI

CHROMATOGRAPHIC DETERMINATION OF THE RATIO
OF BETA/ALPHA LIPOPROTEINS

	Beta/alpha ratio	SD	Number of Determinations
SERUM I			
Ionographically pre-stained	4.1	0.03	5
Chromatographically pre-stained	3.8	0.08	5
SERUM II			
Chromatographically post-stained	3.4	1.0	6
Chromatographically pre-stained	1.8	0.07	6
SERUM III			
Chromatographically pre-stained	3.0	0.08	5

Figure 7 is a schematic representation of a centrifugally accelerated chromatogram. The part labeled D is shown in figure 8 after developing and staining a forty microliter sample of serum. When this chromatogram was developed, ultracentrifugal chylomicrons, beta lipoproteins and alpha lipoproteins were run simultaneously. The farthest moving spot corresponded to the ultracentrifugal alpha lipoproteins. Similarly, the spot closest to the origin corresponded to the low density lipoproteins. While the alpha lipoproteins spot is well defined, only the leading edge of the low density lipoproteins spot could be readily distinguished. The radial type chromatographic development causes the large increase in the final area of the spots.

In an attempt to eliminate the arc type redistribution of the migrant and yet maintain the rapidity of the centrifugally accelerated procedure, slits were cut in the paper. This is represented by E on figure 7. The resulting "spoke" was one inch wide and was free at the peripheral end. Many experiments varying rotational speed and solvent flow were performed, but reproducible separations of the serum lipoproteins were not obtained by this technique. Probably the main factor preventing resolution of the lipoproteins was the rapid flow of developing solvent through the strip. Experiments on dry paper indicated that developing solvent flow through this "spoke" is considerably faster than through the intact paper disc. This phase of the investigation is being continued.
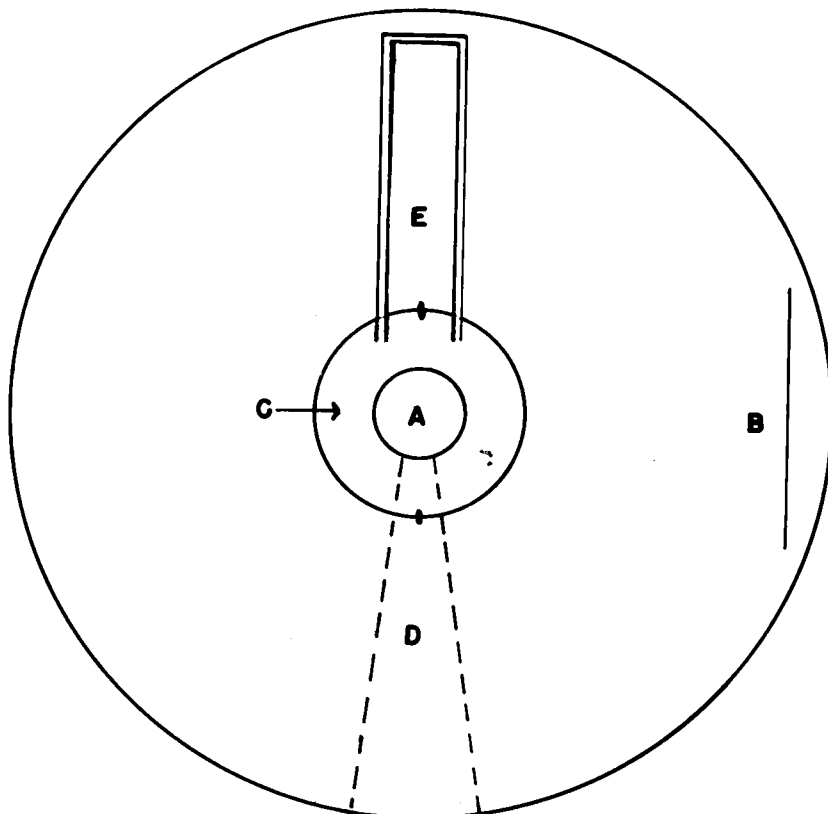
FIGURE 7

SCHEMATIC REPRESENTATION OF CENTRIFUGALLY ACCELERATED CHROMATOGRAM

(A) Circular hole for attachment to chromatofuge head   (B) Machine direction of chromatographic paper   (C) Solvent strikes paper   (D) Area cut out for Sudan Black B staining after development (see figure 8)   (E) Slits inserted in paper to reduce arc-type development.
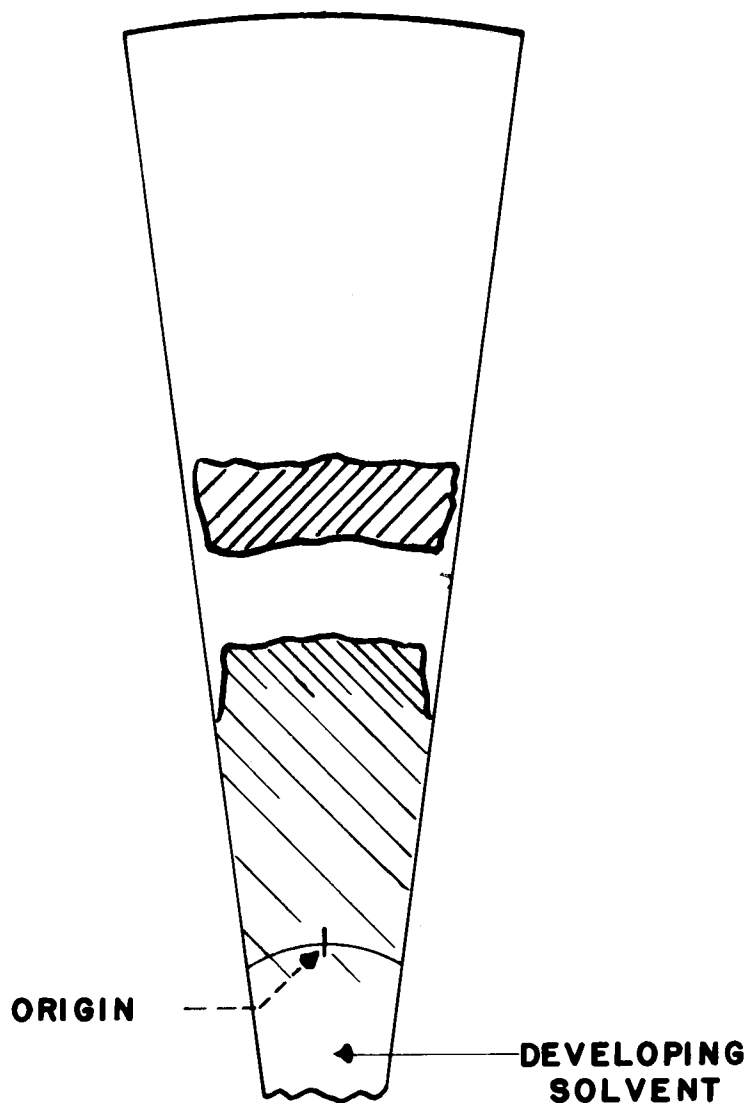
FIGURE 8

CENTRIFUGALLY ACCELERATED LIPOPROTEIN CHROMATOGRAPHIC DIAGRAM

Whatman 3MM paper disc pre-wetted with phosphate buffer. Run for ten minutes at 600 RPM. Forty microliters of serum applied. The chromatogram was developed for fifteen minutes at 600 RPM; the developing solvent was isopropyl alcohol/phosphate buffer, 40/60, volume/volume. Solvent flow was 0.92 ml./min.

Because of the arc-like nature of the final lipopro-
tein spots on the circular sheets, densitometric measurements
were not carried out.

CHAPTER V

THE FADING OF SUDAN BLACK B BOUND TO LIPOPROTEINS

Sudan Black B is a dye insoluble in aqueous solutions and soluble in non-polar solvents. Because of this characteristic solubility and because of the deep blue-black color of Sudan Black B, it has long been used for staining lipid material. Serum lipoproteins stained with Sudan Black B retain the solubility characteristics of the lipoproteins. The ionographic mobility and chromatographic R_b of the serum lipoproteins stained with Sudan Black B appears to be about the same as the unstained lipoprotein. The color of chromatograms of pre-stained serum lipoproteins was observed to fade over a period of several days. Of special interest was the fact that the chromatographic fraction corresponding to the low density lipoproteins appeared to lose the Sudan Black B color faster than the alpha lipoproteins. The fading of chromatograms of Sudan Black B stained lipoproteins was promoted by exposure to air. Scotch Tape placed over the spots of Sudan Black B appeared to reduce the visible fading of the color. It was then thought that perhaps this fading reaction could be accelerated. When the Sudan Black B--lipoprotein chromatograms were placed in a ten per cent hydrogen peroxide solution for two hours no increase in fading was visibly apparent. However, the treatment with hydrogen peroxide des-

troyed the integrity of the paper strip and this technique was abandoned.

Since complexes of lipoproteins and Sudan Black B are soluble in aqueous solutions, it was decided to test the effect of hydrogen peroxide on solutions of Sudan Black B bound to lipoproteins. This permitted photometric measurement of color loss. The results will be presented in this chapter. Beforehand, there are two things which must be considered: (1) The exact nature, of what has been assumed to be an oxidative reaction, is unknown. This assumption is made on the basis that hydrogen peroxide, an oxidizing agent, increases the rate of fading. (2) Commercial Sudan Black B as used in these experiments has been shown to contain a number of colored components (7). It is then likely that this fading reaction is actually multiple in nature. It probably involves some or all of the components of the stain. Hydrogen peroxide treatment for a period of twenty-four hours seems to cause a complete fading of the blue-black color of the Sudan Black B. This indicates that at least all of the blue-black components have been effected.

In figure 9, the absorption spectra of Sudan Black B-- beta lipoproteins and the resulting spectra after treatment with one per cent hydrogen peroxide for twenty-four hours is illustrated. In the hydrogen peroxide treated Sudan Black B--beta lipoproteins spectra, there is almost a complete diappearance of the peak which occurred at 600 millimicrons. There appears to be
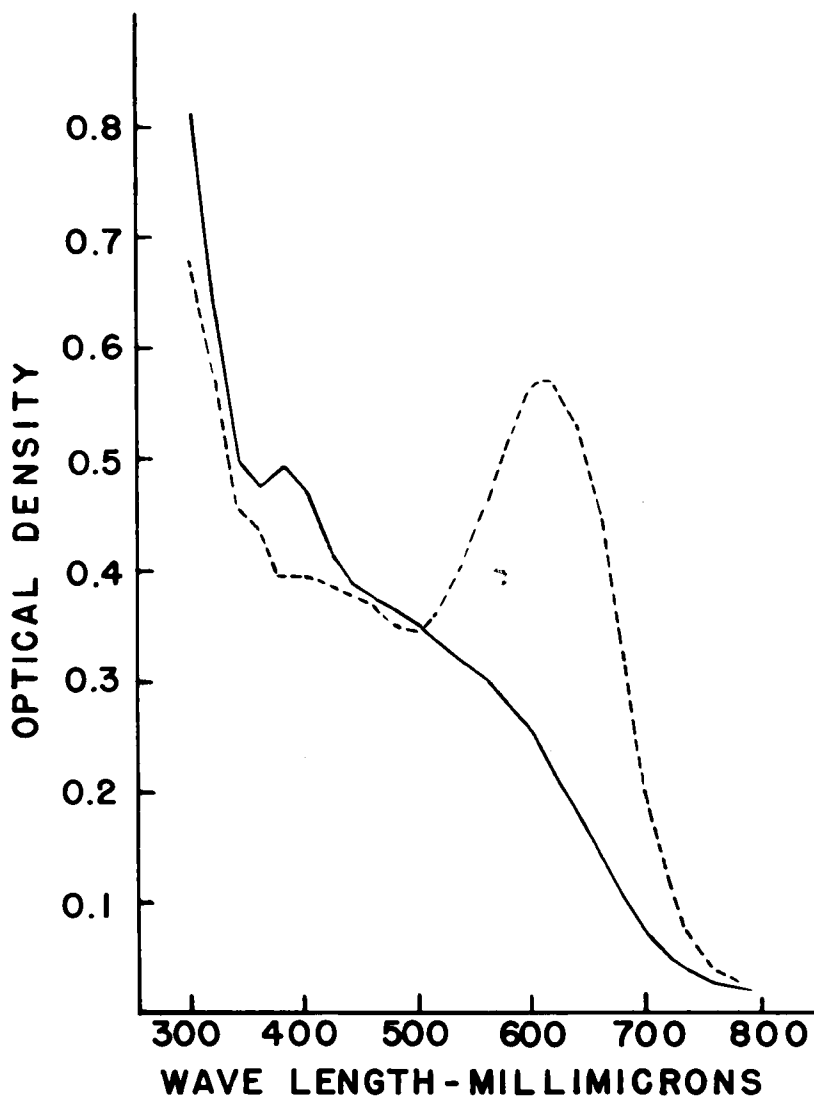
FIGURE 9

ABSORPTION SPECTRA OF SUDAN BLACK B--BETA LIPOPROTEINS

――――Treated with one per cent hydrogen peroxide for twenty-four hours. - - - -Untreated. Both solutions were made up in Michalec buffer. The data is contained in table XII of the appendix.

another peak formed 380 millimicrons. The hydrogen peroxide treatment was done in Michalec buffer, pH 8.6, ionic strength 0.05. The untreated Sudan Black B--beta lipoproteins spectra in figure 9, is the same as the absorption spectra of Sudan Black B above 300 millimicrons. Thus lipoproteins stained with Sudan Black B all have the same absorption spectra in the range 300-800 millimicrons as the dye itself (see table XII, appendix). This determination was necessary since both unstained alpha lipoprotein and beta lipoprotein have absorption peaks in the visible range (8). Apparently enough Sudan Black B is bound to the lipoproteins to completely overshadow the peaks resulting from carotenes and other materials which are contained in the lipoproteins.

Figure 10 illustrates the effect of treatment with three per cent hydrogen peroxide solution in phosphate buffer, pH 8.6, on the ultra--violet absorption spectra of beta lipoproteins. The solution contained 0.50 ml. of the beta lipoproteins, from the ultracentrifugal preparation, in 10.0 ml. of the phosphate buffer. Similar treatment was given to a solution containing 0.50 ml. of the alpha lipoproteins from the ultracentrifugal preparation. After two and one-half hours of hydrogen peroxide treatment, the solutions were placed in dialysis sacks and dialyzed against the phosphate buffer for forty-eight hours. The buffer was changed six times using a total volume of three liters. The untreated lipoproteins solutions were dialyzed in the same manner. The spectrophotometer was set at zero optical
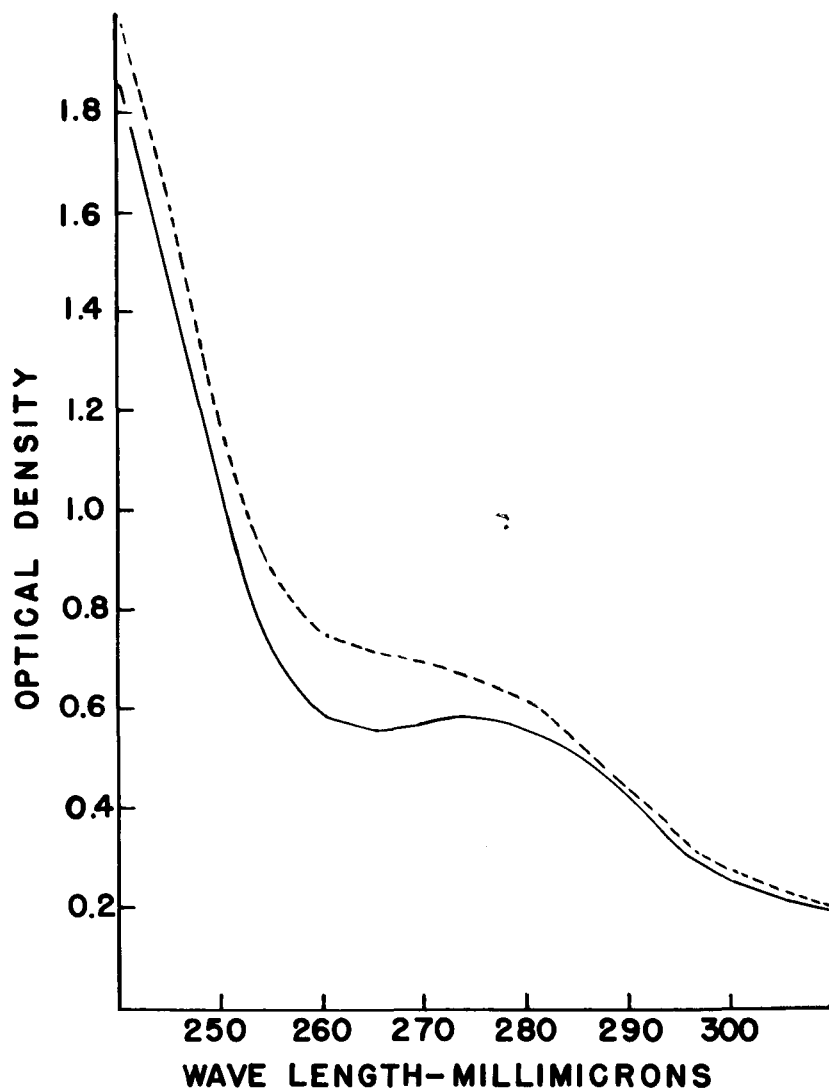
FIGURE 10

ULTRA-VIOLET ABSORPTION SPECTRA OF BETA LIPOPROTEINS

————Untreated and dialyzed beta lipoprotein. – – – –Treated
with three per cent hydrogen peroxide for two and one-half hours
prior to dialysis.

density with the phosphate buffer. No alterations were found in the ultra-violet absorption spectra of alpha lipoproteins (see table XIII, appendix).

The rate of fading of Sudan Black B bound to the ultracentrifugally prepared serum lipoprotein fractions is illustrated in figure 11 and the data contained in tables XIV and XV. Each solution contained 0.50 ml. of the lipoproteins from the ultracentrifugal preparation pre-stained with Sudan Black B. All solutions were diluted with Michalec buffer, pH 8.6. The final volume was 10.0 ml. and it contained one per cent hydrogen peroxide. The Sudan Black B bound to the beta lipoproteins faded rapidly. In table XV, the data indicates that the Sudan Black B bound to the chylomicrons, density less than 1.019, or to the chylomicrons and beta lipoproteins, density less than 1.063, behaves similarly to the stain bound to the beta lipoproteins. No significant loss in Sudan Black B color was detected for the alpha lipoproteins. In each experiment blanks, containing no hydrogen peroxide, were also run but in no case was any significant amount of fading detected. The low readings obtained for Sudan Black B alone indicate the difficulty of dissolving Sudan Black B in aqueous solutions. From the data in table XV, it is difficult to reach any conclusions regarding the fading of Sudan Black B in the absence of lipoproteins. In an attempt to increase the concentration of Sudan Black B in the Michalec buffer, the buffer was made in five per cent ethyl alcohol. Enough
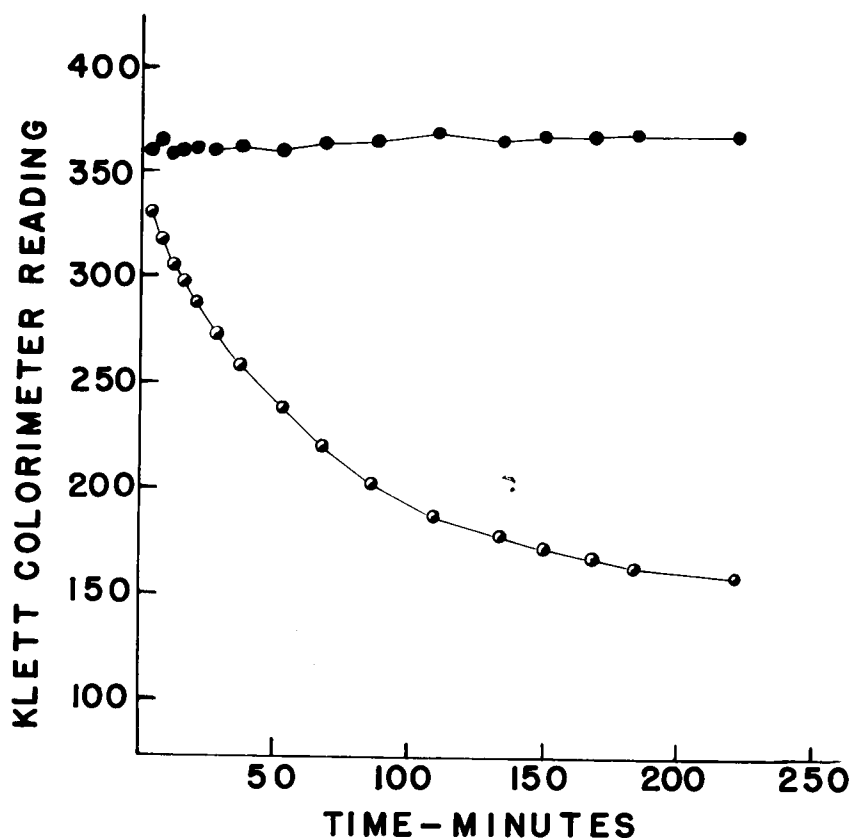
FIGURE 11

THE RATE OF FADING OF SUDAN BLACK B BOUND TO ALPHA
AND BETA LIPOPROTEINS

Solid circles--rate of fading of the Sudan Black B taken up by
0.50 ml. of the alpha lipoproteins from the ultracentrifugal pre-
paration. Half shaded circles--rate of fading of the Sudan Black
B taken up by 0.50 ml. of the beta lipoproteins from the ultra-
centrifugal preparation. Both solutions diluted with Michalec
buffer; the final volume was 10.0 ml. and contained one per cent
hydrogen peroxide.

TABLE XV

THE RATE OF FADING OF SUDAN BLACK B AND SUDAN BLACK B BOUND TO LOW DENSITY LIPOPROTEINS

Klett colorimeter readings

Time minutes	1% H_2O_2 Sudan Black B	1% H_2O_2 chylomicrons	1% H_2O_2 chylomicrons and beta lipoproteins
3	7	325	425
7	7	319	416
11	7	310	410
15	7	299	409
30	7	272	380
48	7	249	360
62	6	237	349
98	6	212	307
140	5	194	264
167	5	187	250
193	4	184	238
218	6	180	228
253	6	173	219

Sudan Black B was dissolved in this solution to give an initial reading on the Klett colorimeter of 435. Hydrogen peroxide was added to a final concentration of one per cent, and after 171 minutes only six per cent of the color had disappeared. In the absence of hydrogen peroxide, there was no loss of color. A solution containing Sudan Black B--beta lipoproteins with the same concentration of hydrogen peroxide, lost forty-eight per cent of the Sudan Black B color in 171 minutes. The zero time Klett colorimeter reading was 625. It appears that Sudan Black B-- beta lipoproteins fades more rapidly than the Sudan Black B alone.

The salt concentration added with the lipoproteins as a result of the ultracentrifugal preparation is not likely to have caused the differences in the fading properties of the Sudan Black B bound to high density and low density lipoproteins. In figure 11, the reaction mixture containing the alpha lipoproteins had a salt concentration of 0.10 molar potassium bromide and 0.021 molar sodium chloride. On the other hand, the reaction mixture containing the beta lipoproteins had a salt concentration of 0.021 molar sodium chloride and 0.025 molar potassium bromide. If the solution containing the beta lipoproteins stained with Sudan Black B was brought to a final salt concentration of 0.125 molar potassium bromide and 0.042 molar sodium chloride, no change in the rate of fading was noted.

In another experiment, fading of Sudan Black B bound

to alpha lipoproteins did occur when the concentration of the
hydrogen peroxide was raised to three per cent or more. The re-
sults are given in table XVI. The per cent color loss is calcu-
lated by using the five minute Klett reading as the initial
color concentration. This was necessary because no zero time
reading was made; the data indicates that the five minute read-
ings are approximately the same. The pH of the solutions at the
termination of the experiment indicated that the higher concen-
trations of hydrogen peroxide had produced a change in the pH
of the Michalec buffer.

TABLE XVI

THE EFFECT OF HYDROGEN PEROXIDE CONCENTRATION ON THE
FADING OF SUDAN BLACK B BOUND TO ALPHA
LIPOPROTEINS

Time minutes	blank	0.9% H_2O_2	3% H_2O_2	6% H_2O_2	15% H_2O_2	24% H_2O_2
		Klett colorimeter readings				
5	134	132	137	141	135	140
231	135	134	131	118	89	81
		Per cent color loss				
	0%	0%	4.4%	16%	34%	41%
		Final pH				
	8.5	8.4	8.3	8.1	7.6	6.9

Unfractionated serum stained with Sudan Black B should
fade at a rate which is a composite of that obtained for the
Sudan Black B bound to the ultracentrifugal fractions. Table XIV

indicates that this was not the case. In an experiment similar
to that described in table XVI, using the Sudan Black B contained
in pre-stained serum, no fading resulted at hydrogen peroxide
concentrations of 2%, 4%, 6% and 8% over a period of two and one-
half hours. Whenever hydrogen peroxide was added to either
serum or pre-stained serum, a gas was evolved. Initially this
was thought to result from trace amounts of catalase in the
serum. The rationale was that the catalase catalyzed the de-
composition of the hydrogen peroxide with a resulting evolution
of oxygen. However, the presence of 0.001 molar and 0.01 molar
potassium cyanide did not stop the evolution of gas. The cyanide
ion has been shown to be an inhibitor of catalase activity. At-
tempts to absorb the evolved gas in a ten per cent barium hydr-
oxide solution were unsuccessful. This indicates that the gas
is probably not carbon dioxide. In another experiment, five
milliliters of serum was dialyzed with stirring ($2^\circ C$) for forty-
eight hours against six changes of Michalec buffer, a total vol-
ume of three liters. Gas was still evolved upon the addition of
hydrogen peroxide to the dialyzed serum. No further evidence
was obtained concerning the nature of the substance contained
in serum which produces the evolution of the gas upon the addi-
tion of hydrogen peroxide .

The addition of serum proteins to Sudan Black B bound
to low density lipoproteins will inhibit the fading of the
Sudan Black B by hydrogen peroxide. For example, if one-tenth

of a milliliter of the serum fraction with practically all of
the lipoproteins removed, density greater than 1.21, is added
to a mixture of the low density lipoproteins stained with Sudan
Black B and contained in Michalec buffer, no fading of the
Sudan Black B occurs. The Sudan Black B concentration was that
resulting from the pre-staining of 0.50 ml. of the lipoproteins
prepared by ultracentrifugation, density less than 1.063. The
final volume was 10.0 milliliters. Each solution contained one
per cent hydrogen peroxide. The results are indicated in table
XVII.

In another experiment, serum diluted one to eight with
Michalec buffer was treated with twelve per cent trichloroacetic
acid. The trichloroacetic acid precipitate from serum was col-
lected by centrifugation and was redissolved in Michalec buffer.
It was necessary to add 2 Normal sodium hydroxide, approximately
two milliliters, to maintain the pH of 8.6. This solution of the
serum proteins was then dialyzed for twenty hours (2°C) with
stirring against four changes of Michalec buffer, total volume
of 1.6 liters. The effect of this protein solution on the fading
of Sudan Black B bound to beta lipoproteins is illustrated in
figure 12 and the data recorded in table XVIII. Each solution
contained one per cent hydrogen peroxide in Michalec buffer and
had a final volume of 10.0 milliliters. The serum protein prepa-
ration contained 1/25 of the protein concentration in serum. The
final reaction mixture contained 0.50 ml. of the protein prepa-

TABLE XVII

EFFECT OF SERUM PROTEINS ON THE FADING OF SUDAN BLACK
B BOUND TO THE LOW DENSITY LIPOPROTEINS

Klett colorimeter readings

Time minutes	chylomicrons	Serum proteins and chylomicrons	beta lipo-proteins	Serum pro-teins and beta lipo-proteins
5	271	277	206	195
9	263	273	204	194
15	254	273	198	194
25	239	271	189	194
40	216	270	175	192
78	170	271	145	191
98	154	271	134	191
123	138	270	117	191
154	128	273	107	190
190	118	270	100	190
224	113	270	96	190
260	109	271	93	190

"serum proteins" signifies the fraction from the ultracentrifuge having a density greater than 1.21.

FIGURE 12

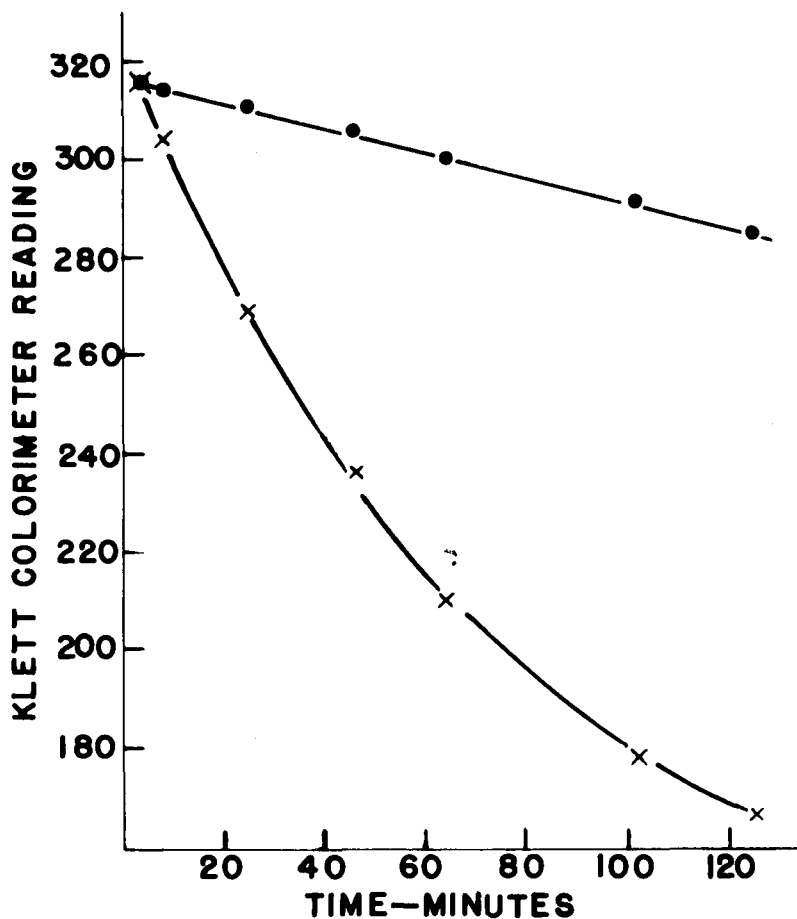THE EFFECT OF SERUM PROTEINS ON THE FADING OF SUDAN
BLACK B BOUND TO BETA LIPOPROTEINS

X - X is the Sudan Black B--beta lipoproteins, O-O contains the
Sudan Black B--beta lipoproteins and the serum proteins prepared
from the trichloroacetic acid precipitate.   The Sudan Black B
concentration is that resulting from the pre-staining of 0.50 ml.
of the beta lipoproteins from the ultracentrifugal preparation.

ration; the blank contained 0.50 ml. of the buffer used in the final dialysis. All the solutions contained 0.50 ml. of the ultracentrifugally prepared beta lipoproteins stained with Sudan Black B. From figure 13, it is apparent that the addition of the serum proteins prepared from a trichloroacetic acid precipitate of serum, reduces the rate of fading of Sudan Black B bound to beta lipoproteins. While this experiment offers no explanation as to the mechanism of the inhibition of the fading, it indicates that other serum proteins are most likely the factors which prevent the fading of Sudan Black B bound to low density lipoproteins and contained in unfractionated serum.

CHAPTER VI

DISCUSSION

Because of the dual nature of the data presented in this thesis, it was decided to divide the discussion into two separate portions. The first part will be a consideration of the paper chromatography of lipoproteins and the second part concerns the fading of Sudan Black B.

THE PAPER CHROMATOGRAPHY OF LIPOPROTEINS

Employing the technique of paper chromatography on paper pre-wetted with a buffer solution, two distinct lipoprotein fractions are resolved from human serum. The necessity of pre-wetting the paper introduces another variable into the technique and negates the possibility of reporting the chromatographic properties in terms of R_f values. Since no positive results were obtained in attempting to locate the solvent front on pre-wetted paper, further discussion on this point is unwarranted. The use of the R_b value results in a numerical representation of a chromatographic property. R_b values or similar quantities are ratios of the distance moved by a given substance divided by the distance moved by some reference substance, in this case bromphenol blue. R_b values can be satisfactorily substituted for R_f values in characterizing the chromatographic migration and separation of lipoproteins

The correlation between the lipoprotein fractions obtained by the paper chromatographic technique and those obtained from ultracentrifugal methods was fairly well established. Table XIX is a summary of the serum lipoprotein fractions obtained from different physico-chemical procedures.

On the paper chromatograms of the lipoproteins, the spot which moves farthest from the origin corresponds to the high density lipoproteins, the alpha lipoproteins. The alpha lipoproteins as prepared by ultracentrifugal procedures separates into at least two well defined spots. Resolution of these two alpha lipoproteins by the paper chromatography of serum was not found. Chromatographic development for considerably longer periods of time and at a lower temperature may result in the separation of the alpha lipoproteins from untreated serum.

The spot closest to the origin corresponds to the low density lipoproteins, beta lipoproteins and chylomicrons. Discrepancies in the R_b values of the ultracentrifugal fractions and the corresponding fraction on serum chromatograms may be attributed to the quantity of lipid or protein material applied to the paper. While no determination of either type of substance has been made, table VI may be taken as indicative of the lipid content of the various fractions.

The limited studies on the effect of alterations in the composition of the developing solvent indicate that resolution of the lipoproteins is due in a large part to their lipid comp-

TABLE XIX

HUMAN SERUM LIPOPROTEINS

ULTRACENTRIFUGE	IONOGRAPHY	PAPER CHROMATOGRAPHY
Density at 25°C		
(1) 0.94	"Origin lipid" but no	Chylomicrons, low den-
density fraction less	true distinction	sity lipoproteins
(2) 0.98 than 1.019	from (3)	(a,b)
(3) 1.03 density fraction		
1.019 to 1.063	beta-1 globulin	beta lipoproteins(a,b)
(4) 1.09	alpha-1 globulin	alpha lipoproteins,
density fraction		high density lipopro-
(5) 1.14 1.063 to 1.21	alpha-1 globulin	teins (c)

(a) The lipoproteins of density less than 1.063 are often referred
    to as low density lipoproteins; those of density greater than
    1.063 are often called high density lipoproteins

(b) No resolution of fractions 1 to 3 was obtained by paper chroma-
    tography; differences in R_b values were noted

(c) Density fraction 1.063 to 1.21 (fractions 4,5) were isolated
    as one fraction by ultracentrifugation. Paper chromatography
    of this fraction indicated two components. Similar resolution
    from untreated serum was not obtained.

osition . Thus changes in the pH or the ionic strength of the
buffer component of the developing solvent had little or no
effect on the resolution of the lipoproteins. These changes may
be expected to alter the nature of the protein moiety. On the
other hand, alterations in the isopropyl alcohol content resulted
in large changes in the distance moved by the lipoproteins. As
has been pointed out previously no real conclusions could be
made from these studies on the varying isopropyl alcohol content
of the developing solvent. Experiments on dry paper had indi-
cated sizeable changes in the distance the solvent front had
moved for the various alcohol concentrations. It seems feasible
that similar results would be obtained on pre-wetted paper.
Therefore, part of the differences noted in the distance moved
by the lipoproteins resulted from the changes in the rate of
developing solvent flow through the paper.

From the general appearance of the chromatograms result-
ing from various developing conditions, a composition of the
developing solution of isopropyl alcohol/buffer, 40/60, volume/
volume appeared to give optimal results. The use of a phosphate
buffer of about the same ionic strength and pH as the citrate-
oxalate-veronal buffer used by Michalec resulted in comparable
chromatograms.

Pre-staining of the serum gave essentially the same
type of chromatographic results as those obtained by the post-
staining procedure. The pre-staining technique eliminates the

rather cumbersome method of staining and rinsing the developed
chromatogram. Thd data presented in tables VIII nad IX indicates
that the pre-staining technique is more adaptable to quantita-
tive procedures such as densitometric scanning.

The paper chromatographic separation of serum lipopro-
teins has been compared to ionographic methods. Table XI indi-
cates that the beta lipoproteins to alpha lipoproteins ratio
determined by paper chromatography agrees well with the results
obtained by ionography. The paper chromatographic procedure has
the advantage of being simpler in form. One disadvantage to the
chromatographic method which has not been pointed out is the
change in the shape of the lipoprotein -- Sudan Black B spots as
they move down the paper. When such chromatograms are scanned,
only a parallel band of the Sudan Black B down the center of the
chromatographic strip is measured. The densitometric recording
is not completely representative of the total Sudan Black B con-
centration. It was not feasible to scan the entire width of the
paper strip with the instruments described in the densitometric
studies. The problem was partially circumvented by streaking the
migrant along the origin rather than simply applying it as one
spot. The difficulties encountered with the densitometric meas-
urement of chromatograms post-stained with Sudan Black B has been
discussed. Most likely, the problem results from the uneven
background color on the chromatograms.

The adaptation of centrifugally accelerated chromato-

graphy to the paper chromatographic procedure for the separation

of lipoproteins was only partially successful. The radial type

of development on the whole circular sheet, resulted in developed

spots of an arc-type shape. This prevented ordinary densitometric

scanning. Modification of the paper chromatographic disc, to

eliminate the arc-like development, was largely unsuccessful.

THE FADING OF SUDAN BLACK B

Data presented in Chapter V indicates that there is a

difference in the rate of fading of Sudan Black B bound to high

density and low density lipoproteins. This was observed initially

on paper chromatograms. Hydrogen peroxide at concentrations of

about one per cent accelerates the disappearance of Sudan Black B

color. The changes produced in Sudan Black B and in the various

lipoprotein fractions following hydrogen peroxide treatment has

been partially described by examining the light absorption spec-

tras. The fading of the Sudan Black B bound to the low density

lipoproteins occurs at a much faster rate than that of the stain

bound to high density lipoproteins. Similarly, the Sudan Black B

bound to low density lipoproteins fades faster than the dye it-

self. In order to examine the rate of fading of Sudan Black B

alone, it was necessary to perform the experiment in a solution

containing five per cent ethyl alcohol. The presence of the alco-

hol may have had an effect on the rate of the fading. At present

no explanation can be offered for the differences in the rates of

fading.   Sudan Black B bound to the lipoproteins contained in untreated serum does not fade under conditions similar to those which resulted in disappearance of Sudan Black B color when bound to the low density lipoproteins prepared ultracentrifugally. Furthermore, the addition of small quantities of a serum protein preparation "inhibits" the fading of Sudan Black B bound to low density lipoproteins prepared ultracentrifugally.

CHAPTER VII

SUMMARY

An improved paper chromatographic procedure for the separation and determination of human serum lipoproteins has been presented. The fractions obtained by paper chromatography were correlated with those obtained by ultracentrifugal techniques. Densitometric scanning was used to measure the concentration of Sudan Black B bound to the lipoproteins which were separated by conventional paper chromatography. Chromatograms of serum lipoproteins were also developed using centrifugally accelerated techniques. The results indicated that separations similar to those obtainable by conventional descending chromatography were possible. The radial type of development of the migrants in the centrifugally accelerated procedure made photometric scanning difficult. Possible modifications of the centrifugally accelerated procedure which would permit photometric scanning were introduced but were largely unsuccessful. Investigations along this line are being continued.

An investigation of the fading properties of Sudan Black B bound to different lipoprotein fractions has been initiated. The nature of these experiments was such as to indicate the differences in the rates of fading of the stain bound to the different lipoprotein fractions and contained in aqueous

hydrogen peroxide solutions. Serum protein preparations were
demonstrated to reduce the rate of fading of Sudan Black B
bound to low density lipoproteins. Further investigation will
be necessary to present a satisfactory explanation for this
phenomena.

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APPENDIX

TABLE VIII

RELATIONSHIP BETWEEN THE AREA UNDER THE DENSITOMETRIC RECORDING
AND THE APPLIED PRE-STAINED SERUM

Area under the curve-centimeters^2

Microliters of serum	Alpha lipo-proteins	Beta lipoproteins and chylomicrons	Total
10	5.7	12.1	17.8
20	10.8	19.3	30.1
30	15.0	27.1	42.1
40	20.2	35.4	55.6
50	27.8	37.8	65.6

TABLE IX

RELATIONSHIP BETWEEN THE AREA UNDER THE DENSITOMETRIC RECORDING
AND THE APPLIED SERUM--POST-STAINED CHROMATOGRAMS

Area under the curve-centimeters^2

Microliters of serum	Alpha lipo-proteins	Beta lipoproteins and chylomicrons	Total
10	5.6	19.9	25.5
20	8.6	43.7	52.3
30	14.8	44.4	59.2
40	14.9	37.2	52.1
50	16.6	52.9	69.5
60	23.8	54.0	77.8

TABLE XII

ABSORPTION SPECTRA OF SUDAN BLACK B (SB-B)--SERUM LIPOPROTEINS

Optical Density-Beckman DU Spectrophotometer

Wave length mu	SB-B	SB-B--alpha lipoproteins	SB-B--beta lipoproteins	SB-B--beta lipo-proteins treat-ed with 1% H_2O_2
300	0.075	0.535	0.655	0.811
320	0.083	0.469	0.569	0.636
340	0.076	0.369	0.452	0.498
360	0.055	0.356	0.433	0.473
380	0.046	0.316	0.389	0.491
400	0.045	0.331	0.389	0.469
420	0.044	0.336	0.381	0.419
440	0.042	0.325	0.373	0.389
460	0.049	0.309	0.364	0.374
480	0.043	0.292	0.346	0.362
500	0.041	0.298	0.340	0.348
520	0.041	0.327	0.362	0.330
540	0.046	0.376	0.401	0.313
560	0.040	0.432	0.454	0.297
580	0.044	0.499	0.512	0.274
600	0.043	0.533	0.558	0.251
620	0.047	0.533	0.560	0.214
640	0.035	0.475	0.521	0.177
660	0.031	0.391	0.444	0.143
680	0.027	0.274	0.314	0.103
700	0.023	0.171	0.194	0.071
720	0.016	0.099	0.113	0.050
740	0.013	0.052	0.068	0.037
760	0.008	0.025	0.032	0.026
780	0.007	0.016	0.024	0.023
800	0.006	0.011	0.006	0.018

TABLE XIII

ULTRA-VIOLET ABSORPTION SPECTRA OF SERUM LIPOPROTEINS

Optical density--Beckman DU Spectrophotometer

Wave length mu	alpha lipo-proteins	alpha lipo-proteins treated with 3% H_2O_2	beta lipo-proteins	beta lipo-proteins treated with 3% H_2O_2
240	1.942	1.950	1.855	2.070
245	1.036	1.061	1.463	1.633
250	0.738	0.769	1.043	1.169
255	0.652	0.683	0.707	0.890
260	0.723	0.751	0.582	0.751
265	0.845	0.875	0.559	0.711
270	0.999	1.008	0.571	0.691
275	1.118	1.129	0.585	0.658
280	1.149	1.156	0.550	0.611
285	1.054	1.053	0.504	0.539
290	0.765	0.766	0.418	0.443
295	0.408	0.415	0.316	0.348
300	0.208	0.220	0.250	0.280
305	0.126	0.138	0.211	0.237
310	0.091	0.102	0.188	0.203
315	0.079	0.088	0.169	0.181
320	0.073	0.079	0.157	0.163
325	0.063	0.073	0.148	0.148

TABLE XIV

THE RATE OF FADING OF SUDAN BLACK B BOUND
TO ALPHA AND BETA LIPOPROTEINS

Klett colorimeter readings

Time minutes	1% H$_2$O$_2$ Alpha lipoproteins	1% H$_2$O$_2$ beta lipoproteins	1% H$_2$O$_2$ serum
3	361	331	336
7	366	319	327
11	257	307	334
15	361	299	334
20	363	289	333
27	361	273	334
37	363	259	333
53	362	237	328
68	367	222	333
87	366	205	334
110	369	189	331
134	366	179	331
150	368	174	335
168	368	168	331
183	368	164	330
221	368	160	331

TABLE XVIII

THE EFFECT OF SERUM PROTEINS ON THE FADING OF SUDAN BLACK B BOUND TO BETA LIPOPROTEINS

Klett colorimeter readings

Time minutes	Beta lipoproteins	Serum proteins and beta lipoproteins
4	316	316
8	304	314
25	269	311
46	236	306
64	210	300
102	178	292
125	167	285

"serum proteins"--TCA precipitate of serum redissolved in Michalec buffer; dialyzed against the buffer.

<u>APPROVAL SHEET</u>

The thesis submitted by Leonard J. Banaszak has been read and approved by three members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

December 15, 1959
Date

Signature of Adviser