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A BIOCHEMICAL AND GENETIC STUDY OF TYPE III AND TYPE IV HYPERLIPOPROTEINEMIA

by

© ROBERT DAVID BAYNTON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "A BIOCHEMICAL AND GENETIC STUDY OF TYPE III AND TYPE IV HYPERLIPOPROTEINEMIA", submitted by Robert David Baynton in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

This research had three major objectives. First was the development of a means of determining plasma lipoproteins in a quantitative manner. The purpose here was to make possible the detection of abnormal levels in patients suspected of having coronary artery disease. Ability to measure lipoprotein levels was also necessary for genetic studies of affected families. Quantitative measurements of plasma lipoproteins were accomplished by agarose gel electrophoresis utilizing a correlation established between dye uptake by lipoprotein fractions and total lipid analysis of plasma.

Secondly, the inheritance pattern of three Types of primary hyperlipoproteinemias was investigated using agarose gel electrophoresis and serum lipid measurements as genetic markers. Three Type II hyperlipoproteinemic families, one consisting of fifty members over four generations were investigated. It was concluded that this disorder was transmitted in an autosomal dominant mode; however, a variable degree of penetrance of the mutant gene appeared within two of the three families. Only one Type III hyperlipoproteinic family was available for biochemical study and as the propositus was the sole individual exhibiting Type III hyperlipoproteinemia, no conclusion as to the mode of heritance could be made.

Six families with Type IV hyperlipoproteinemia were also investigated. In each family only the propositus

exhibited the characteristic Type IV lipid pattern. These results suggested an autosomal recessive mode of inheritance, although the possibility of a dominant mutant gene of incomplete penetrance could not be ruled out.

Of all the family members investigated in the inheritance studies, only one Type of hyperlipoproteinemia was detected within each family and thus the possibility of detecting Types II and IV, Types II and III, or Type III and Type IV within a family was not realized.

The third and most extensive aspect of this investigation was a biochemical and physical investigation of the very low density lipoproteins (VLDL) of a Type III and a Type IV individual. These studies of native VLDL, the apolipoproteins and respective polypeptide composition yielded the following new information:

1. A protein component, SF1, of Type III VLDL contains at least four polypeptides not found in the SF1 component of the corresponding Type IV apolipoprotein.

2. When compared to normal, Type III apo VLDL contain a greater amount of SF1 component as well as having an extra fraction eluting as a shoulder after gel chromatography of delipidated very low density lipoproteins. As SF1 has a great affinity for cholesterol and migrates as a beta apolipoprotein by acrylamide gel electrophoresis, this may explain the high cholesterol content and beta-migrating characteristics of Type III very low density lipoproteins.

3. The VLDL of Type III and Type IV differ in

electrophoretic migration properties in agarose gel in that Type III VLDL migrates as a "broad-beta" lipoprotein. In acrylamide gel, the VLDL in both Types possess similar electrophoretic characteristics.

The conclusions are:

1. The different peptide composition (increased SF1) of Type III VLDL explains the beta-migrating, cholesterol rich nature of Type III very low density lipoproteins.

2. Type III VLDL is more complex than normal VLDL due to the presence of previously unreported peptides. These findings may be related to the severity of peripheral vascular disease in Type III patients.

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I. INTRODUCTION

Knowledge and understanding of lipoproteins has grown immensely since the concept of a specific lipid-carrying protein was introduced by Nerking in 1901 (1). Application of chemical, ultracentrifugal, and electrophoretic techniques in the 1950's with subsequent refinements of these methods has aided the biochemist and clinician in investigating the biochemical and metabolic properties of lipoproteins in health and disease.

The clinician's interest stems from the implication that serum lipids are the main blood constituents responsible for atherosclerosis (2). The later discovery that serum lipids are transported by distinct proteins, the complex being termed a "lipoprotein", also generated the clinician's interest in studying lipoproteins.

Of current interest is the finding that the primary dyslipoproteinemias are due to inheritable defects in lipid or lipoprotein metabolism (3-7). The hyperlipoproteinemias are of particular interest because of the accompanying high incidence of coronary artery disease. Detection and treatment has been aided by the classification system set out by Fredrickson and Lees in 1965 (8). Five different hyperlipoproteinemic states are defined on the basis of the electrophoretic pattern of serum lipoproteins observed in a twelve to sixteen hour fasting blood specimen. Classification assists the clinician in treating the patient because the mode of treatment differs with the Type observed.

In the past five years, a greater understanding of lipoprotein biochemistry has resulted from investigations of the protein components of lipoproteins--the apolipoproteins. Three apolipoproteins, designated as A, B, and C have been isolated from human plasma lipoproteins. Apolipoprotein A is characteristic of alpha (α) lipoproteins, apolipoprotein B characteristic of beta (β) lipoproteins, and apolipoprotein C is characteristic of pre-beta lipoproteins. Pre-beta lipoproteins also contain a significant amount of apolipoprotein B and some of apolipoprotein A.

Shore and Shore (9) have demonstrated that apolipoprote A consists of two different polypeptides. As identified by their carboxyl terminal amino acid residues, these are apolipoprotein-Glutamine (apoLp-Gln) and apolipoprotein-Threonine (apoLp-Threo). Recently, they have also reported minor amounts of apolipoprotein-Alanine (apoLp-Ala) present in the A protein (10).

At present, it is generally agreed that apolipoprotein B consists mainly of apolipoprotein-Serine (apoLp-Ser) (11). Pre-β lipoproteins have been shown to contain apolipoprotein A, apolipoprotein B, and apolipoprotein C. The C protein, isolated as a phospholipid complex, was shown to possess two N-terminal amino acids, threonine and serine (12). Recently, Fredrickson, et al (13, 14) were able to completely remove phospholipid from the C protein and subsequently demonstrate the presence of three polypeptides, apolipoprotein-Valine (apoLp-Val), apolipoprotein-Glutamic acid (apoLp-Glu), and

apoLp-Alanine. The amino terminal residue of both apoLp-Glu and apoLp-Val is threonine, whereas apoLp-Ala is serine.

It is not surprising that research workers then turned their attention to the status of the polypeptides found in the apolipoprotein in various hyperlipoproteinemic states. The detection of differing polypeptides might reveal the biochemical defect in the disease state, however preliminary reports have not indicated this, at least in the more commonly occuring Type II and Type IV conditions.

In view of these recent advances in knowledge, an investigation of the inheritance patterns of some hyperlipoproteinemias using a quantitative lipoprotein electrophoretic method with established normal range values of the plasma lipoproteins was carried out. In particular, due to the enigma that surrounds the "floating broad-beta lipoprotein", characteristic of Type III hyperlipoproteinemia, it was proposed that a reexamination of the inheritance pattern of Type III hyperlipoproteinemia using agarose gel electrophoresis would be of value. Of special interest was the possibility of Type III and Type IV individuals being detected within one family, as reported by Matthews (15) and possibly detecting a family containing Types II and IV, or Types II and III individuals.

Also it was hoped that the genetic studies would reveal suitable Type III individuals such that the "broad-β" lipoprotein could be further investigated with respect to

its apolipoprotein composition. This has not been done, and such a study might provide an explanation of the peculiar physical properties characteristic of "broad-β" lipoprotein.

II. LITERATURE REVIEW

A. Human Serum Lipoproteins

Although the presence of protein-bound lipids in plasma or serum was observed as early as 1901 (1), it was not until 1929 that separation of a lipoprotein from serum was accomplished (16). The complex isolated by Macheboeuf was obtained from horse serum by salt precipitation and was later shown to be an alpha globulin (17). In 1951, Blix, Tselius, and Svensson (18) showed that large amounts of lipid were associated with alpha and beta globulin fractions which had been electrophoretically separated from normal human plasma. In 1943, Adair and Adair (19) isolated a lipoprotein by salt precipitation and demonstrated the complex to be a beta globulin by electrophoresis. This globulin appeared to have a density of 1.10 grams/milliliter.

The "X-protein" of plasma described by McFarlane (20) in 1935 was later shown by Pedersen (21) to be a beta globulin having a density of 1.030 to 1.035 grams/milliliter. In 1946, Cohn, et al (22) were able to demonstrate two distinct types of lipoproteins by precipitation of plasma lipoproteins using different ethanol-water mixtures. These lipoproteins differed in solubility in water and ethanol-water mixtures, lipid content, molecular size and shape, and electrical charge. Russ, Eder, and Barr (23) verified that essentially all the cholesterol and phospholipid of plasma is combined with protein in the form of alpha and beta lipoproteins. Approximately the same amount of phospholipid

present in each of the two fractions, whereas most of the cholesterol was bound to the β globulin.

Analytical ultracentrifugal work of Gofman and co-workers in 1949 (24) offered a new approach to characterizing the lipoproteins. They showed that the "X protein" of McFarlane (20) could be separated from albumin if an appropriate adjustment of the sample density was made. In a subsequent paper (25), Gofman, et al found β lipoproteins to contain two classes of molecules. These lipoprotein molecules had flotation coefficients between 3-8 and 10-20 when ultracentrifuged in a medium of density 1.063 g/ml at 27° Centigrade. They also reported the frequent occurrence of low density lipoproteins (LDL) which had flotation coefficients (S_f)* between 40 and 70 and serum components of flotation rates greater than S_f 70. Green, et al (26) suggested the use of a medium of density 1.21 g/ml and thus were able to obtain flotation coefficients for the α lipoproteins. Employing the 1.21 g/ml medium, Lindgren, et al (27) showed heterogeneity with the α lipoprotein fraction, isolating three classes of molecules with hydrated densities of 1.05, 1.075, and 1.145 grams/milliliter. Modern day terminology refers to these α lipoproteins as α_1, α_2, and α_3 lipoproteins.

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*S_f units describe the flotation rate of lipoproteins in a sodium chloride solution of density 1.063 g/ml at 26° Centigrade. They are referred to as Svedberg units of flotation where 1 unit equals 10^{-13} cm/sec/dyne/gram.

B. The Development of Electrophoretic and Ultracentrifugal Methods for Characterizing and Isolating Lipoproteins

Many methods are available for investigating serum lipoproteins. Precipitation, chrmatographic, immunochemical, and membrane filtration techniques have been used; however, the application of electrophoretic and ultracentrifugal techniques have dominated lipoprotein research.

The use of electrophoresis for separating lipoproteins was introduced by Blix, Tiselius, and Svensson in 1941 (18). They noted that the largest amounts of cholesterol and phospholipid were associated with the alpha and beta globulins. Few studies on the electrophoretic properties of the lipoproteins were reported during the next ten years. This was probably due to the fact that is was very difficult to determine exactly where the lipoproteins migrated when patterns of whole serum were obtained by the classical Tiselius method of "free" electrophoresis. Kunkel and Slater (28) introduced a new technique of zone electrophoresis for separating serum lipoproteins. This method possessed the advantage of electrophoretic components being isolated directly so that lipid analysis could be carried out. Their results indicated that lipoprotein patterns could be obtained on whole serum and could be correlated in terms of relative elctrophoretic mobility with the other serum proteins. Of interest is their obsercation that pathological sera with high lipid concentrations showed many variations of lipoprotein patterns. Besides detecting the

usual β and α lipoproteins, they noticed a "trail left behind as the β component migrated from the origin" and that this "vague peak contained considerably more neutral fat than the β or α peaks". Also of interest was the occasional detection of a lipid stainable component in the region between the α and β lipoprotein peaks.

In 1955, Jencks and Durram (29) described a paper electrophoretic technique capable of separating lipoproteins from 0.20 ml of serum. More importantly, the staining properties of the lipoprotein fractions were studied in detail and by adjusting the staining and destaining conditions they were able to obtain a semi-quantitative estimation of the amount of lipoprotein fraction present in serum. Dangerfield and Smith (30) in an investigation of serum lipids and lipoproteins employing paper electrophoresis noted a "pre-β lipid" band which migrated faster than the β lipoprotein band. This "pre-β lipid" band was observed to be a prominant feature of the nephrotic syndrome and also occurred frequently in coronary thrombosis. Dangerfield and Smith suggested that the "pre-β lipid" band might correspond to the α globulin band observed by Kunkel and Slater (28). It is also interesting to note that in three cases of hyperlipemia, cholesterol was distributed more or less uniformly throughout the β zone, with slight staining at the origin. This may have been the first report of what was later described as a "broad-β" lipoprotein.

An important advance in lipoprotein methodology was

made by Gofman and his associates when they demonstrated that flotation values of serum lipoproteins were best measured by centrifugation at a density of 1.063 g/ml instead of sedimenting the lipoprotein concentrate at lower densities in the analytical ultracentrifuge (24,25).

The ultracentrifugal method of Lindgren, Elliott, and Gofman (27) achieves separation of lipoproteins with densities less than 1.006 g/ml by differential rate separation of serum at that density. Two main fractions are obtained: S_f 70-10,000 lipoproteins and S_f 17-70 lipoproteins.

(1) S_f 70-10,000. This fraction included the chylomicrons and some aggregates of much smaller dimensions than the chylomicrons.

(2) S_f 17-70. This fraction was then subfractionated by differential rate separation into S_f 20-30 and S_f 30-70 lipoprotein species.

When a two-stage differential density preparative isolation was performed on the S_f 17-10,000 infranatant, two additional lipoprotein fractions were obtained:

(1) S_f 13 lipoproteins resulted from an adjustment of the S_f 17-10,000 infranatant to a density of 1.019 g/ml with appropriate ultracentrifugation.

(2) S_f less than 13 lipoproteins which were contained in the S_f 13 infranatant. These could be further separated by differential density ultracentrifugation.

In 1952, Lewis, Green, and Page (31) proposed the

classification of lipoproteins by ultracentrifugation at a density of 1.21 grams/milliliter. This method enabled separation and estimation of α as well as β lipoproteins. Since the S_f terminology of Gofman had been based on a solvent density of 1.063 g/ml, the flotation rates of α and β lipoproteins were represented by $-S_{1.21}$ to indicate the density employed and the flotation rate in terms of negative Svedberg units. A comparison of the fractionation obtained by the Gofman and Lewis systems is illustrated in Table I.

In 1955, Havel, Eder, and Bragdon (32) proposed a method for lipoprotein fractionation by repeated ultracentrifugations after progressively raising the solvent density. They were able to obtain three fractions: one of densities less than 1.019 g/ml, one between densities 1.019-1.063 g/ml, and a third of densities greater than 1.063 grams/milliliter. These three fractions corresponded roughly to the Gofman S_f 12-400, the Gofman 0-12, and the Gofman less than 0 classes of lipoproteins.

Development of preparative ultracentrifugal techniques lent itself to isolating quantities of the lipoproteins suitable for chemical analysis. In a study of the chemical composition of lipoprotein fractions, Lindgren and co-workers (33) demonstrated an increasing glyceride content as the densities of the lipoproteins decreased. Bragdon, Havel, and Boyle (34) showed that all lipoprotein fractions contained free and esterified cholesterol, lipid phosphorus, glyceride, and protein nitrogen. However, the constituents

TABLE I

RELATIONSHIP BETWEEN LIPOPROTEINS ISOLATED BY THE LEWIS AND GOFMAN SYSTEMS OF ULTRACENTRIFUGATION

	Lewis	Gofman
Density	1.21 g/ml	1.063 g/ml
Symbol	$-S_{1.21}$	S_f
Comparative Fractions	70	20-100 plus
	40-70	10-20
	25-35	3-8
	20-25	1-3
	22-8	--

of the lipoproteins varied in proportion from one class to another as seen in Table II.

Due to the simplicity of electrophoretic methods for separating lipoproteins, such methods have become widely used. This is probably due to the practicality of the method for the clinical laboratory. However, the relationship between the electrophoretically obtained lipoprotein fractions and those isolated by ultracentrifugation was not clear.

Lewis and Page (35) prepared a total lipoprotein concentrate by preparative ultracentrifugation and compared its behaviour in the analytical ultracentrifuge and in the Tiselius electrophoretic apparatus. Isolation of lipoprotein fractions for direct comparison of flotation values and electrophoretic mobilities, however, was not accomplished. In 1957, Smith (36) attempted to relate the lipoprotein fractions isolated by both procedures. Using the preparative ultracentrifuge, he separated the low density and very low density lipoproteins into five groups by utilizing varying combinations of density, centrifugal force, and time. These fractions were further characterized by analytical ultracentrifugation and the relationship to paper electrophoretic patterns was established as shown in Table III. This work also revealed the following new information:

(1) In paper electrophoresis, the pre-β lipid is a separate kind of lipoprotein and is not a result of overloading the paper with β lipoprotein.

TABLE II

CHEMICAL COMPOSITION OF HUMAN SERUM LIPOPROTEINS
ISOLATED BY ULTRACENTRIFUGATION (34)

Ultracentrifuge Nomenclature	Electrophoretic Nomenclature	Protein (% weight)	Cholesterol (% weight)	Glycerides (% weight)	Phospholipids (% weight)
S_f 400	chylomicrons	2	8	81	9
S_f 20-400	pre-β lipoprotein	7	22	52	18
S_f 0-20	β lipoprotein	21	47	9	23
S_f 0	α lipoprotein	47	18	8	27

TABLE III

RELATIONSHIP BETWEEN ULTRACENTRIFUGAL AND PAPER ELECTROPHORETICALLY SEPARATED S_f 0-400 LIPOPROTEINS (36)

Smith Fraction	Paper Electrophoretic Component	Ultracentrifugal Component	Average Ultracentrifugal Peak Value
1	Deposit on origin	S_f 400 plus	not visible
2	Trailing lipids (origin to β)	S_f 50-400	S_f 74.0
3	"Pre-β lipid" with trail to origin	S_f 20-100	S_f 28.5
4	"Fast" β lipoprotein	S_f 10-30	S_f 14.0
5	β lipoprotein	S_f 0-10	S_f 6.0

(2) The "trail" extending from the point of sample application to the β lipoprotein position does not consist of adsorbed β lipoprotein but is mainly lipoproteins of very light density, S_f 100-400.

Lees and Hatch (37) demonstrated a sharper delineation of the major lipoprotein components and a partial resolution of the very low density lipoproteins. This significant improvement in paper electrophoretic separation of plasma lipoproteins was achieved by using a buffer system containing human serum albumin.

The interest in lipoprotein fractionation by electrophoresis was further generated when Bierman, et al (38) demonstrated that plasma chylomicrons were composed of exogenous fat particles whereas the S_f 20-400 lipoproteins were composed of endogenous fat particles. Also of interest was the finding by Ahrens and colleagues that the endogenous source of S_f 20-400 lipoproteins were carbohydrate-inducible.

The findings by Bierman, et al (38) and Ahrens, et al (39) led Lees and Fredrickson to reexamine their above-mentioned paper electrophoretic method. They demonstrated that paper electrophoresis in buffer containing albumin was capable of separating chylomicrons from the very low density lipoproteins. They showed that chylomicrons (exogenous glycerides) remained at the sample application point and corresponded to both primary and secondary particles as defined by starch block electrophoresis and polyvinylpyrrolidine precipitation. Very

low density lipoproteins (endogenous glycerides) migrated to the pre-beta position on paper (40).

The time consuming procedure and poor resolution of paper electrophoresis has discouraged its widespread use. This has prompted the evaluation of electrophoresis on other media such a polyacrylamide, cellulose acetate, and agarose.

Narayan, Narayan, and Kummerow (41) introduced polyacrylamide gel disc electrophoresis as a means of separating serum and plasma lipoproteins. It demonstrated better resolution of low and very low density lipoproteins, plus the finding of more lipoprotein bands. Although the advantages of disc gel electrophoresis are several (42), the additional lipoprotein bands occasionally observed may introduce difficulties in phenotyping the hyperlipoproteinemias. A more complete evaluation of disc electrophoresis in terms of ultracentrifugal characterization of the lipoprotein bands and clinical interpretation of electropherograms is necessary.

The use of cellulose acetate as an electrophoretic medium for separating lipoproteins was first introduced by Scherr (43) in 1961. Little development of this method occurred until 1967 when Charman and Landowne (44) outlined a method by which chylomicrons, beta, pre-beta and alpha lipoproteins could be satisfactorily resolved on this medium. Characterization of cellulose acetate electrophoretic resolution of lipoprotein fractions in terms of those obtained by paper electrophoresis and preparative ultracentrifugation was

achieved by Chin and Blankenhorn (45).

Agarose gel has been shown to serve as an excellent medium for electrophoresis of lipoproteins (46-51). An extensive study of the method by Irwin (52) has shown that:

(1) A 0.5 percent agarose gel is optimum in that clear resolution of β and pre-β lipoproteins is obtained. Chylomicrons are clearly resolved from pre-β lipoproteins in that chylomicrons do not enter the gel matrix and are thus retained in the sample application slot.

(2) Pre-β lipoproteins electrophoretically migrate as an α_2 globulin, as in starch block electrophoresis, and not as a β globulin as in paper electrophoresis.

(3) The S_f 20-400 lipoproteins are all present as a distinct pre-β lipoprotein band. This is contrary to paper electrophoresis in which the S_f 100-4,000 lipoproteins present as a smear from the pre-β position back to the origin (towards the cathode).

(4) Lipoprotein staining is accomplished in 0.5 hr using Sudan Black B dye. Oil Red O staining (used in paper and cellulose acetate electrophoresis) requires up to 12 hours.

(5) The clear background of the agarose gel electropherogram facilitates semi-quantitation of the lipoprotein fractions by densitometric scanning.

Irwin's work has been confirmed by others (51,53), and it is obvious that agarose gel electrophoresis offers many advantages to the modern clinical laboratory because of

reproducibility and excellent resolution of lipoprotein fractions.

C. Relationship of Lipids and Lipoproteins to Atherosclerosis

The suggestion by Gofman and co-workers in 1950 (25) that changes in plasma lipoproteins are implicated in atherosclerosis increased the popularity of lipid and lipoprotein studies. Dangerfield and Smith (30), Lewis and Page (35), and Smith (36) also suggested the involvement of one or more elevated lipoprotein fractions in coronary artery disease.

The best evidence for the involvement of lipids in atherosclerosis has resulted from extensive epidemiological studies. One of the more notable of these is the Framingham Study by Kannel, et al (54). They conclude that hypercholesterolemia precedes the development of atherosclerosis because cholesterol levels over 245 mg/100 ml were associated with a three-fold increase in the risk of development of the disease. A study by Chapman (55) and also by Keys (56) concludes that the highest incidence of myocardial infarctions occurs in individuals with the highest cholesterol levels.

More recently, serum triglyceride levels have also been implicated. Albrink and Man (57) were the first to associate elevated serum triglycerides with coronary artery disease. They found that serum triglycerides above 160 mg/100 ml were present in 85 of 100 coronary artery diseased patients between 20 and 78 years of age. This was compared to the finding that only five percent of young non-affected

males between 20 and 30 years of age and thirty percent of males of 50 years of age displayed triglyceride levels over 160 milligrams/100 milliliters. In the same group of patients, only eighteen percent exhibited abnormal serum cholesterol levels. Subsequent studies by Carlson (58) and Albrink (59) stressed the important role of triglycerides in the development of atherosclerosis.

An investigation of blood lipids and lipoproteins by Havel and Carlson (60) concluded that serum triglyceride levels correlated more closely than cholesterol as an indicator of atherosclerosis, however, neither lipid measurement alone provided adequate correlation.

Certainly the overwhelming accumulation of evidence in the study of atherosclerosis would indicate that no single cause of the disease can be designated. However, it remains that most of these studies have indicated serum lipids to play a key role in the genesis and perpetuation of the disease.

In an attempt to clarify the variety of hyperlipoproteinemic conditions observed and their clinical and biochemical characteristics, Fredrickson and Lees set out a system for classifying these disease states. The system involves five basic phenotypes of hyperlipoproteinemias as defined by paper electrophoresis (5-8). Phenotyping of hyperlipoproteinemic patients has proved advantageous in regards to the severity of atherosclerotic involvement, mode of treatment and the inheritance pattern of the conditions.

The different phenotypes, as distinguished by paper electrophoresis, are illustrated in Figure I and outlined below.

Type I: This exogenous lipemia is characterized by the presence of chylomicrons (S_f>400) in the plasma fourteen to sixteen hours after the last meal of a normal diet. Fasting plasma samples are creamy, the cholesterol is elevated, and triglycerides levels usually exceed 1,000 mg/ 100 milliliters. It is thought to be caused by a deficiency in post-heparin lipolytic activity as a result of a deficiency in a serum enzyme lipoprotein lipase. The occurrence of Type I is rare and is believed to be inherited as a simple recessive trait. Clinical features of the disease include eruptive xanthomata, hepatosplenomegaly, lipemia retinalis, abdominal pain, and occasionally pancreatitis.

Type II: This syndrome is characterized by an increased plasma concentration of β lipoprotein (S_f 0-20) and thus elevated levels of cholesterol. The plasma sample is clear due to the solubility of β lipoprotein in plasma. This disorder is inherited as an autosomal dominant characteristic and clinical features include tendonous and tuberous xanthomata. It is associated with severe and premature atherosclerosis.

Type III: In Type III, a peculiar type of lipoprotein exists. It possesses the electrophoretic migration characteristics of a β lipoprotein, yet the flotation characteristics of a pre-β lipoprotein. This unusual

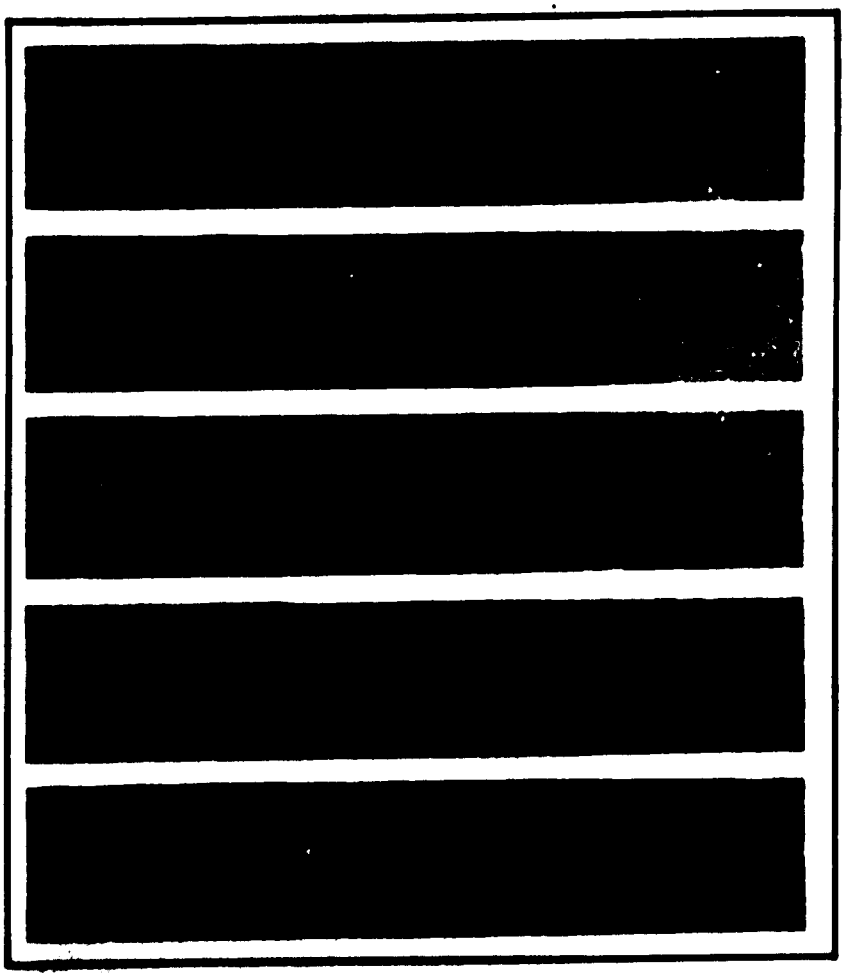

FIGURE I. Paper electropherograms showing the five Types of plasma lipoprotein patterns as described by Fredrickson (3-7).

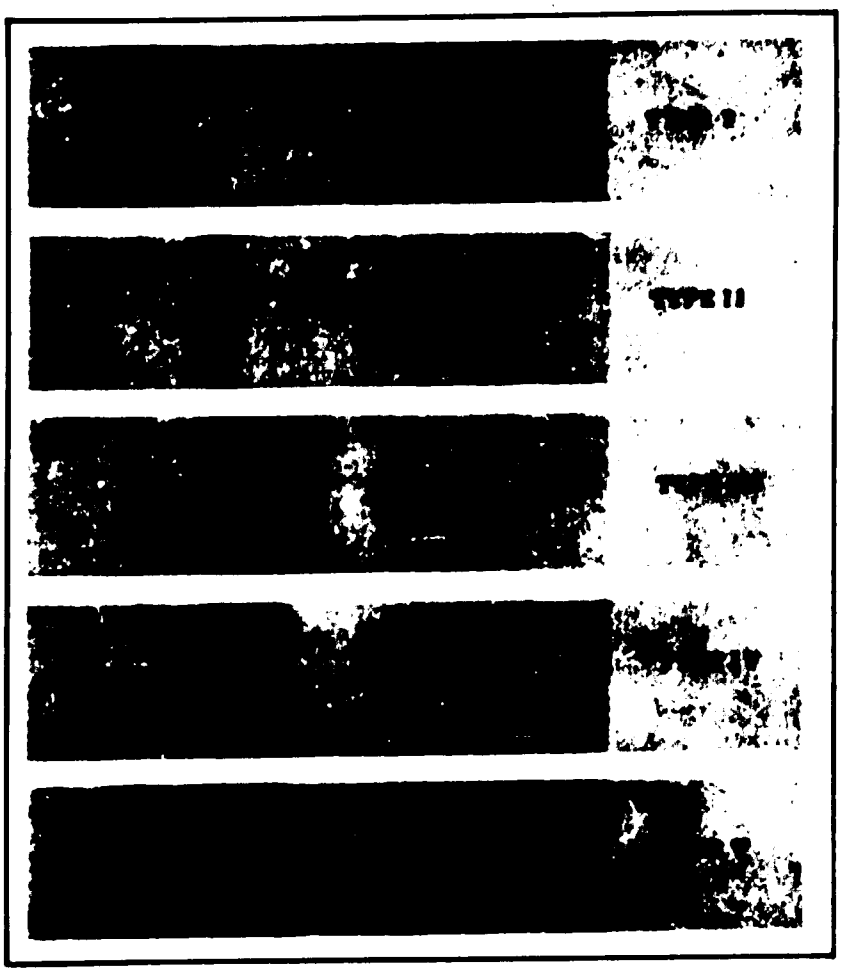

FIGURE I. Paper electropherograms showing the five Types of plasma lipoprotein patterns as described by Fredrickson (3-7).

lipoprotein is often referred to as "broad-β" lipoprotein because its electrophoretic band, although β-migrating, smears towards the pre-β fraction. The plasma may be clear or turbid and contains elevated cholesterol and triglyceride levels. Clinical features include palmar xanthomata in addition to xanthomatosis and severe premature atherosclerosis. The disorder is relatively uncommon and thought to be inherited as an autosomal recessive trait. Conclusive diagnosis of Type III requires preparative ultracentrifugation combined with lipoprotein electrophoresis.

Type IV: This syndrome is perhaps the most common abnormality of lipoproteins in the American population. Elevated levels of triglycerides which are endogenous in nature are characteristic of the disorder. Thus, elevated pre-β lipoprotein levels are noted. Serum cholesterol levels are normal or slightly elevated. Clinically, the patients exhibit premature atherosclerosis, xanthomatosis, lipemia retinalis, and hepatosplenomegaly. Most patients have some abnormalities of glucose tolerance and their triglyceride levels are markedly carbohydrate-induced. The inheritance pattern appears to be one of autosomal recessive nature.

Type V: The Type V condition is associated with the presence of both exogenous and endogenous glycerides in the plasma, with increased levels of serum cholesterol. The plasma specimen is usually turbid or creamy. Paper electrophoresis reveals the presence of chylomicrons and increased

pre-β lipoprotein levels. The inherited form is rare and thought to be transmitted as an autosomal recessive trait. Clinical features are eruptive xanthomatosis, lipemia retinalis, and glucose intolerance.

An important aspect of phenotyping hyperlipoproteinemias is to recognize whether the disorder is primary (familial) or secondary in nature. Disorders such as diabetes*, nephrotic syndrome, hypothyroidism, obstructive hepatic disease, pancreatitis, glycogen storage disease, and alcoholism may give rise to secondary hyperlipoproteinemias in a variety of electrophoretic patterns.

D. The Characterization of the Protein Moiety of the Serum Lipoprotein

As mentioned previously, the plasma lipoproteins consist of three major protein moieties designated as apolipoproteins A, B, and C. Recent studies of the polypeptide composition of the apolipoproteins have provided information as to the metabolic and biochemical relationships between the different plasma lipoproteins.

1. Alpha apolipoprotein

The high density lipoprotein class (HDL) which floats at a solvent density between 1.063 and 1.21 g/ml was shown by Delalla and Gofman (61) to consist of two major subfractions called α_2 and α_3 lipoprotein. Shore (11) demonstrated that the α_2 lipoprotein contained two amino-terminal-aspartic acids: carboxyl terminal-threonine peptide chains, each of approximate molecular weight 95,000. The α_3 lipoprotein

* diabetes mellitus

was shown to contain a single amino terminal-aspartic acid; carboxyl terminal-threonine chain of approximate molecular weight 95,000. Homogeneity of the high density lipoprotein class in terms of protein moiety was also indicated by immunochemical studies (62). Based on the above studies combined with additional ultracentrifugal and isotope labelling experiments, Scanu and Hughes (63) postulated that the human serum alpha lipoproteins represent a single family of lipoproteins with identical protein components able to carry various amounts of lipids.

Although the intact alpha$_3$ lipoprotein had not been dissociated into subunits, apparently the protein moiety is comprised of subunits whose dissociation in the absence of lipid is favoured by the presence of detergent. After removal of lipids, the protein moiety in aqueous solution was found by Scanu (62) to have a molecular weight of 75,000 and by Shore and Shore (64) to be comprised of a major and a minor component. On addition of sodium dodecyl sulfate to the protein solution, subunits of molecular weight 36,000 (64) and 21,500 (65) were found by sedimentation equilibrium. In a subsequent study of the protein moiety of alpha$_3$ lipoprotein, Shore (66) stated a corrected molecular weight of 30,000 for the protein and showed the protein moiety to be homogeneous. Shore (9) later developed an 8M urea solvent system for polyacrylamide gel disc electrophoresis and diethylaminoethyl cellulose chromatography (DEAE) and demonstrated heterogeneity in the protein moiety

α_2 and α_3 lipoproteins. Total and terminal amino acid analysis revealed two major polypeptides designated as apoLp-Glu and apoLp-Threonine. Molecular weight measurements by sedimentation equilibrium ultracentrifugation in the presence of 8M urea and by total amino acid composition resulted in molecular weights of approximately 15,000 for both polypeptides. The strong tendency for the polypeptides to associate as dimers explained previous molecular weight measurements of 30,000 (67).

Of recent interest is the finding of variable amounts of apoLp-Ala in α lipoprotein (9,10). La Rosa, et al (68) have shown this polypeptide to be identical and metabolically related to the apoLp-Ala found in pre-β lipoproteins. The polypeptide appeared to shift from pre-β lipoprotein to α-lipoprotein during heparin-induced lipolysis.

2. Beta apolipoprotein

Early studies of the low density lipoprotein class revealed the apolipoprotein to contain an amino-terminal glutamic acid residue (69). Shore (11) confirmed the presence of the amino-terminal glutamic acid residue and additionally demonstrated the carboxyl-terminal amino acid to be serine. From the total amino acid and end group analysis, Shore concluded that two identical polypeptides, of molecular weight 380,000, made up the β apolipoprotein.

The first chemical evidence for possible heterogeneity of β apolipoprotein was reported by Rodbell (70), who demonstrated the presence of three amino-terminal residues--

glutamic acid, threonine, and serine. The latter two were present in small but significant amounts. Further support came from Bobbitt and Levy (71) who not only confirmed Rodbell's findings but added one more possible amino-terminal amino acid--aspartic acid.

The problem of clearly elucidating the polypeptide composition of the β apolipoprotein is two-fold:

(1) Ultracentrifugal isolation of the low density lipoproteins presumably results in a preparation free of very low density and high density (particularly α_1) lipoproteins which are found on either side of the low density lipoprotein density spectrum. Contamination could be a problem and in fact would explain the presence of trace amounts of amino-terminal aspartic acid (from HDL) and amino-terminal serine and threonine (from VLDL). This problem may be overcome by narrowing the density range over which the β lipoprotein is ultracentrifugally isolated. If one assumes homogeneity of the β lipoproteins with respect to polypeptide composition, then this approach would be valid.

(2) In order to effectively study the polypeptide composition, one must be able to completely delipidate the β lipoprotein and obtain the resulting apoprotein in an aqueous soluble form. In contrast to HDL, delipidation of native β lipoprotein by solvent extraction yields a protein product virtually insoluble in neutral aqueous buffers.

Several different approaches have been used to obtain a lipid-free, soluble protein. Granda and Scanu (72)

delipidated β lipoprotein by an ethanol-ether or ether extraction in the presence of 0.2M sodium dodecyl sulfate. The resulting product is soluble in alkaline aqueous buffers. Gotto, et al (73) found that a preliminary succinylation of the lysine amino acid residues of β lipoprotein enabled complete solubilization of the ethanol-ether extracted apoprotein when sodium decyl sulfate was present in the buffer. Although the succinylation step was first proposed by Scanu, et al (74), the use of sodium decyl sulfate instead of sodium dodecyl sulfate enabled excess detergent to be dialyzed from the protein solution. Gotto and colleagues suggested that excess detergent could have profound effects on the β apoprotein structure. Gotto, et al (75) were later successful in achieving solubilization of unmodified β apoprotein in a sodium decyl sulfate solution. Shore and Shore (76) found that reduction and alkylation of the lipid-free protein rendered a protein that was soluble in an aqueous buffer containing sodium dodecyl sulfate.

Application of the above delipidation methods has resulted in detection of subunit heterogeneity of β lipoprotein. Gel filtration chromatography (77,78) and ion-exchange chromatography (10) of modified β apoprotein have revealed at least two polypeptides which differ in molecular weight and amino acid composition. Whether or not chemical modifications produced these polypeptides is not yet known and it is still not clear if apoLDL consists of more than one type of polypeptide.

3. Pre-beta apolipoprotein

As with β apolipoprotein, early studies of pre-β apolipoprotein emphasized end group amino acid analysis. In addition to amino-terminal glutamic acid found in β apolipoprotein, several investigators demonstrated significant amounts of aspartic acid, serine, and threonine (11,70,79). Shore (11) reported the presence of two carboxyl-terminal amino acids, alanine, and serine.

The problem of obtaining a soluble, delipidated pre-β-apoprotein hampered investigations of the probable heterogeneity of this protein. In 1964, Gustafson (80) reported that partial delipidation of pre-β lipoprotein resulted in two phospholipid-protein complexes that were soluble in phosphate buffer. In a later report (81) the separation of partially delipidated pre-β lipoproteins by Pevikon zone electrophoresis and subsequent ultracentrifugation revealed three distinct phospholipid-protein residues:

(1) Amino-terminal glutamic acid, shown to be identical to the main polypeptide of β apolipoprotein.

(2) Amino-terminal aspartic acid, which was identical to one of the polypeptides found in α apolipoprotein.

(3) Amino-terminal serine and threonine. This phospholipid-protein residue differed from both α and β apolipoproteins and was designated as apolipoprotein C.

In 1969, Brown, et al (13) obtained an aqueous soluble lipid-free protein from pre-β lipoproteins. This was accomplished by an ethanol-ether (3:1) extraction of

lyophilized pre-β lipoproteins, whereupon the protein was completely soluble in an aqueous buffer containing 0.1M sodium decyl sulfate. Three fractions were obtained when this protein preparation was subjected to gel filtration. Two of these corresponded to the proteins found in α and β lipoproteins, whereas the third fraction revealed four polypeptides which were later characterized (14). The polypeptides found were apoLp-Val, apoLp-Glu, and apoLp-Alanine. The amino-terminal residue of both apoLp-Val and apoLp-Glu was threonine, while that of apoLp-Ala was serine. ApoLp-Ala was shown to exist in three forms which differed only in sialic acid content (82). Scanu, et al (83) have recently demonstrated by isoelectric focusing in a narrow pH gradient that apoLp-Ala may exist in a third form which is completely free of sialic acid. Thus, it seems that the C apolipoprotein of Gustafson consists of three completely different polypeptides. It is interesting to note that Shore and Shore (10) encountered greater heterogeneity of pre-β apolipoprotein than did Brown and co-workers (13). This has been suggested to be a result of several differences in techniques and their results have yet to be reconciled.

4. The Protein Moiety of Chylomicrons

At the present time, the composition of the chylomicron protein moiety is not certain. Several problems have prevented elucidation of the protein structure. The protein content of chylomicrons varies from 0.5 to 2.5 percent of their total weight (4). Thus, suitable yields of protein

from plasma chylomicrons are difficult. Also, a precise method of separating chylomicrons from the pre-β lipoproteins is difficult. This is because a continuum of molecules over the S_f 20-10,000 range and contamination of chylomicrons with other serum proteins is a problem. Even when chylomicrons have been purified by repeated ultracentrifugations, several different serum proteins, including gamma globulin and albumin, can often be immunologically detected (84). Thus, amino acid or polypeptide analysis on such small quantities of protein is subject to errors compounded by the probability that a mixture of protein is usually present.

The few studies of the chylomicron protein component are conflicting and inconclusive. Amino-terminal aspartic and glutamic acids are often obtained and the amino-terminal serine and threonine residues, typical of apolipoprotein C, are inconsistently present (11,70,85). It appears that the β and α apoproteins are most frequently reported as the apoprotein moiety present in chylomicrons.

The major polypeptides found in human plasma lipoproteins are listed in Table IV. Future studies of the plasma apolipoproteins may reveal several additional different polypeptides and heterogeneous forms found in human plasma lipoproteins.

TABLE IV

THE POLYPEPTIDES FOUND IN HUMAN PLASMA APOLIPOPROTEINS

Nomenclature	C-Terminal Amino Acid	N-Terminal Amino Acid	Source	Molecular Weight
apoLp-Ser	Serine	Glutamic acid	β, pre-β Lp	95,000
apoLp-Gln	Glutamine	Aspartic acid	α, pre-β Lp	15,000
apoLp-Threo	Threonine	Aspartic acid	α, pre-β Lp	15,000
apoLp-Val	Valine	Threonine	pre-β Lp	7,000
apoLp-Glu	Glutamic acid	Threonine	pre-β Lp	10,000
apoLp-Ala	Alanine	Serine	α, pre-β Lp	10,000

III. EXPERIMENTAL

A. Agarose Gel Electrophoresis of Plasma Lipoproteins

In order to identify hyperlipoproteinemia, it is necessary to be able to reproducibly observe abnormal changes in plasma lipoprotein fractions. Quantitation of these fractions would also be useful rather than observations made from visual inspection of electropherograms. From quantitation, normal ranges of the plasma lipoprotein concentrations could be obtained and when combined with plasma triglyceride and cholesterol estimations, more exact statements about the inheritance patterns of the hyperlipoproteinemias become possible.

A method of agarose gel electrophoresis developed by Irwin was used to detect hyperlipoproteinemic individuals (32). Minor modifications of the original method have been made to increase the usefulness of the method as a quantitative tool for measuring plasma lipoproteins.

1. Electrophoretic procedure.

(a) Materials

Plasma sample: Blood specimens were obtained by venipuncture and collected in Vacuutainer tubes (Becton, Dickinson and Co., Clarkson, Ontario) containing the sodium salt of ethylenediaminetetra-acetic acid (EDTA). Plasma was removed by centrifugation at room temperature. If electrophoresis was not carried out the same day as specimen collection, it was stored at 4^{o} Centigrade.

Agarose: "Sea Kem" agarose manufactured by Marine Colloid Inc. and sold by Bausch and Lomb, Rochester, New York.

Buffer: Barbital, ionic strength 0.05, pH 8.6 was prepared according to Hatch (86). The buffer consisted of:

Barbital (5,5-diethylbarbituric acid)	2.8 g
Sodium Barbital	20.6 g
EDTA (disodium salt)	0.372 g

The solids were dissolved in two liters of deionized water at room temperature. The pH was checked and adjusted, if necessary.

Agarose gel film support: Dupont P.40 B 35 mm safety motion picture film leader was purchased from Dupont of Canada Ltd., #700 Chinook Professional Building, Chinook Shopping Center, Calgary, Alberta. A resin located on the "inner" side of the film is instrumental in obtaining satisfactory adherence of the agarose gel to the film. Some batches of film absorb excessive amounts of Sudan Black B stain, probably related to the variable thickness of the resin coating from batch to batch. It was found that pre-treatment of the resin side with cyclohexanone (Fisher cat. #C-550) renders the film suitable for agarose gel electrophoresis. Pre-treatment consists of moistening a gauze pad with cyclohexanone and wiping the resin-coated side with five longitudinal strokes. Excess cyclohexanone is removed with a clean, dry gauze pad. The technique used in wiping the tape is important. Three or four strokes are

insufficient to remove enough resin, whereas six or seven strokes remove too much resin with resulting poor adherence of the dried agarose gel to the film. Removal of excess cyclohexanone is critical in that dye "streaks" develop in the film if this step is not performed properly.

Electrophoretic cell: A model E800-2 cell from Research Specialties, Berkeley, California was used. This cell was cooled to 4°C during electrophoresis by a circulation water bath (P.M. Tamson, Holland) equipped with a portable bath cooler (Neslab Instruments, Durham, New York). A model 1910 power supply available from Research Specialties, Berkeley, California was used.

Reservoir cutter: A reservoir cutter was constructed by gluing 20 mm length razor blades on either side of a 1 mm thick plastic strip as described by Irwin (52).

Sudan Black B staining solution: Five g of Sudan Black B (Fisher Cat. #S-668) was added to 400 ml of absolute methanol (Fisher Cat. #A-412) and stirred on a magnetic stirrer for ten minutes. One hundred ml of deionized water was added with continuous stirring and the mixture heated until boiling began. The solution was removed from heat, covered, and stored at room temperature overnight. The staining solution was filtered (Whatman #1) and stored at room temperature an additional two days before use.

(b) Preparation of agarose gel strips

Agarose (0.5 g) is added to 100 ml of barbital

buffer and the mixture heated in a boiling water bath for at least 0.5 hour. During this period, the preparation is frequently mixed by swirling the container. Following this, the solution is put through a No. 40 Whatman filter in a previously warmed (80°C) Buchler funnel. A pre-heated flask (80°C) containing the agarose solution is placed in a hot water bath (80°C) to maintain the agarose preparation in solution until pouring the strips. Approximately 33 ml of the above solution is poured onto the resin-treated side of 55 cm strips of leader tape and evenly spread. It is important that the leader tape strips are on a level surface. The poured strips are allowed to set without being disturbed for at least forty-five minutes. Up to four of the above strips may be accommodated in the Model 800 electrophoretic cell.

(c) Preparation of samples and electrophoresis

The prepared agarose strip(s) is placed on the electrophoresis cell bed. Six sample reservoirs (1 mm by 20 mm) are cut at 5 cm intervals along the agarose strip. The agarose remaining in the reservoir is carefully removed by a toothpick. Ten microliters (ul) of the desired plasma specimens are transferred to the respective reservoirs. Each specimen is run in duplicate. Constant voltage electrophoresis is performed for forty-five minutes at 600 volts (approximately 20 ma/strip). After electrophoresis is complete, the strip(s) is dehydrated in absolute methanol for fifteen minutes. Following this, the agarose is dried by use of a hair dryer. The agarose remains as a thin

film which adheres to the leader tape.

If the plasma specimen is suspected of containing chylomicrons, the reservoir is filled with one or two drops of warm agarose solution after electrophoresis is complete. If this is not performed, chylomicrons will be washed out of the reservoir when the strip(s) is dehydrated and will not be readily noted when interpreting the lipoprotein strip.

(d) Staining and destaining

The dried strips are wound onto the developing reel and immersed in the developing container containing 400 ml of the Sudan Black B staining solution. Staining is carried out for thirty minutes. After staining is complete, the reel is removed from the staining container and gently rinsed under deionized water. The strip(s) is removed from the reel and washed under running deionized water while the soft gel surface is rubbed gently with the fingertip. The strip(s) is allowed to dry at room temperature and then placed, gel side down, on blotting or filter paper as the under surface of the strip(s) is cleaned with a cloth moistened with absolute methanol or acetone.

(e) Quantitation

This is done by densitometric scanning of the lipoprotein electropherogram and the area under each obtained by use of a suitable integrator. A Photovolt Densicord Recording Electrophoresis Densitometer (Photovolt Corp., New York, New York) was employed. A filter which transmits energy at a wavelength of 590-600 nanometer (nm) is used.

A defining slit of approximately 1 x 15 mm gives good resolution and sensitivity.

The expression of electrophoretically obtained plasma lipoprotein fractions as a percent of the total dye uptake has limited application. For this study it was necessary to express lipoprotein fractions in a quantitative manner as lipoprotein-lipid per 100 milliliters. This was accomplished by multiplying the total lipid value of the plasma specimen by the percent dye uptake of the lipoprotein fraction. For this approach to quantitative expression of lipoprotein fractions to be valid, it is necessary to demonstrate that Sudan Black B stain is bound equally by the lipids in the lipoprotein fractions.

To demonstrate this, plasma specimens were obtained from twenty individuals and analyzed for triglycerides, cholesterol, total lipids (determined as described below), and the percentage of each lipoprotein fraction (as obtained by densitometric scanning). The individuals used for this study represented wide ranges of plasma lipid values.

The results of this study, summarized in Table V, indicate that total stain uptake by the plasma lipoproteins is best correlated with plasma total lipids. Note that summed plasma triglyceride and cholesterol levels correlated better with total stain uptake than either lipid alone.

(f) Plasma total lipids

As mentioned above, this value is required to express plasma lipoproteins in terms of quantitative amounts.

TABLE V

THE CORRELATION* OF PLASMA LIPIDS WITH TOTAL DYE UPTAKE BY ELECTROPHORETICALLY ISOLATED PLASMA LIPOPROTEINS

	Plasma Cholesterol	Plasma Triglycerides	Plasma Cholesterol and Triglycerides	Plasma Total Lipids
Total Dye Uptake	0.25	0.75	0.84	0.98

*Correlations expressed by Pearson Correlation Coefficients (r).

Plasma total lipids are measured by the sulfophosphovanillin colorimetric method of Drevon and Schmit (87) as modified by Postma and Stroes (88). The plasma sample (100 ul) is first mixed with concentrated sulfuric acid (2.0 ml) and heated at 100°C for ten minutes. After the mixture is cooled, the phosphoric acid-vanillan reagent (5.0 ml) is added. Development of a pink colour occurs over a fifteen minute period when the mixture is incubated at 37° Centigrade. The resulting complex absorbs energy at a wavelength of 525 nanometers. The exact chemistry involved in the method is not completely understood. Although gravimetric analysis of total lipids is thought to be more accurate, it has been shown that the colorimetric method is accurate and more precise when compared to the gravimetric method (88,89).

To determine the precision of the method, total lipid measurements on five replicate specimens were carried out for five consecutive days. The standard deviation (S.D.) of the total lipid analysis was calculated by the formula

$$S.D. = \pm\sqrt{\frac{x^2}{N-1}}$$

where x is the difference of the individual test from the mean and N is the number of tests. The coefficient of variation (C.V.) is expressed as a percentage and was calculated using the formula

$$C.V. = \frac{S.D.}{M} \times 100$$

where M is the mean total lipid value.

The results of this study indicate the total lipid method is precise. The mean total lipid value obtained was 548 mg/100 ml and 1 S.D. was 14 mg/100 ml, giving a 2.6 percent coefficient of variation.

(g) Plasma triglycerides and cholesterol

Plasma analysis for triglyceride and cholesterol levels was necessary for evaluating the quantitative lipoprotein electrophoretic method and for genetic and further biochemical studies.

A semi-automated method for the simultaneous determination of cholesterol and triglycerides was used (90,91). An isopropanol extract of plasma is treated with a slurry containing Lloyd Reagent, zeolite, cupric sulfate, and calcium hydroxide. Lloyd Reagent removes serum chromogenic material, including bilirubin, while the zeolite insures complete removal of all phospholipids. The copper-lime reagent eliminates glucose that might interfere with the oxidation of glycerol with periodate in the triglyceride method.

The isopropanol extract is automatically added to an air-segmented sodium hydroxide reagent and saponification of triglycerides to glycerol occurs on stream in a 50°C heating bath. After saponification periodate is added to the reaction mixture to oxidize the glycerol to formaldehyde. Formaldehyde then condenses with diacetylacetone and ammonia to give a fluorescent product, 3,5 diacetyl 1,4 dihydrolutidene. The reaction mixture then enters the fluorometer where

fluorescence is activated. The activating wavelength is 400 nm and the fluorescent wavelength is 485 nanometers.

For cholesterol determination, the same isopropanol extract is reacted with a solution of sulphuric acid, glacial acetic acid, and ferric chloride. Derivatives of cyclopentanoperhydrophenanthrene having a 5-ene 3-ol grouping react with this reagent to produce a purple colour which is measured at 550 nanometers.

2. Normal ranges of plasma lipids and lipoprotein fractions.

The establishment of normal ranges was necessary in order that the genetic studies of the hyperlipoproteinemias could be done. It was also important that normal values be available to facilitate the screening of abnormal plasmas suitable for further biochemical studies.

It was proposed to determine the normal range of lipoproteins found in normal males of various age groups. Volunteers included the City of Edmonton Police Force, laboratory personnel, individuals from genetic studies who were related to the family through marriage, and miscellaneous individuals. Individuals with a previous history of heart, liver, or kidney disease were excluded from the study. The volunteers were instructed to observe a twelve to sixteen hour fasting period prior to blood-letting. The blood was collected in EDTA tubes (Vacuutainer) and the plasma and cells separated by centrifugation at room temperature. Analysis of the plasma specimens for triglycerides,

cholesterol, total lipids, and lipoproteins was carried out as soon as possible. Plasma specimens were stored at $4^{o}C$ when necessary.

The method used to calculate the normal ranges was that of Desmond and Smith (92). In order that normal ranges could be derived, it was first necessary to group the data into convenient intervals, as listed below:

1.	Triglycerides	10 mg/100 ml
2.	Cholesterol	10 mg/100 ml
3.	Total lipids	25 mg/100 ml
4.	β lipoprotein	20 mg/100 ml
5.	Pre-β lipoprotein	10 mg/100 ml
6.	α lipoprotein	10 mg/100 ml

Using probability x linear graph paper, the cumulative frequency of individuals within an interval was plotted versus increasing concentration of the plasma constituents. An example of this plot is illustrated in Figure II in which the cumulative frequency of individuals, aged 30 - 40 years of age, is plotted against increasing levels of plasma triglycerides. If this plot yielded a straight line, then the frequency distribution of the plasma values follows a Gaussian Curve and the normal range could be obtained by including plasma values that fell within 2.5 and 97.5 percent levels. However, if the probability x linear plot was not a straight line, as seen in the example illustrated in Figure II, but fell off at higher values, then a replot is made using probability x log graph paper. A replot of

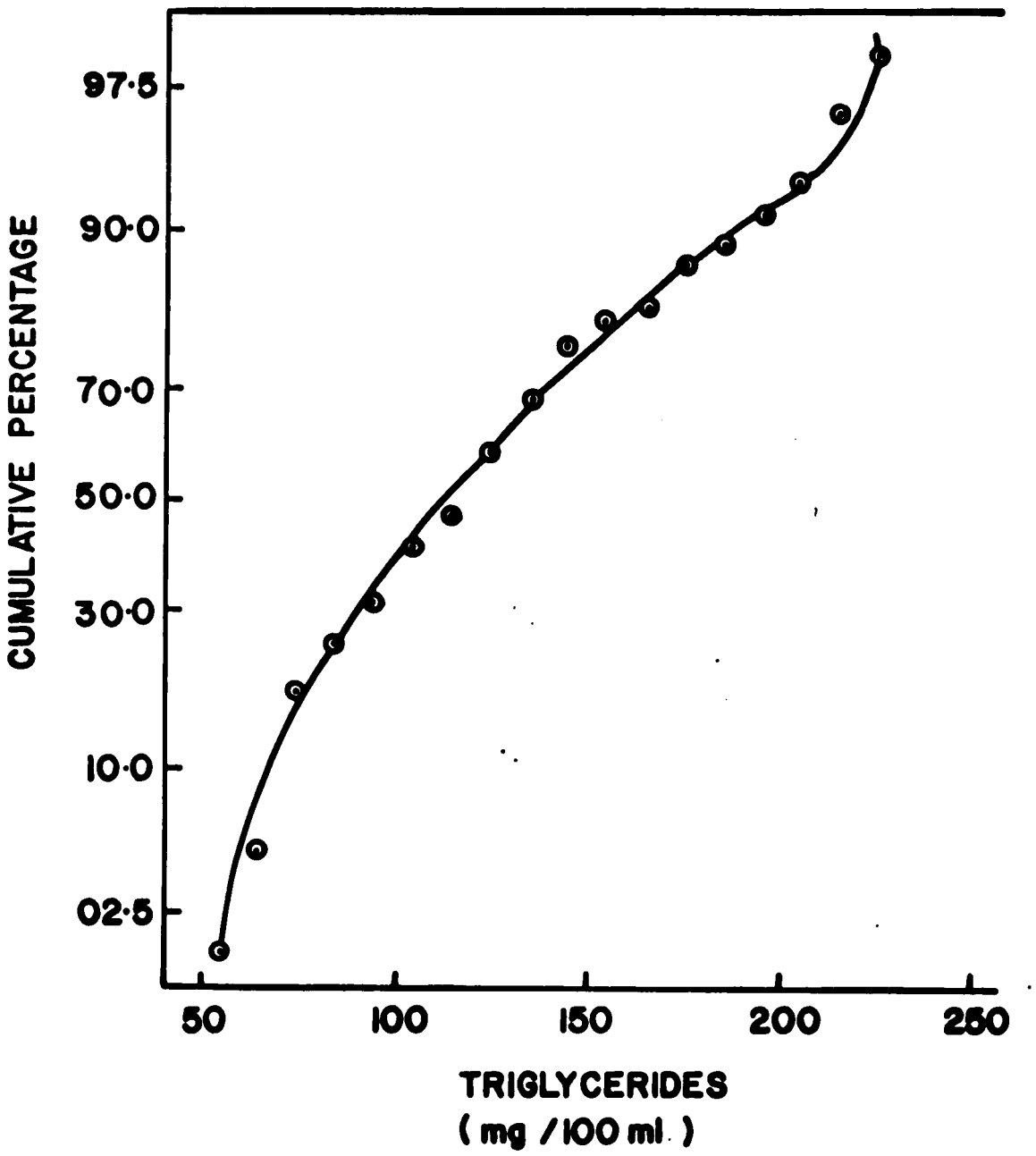

FIGURE II. The cumulative frequency x linear plot of plasma triglyceride levels obtained from 61 normal, male volunteers aged 31-40 years.

the data from Figure II is illustrated in Figure III. The Normal range is derived by including values between 2.5 and 97.5 percent cumulative frequency levels.

It was found that plasma triglycerides and pre-β lipoprotein-lipid levels of each age group best suited a log normal distribution curve and were therefore plotted on probability x log graph paper. Plasma cholesterol, total lipid, β lipoprotein-lipid, and α lipoprotein-lipid levels fit either a normal or log normal distribution curve.

The results of the normal range determinations for the plasma constituents previously mentioned as illustrated in Figures IV to IX and summarized in Table VI. It can be seen that the concentrations of plasma lipids and lipoproteins found in "normal" individuals vary over a wide range of values. A general upward trend of the average lipid and lipoprotein-lipid values is observed. With minor exceptions, the normal ranges do not increase with age. It should be noted that normal ranges for the 51-60 year age group could not be calculated due to the small number of individuals (n=16) studied.

It is difficult to compare the normal ranges obtained in this study with those obtained by others. The variability of lipid methodology used by different laboratories and the geographical location have marked effects on the results obtained in each study. Aside from this, it is interesting to attempt a comparison of normal plasma lipoprotein levels obtained by agarose gel electrophoresis with those obtained

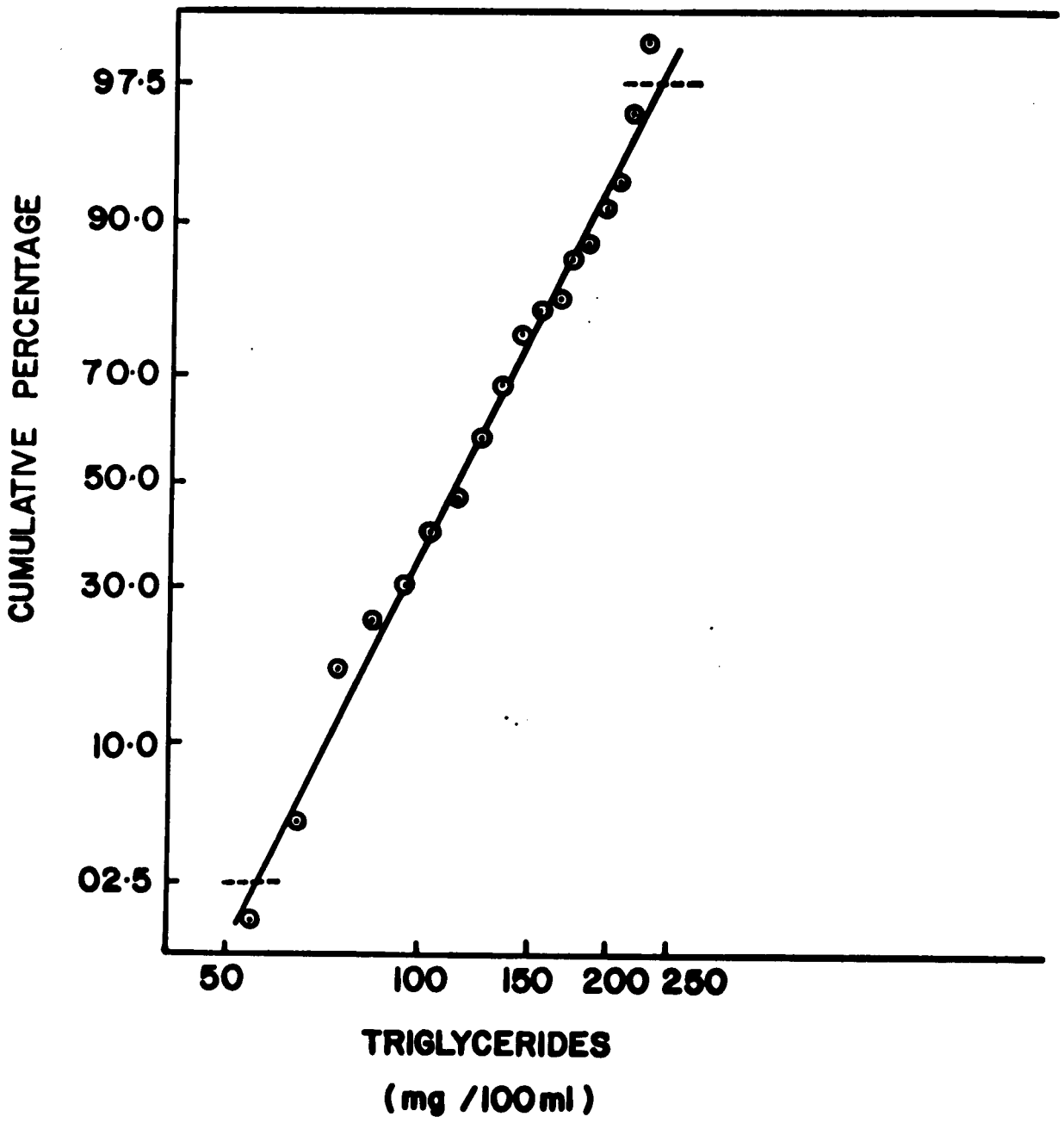

FIGURE III. The cumulative frequency x log plot of plasma triglyceride levels obtained from 61 normal, male volunteers aged 31-40 years.

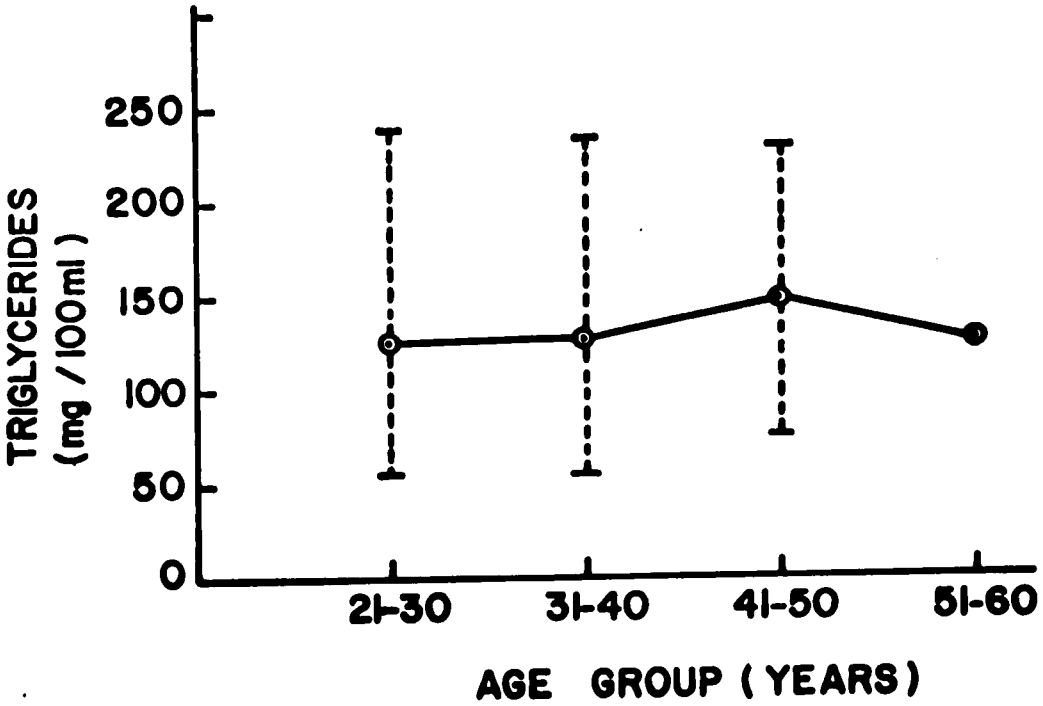

FIGURE IV. The average and range of plasma triglyceride levels found in normal male volunteers. Age groups were 21-30 (n=49), 31-40 (n=61), 41-50 (n=34) and 51-60 years (n=16).

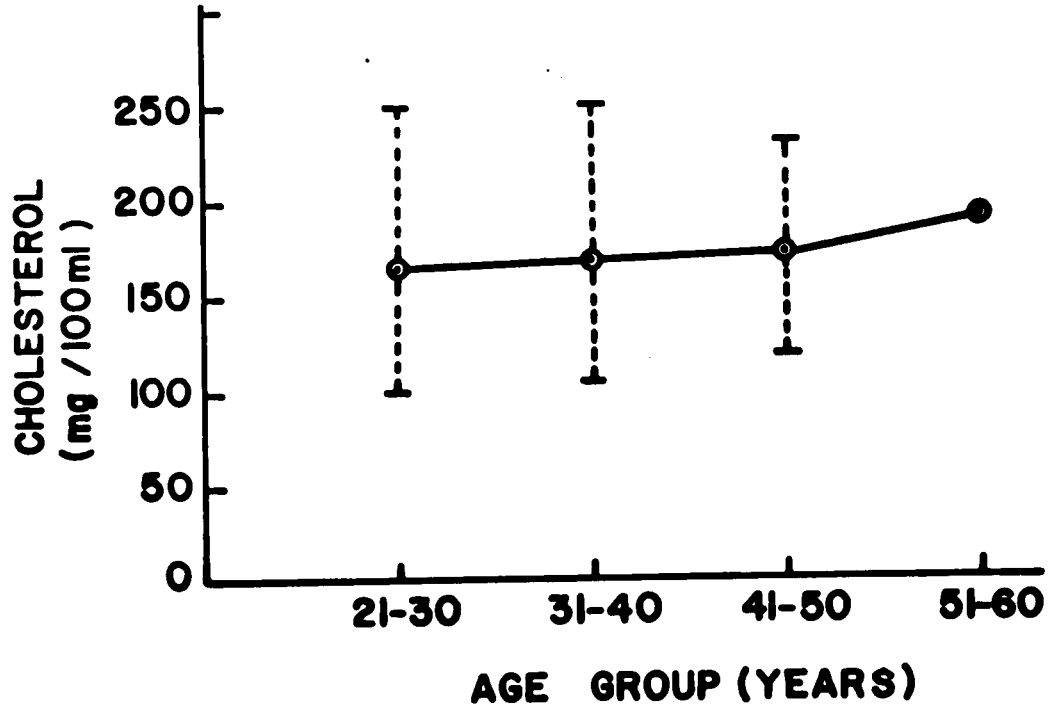

FIGURE V. The average and range of plasma cholesterol levels found in normal, male volunteers. Age groups were 21-30 (n=49), 31-40 (n=61), 41-60 (n=34) and 51-60 years (n=16).

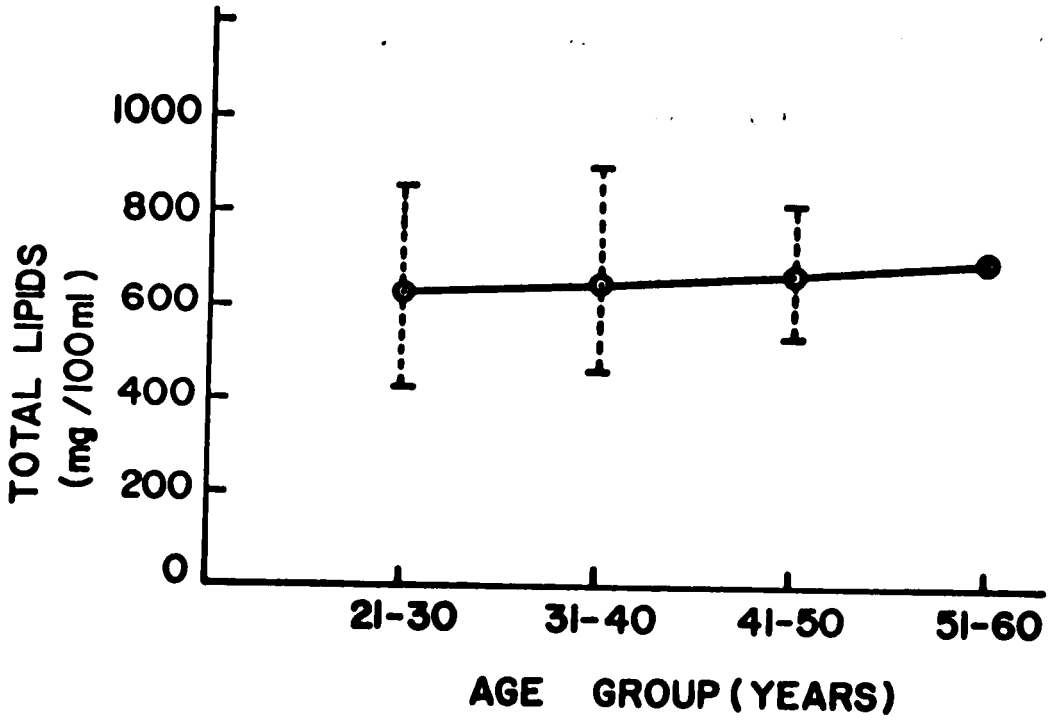

FIGURE VI. The average and range of plasma total lipid levels found in normal, male volunteers. Age groups were 21-30 (n=49), 31-40 (n=61), 41-50 (n=34) and 51-60 years (n=16).

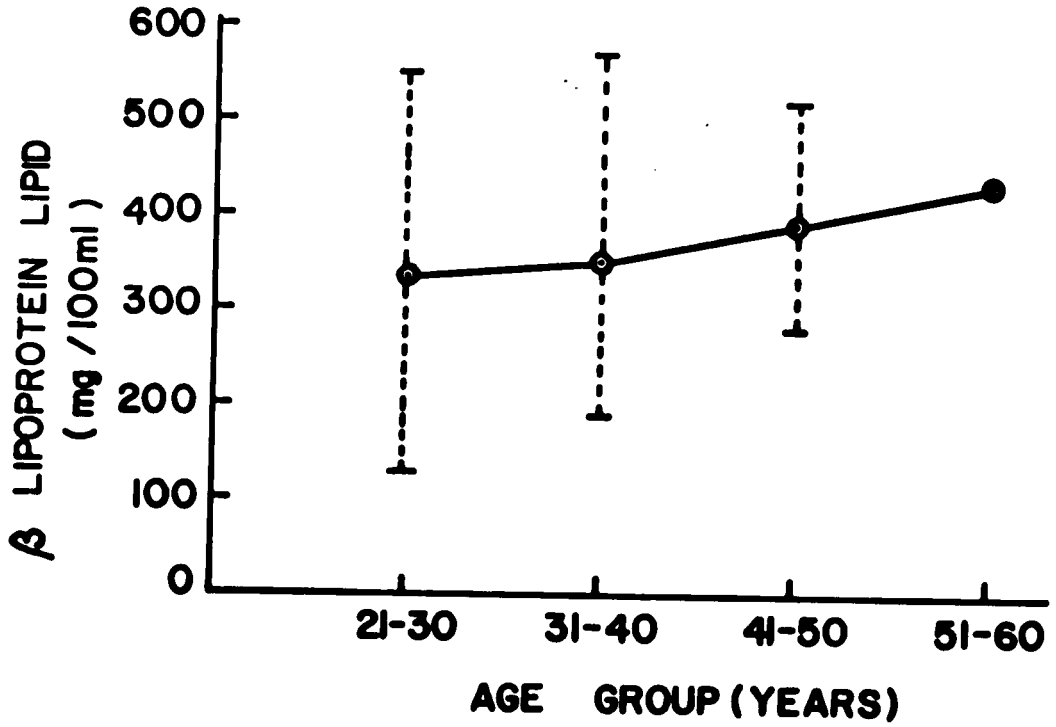

FIGURE VII. The average and range of plasma β lipoprotein levels found in normal, male volunteers. Age groups were 21-30 (n=49), 31-40 (n=61), 41-50 (n=34) and 51-60 years (n=16).

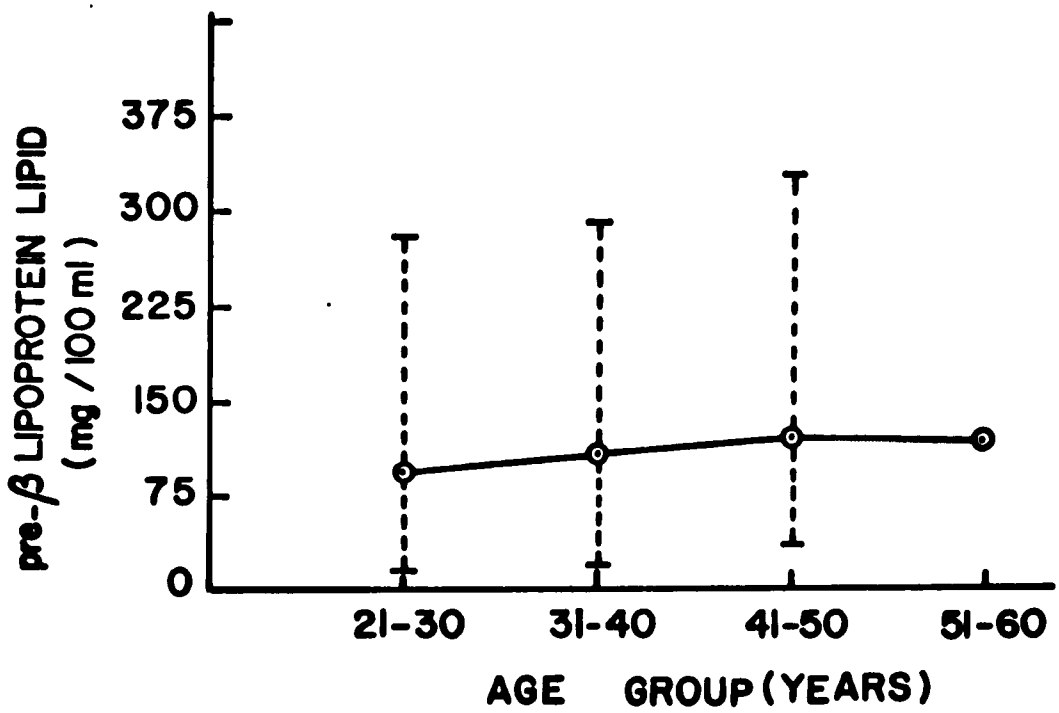

FIGURE VIII. The average and range of plasma pre-β lipoprotein-lipid levels in normal, male volunteers. Age groups were 21-30 (n=49), 31-40 (n=61), 41-50 (n=34) and 51-60 years (n=16).

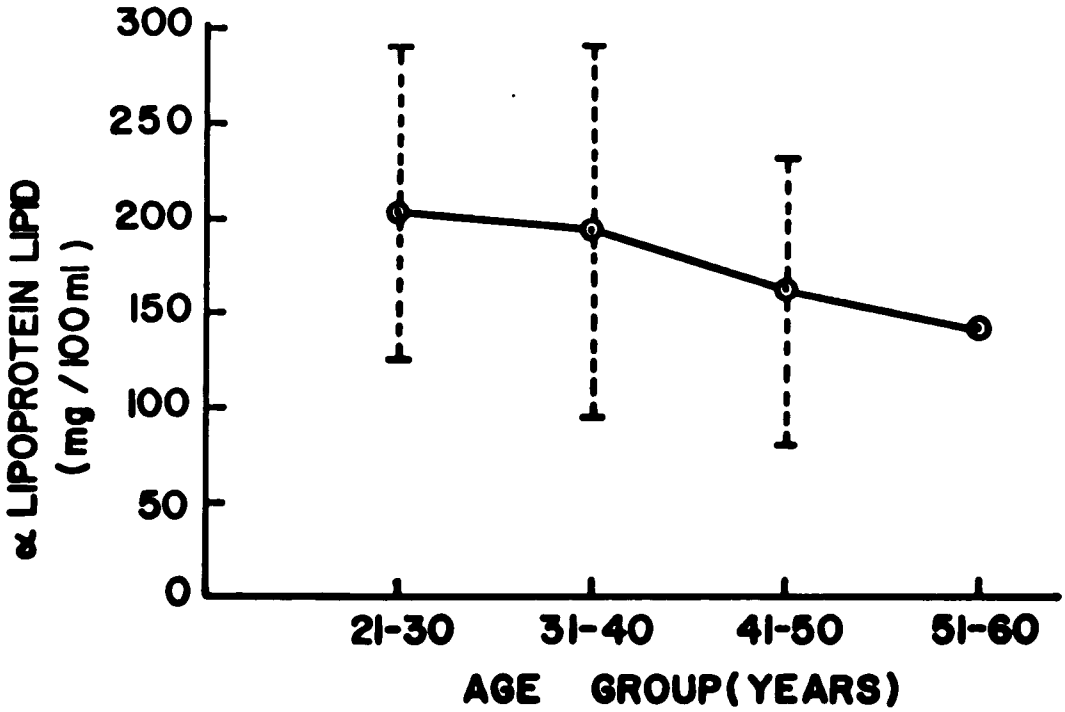

FIGURE IX. The average and range of plasma α lipoprotein-lipid levels found in normal, male volunteers. Age groups were 21-30 (n=49), 31-40 (n=61), 41-50 (n=34) and 51-60 years (n=16).

TABLE VI

NORMAL RANGES OBTAINED FOR PLASMA LIPIDS AND LIPOPROTEIN-LIPIDS FROM NORMAL MALE INDIVIDUALS AGED 21-50 YEARS

Age Group	Cholesterol (mg/100 ml)	Triglycerides (mg/100 ml)	Total Lipids (mg/100 ml)	β Lipoprotein-Lipids (mg/100 ml)	Pre-β Lipoprotein-Lipids (mg/100 ml)	α Lipoprotein-Lipids (mg/100 ml)
21-30	100-250	55-240	430-850	130-550	15-280	125-290
31-40	105-250	55-235	460-895	190-570	20-290	95-290
41-50	120-215	75-230	530-810	280-520	35-330	80-230

by analytical ultracentrifugation. The normal ranges of S_f 0-20 and S_f 20-400 lipoprotein obtained by analytical ultracentrifugation from fasting, male individuals were reported for two age groups which averaged 44.4 and 54.3 years of age (93). These were recalculated as lipoprotein-lipid in order that a comparison could be made with the electrophoretic results from the present study. The results (Table VII) indicate that the electrophoretically quantitated β and pre-β lipoprotein-lipid range closely approximates that obtained by analytical ultracentrifugation. Despite the completely different methods of analysis and sample population used in the two studies, the agreement between the two procedures is remarkably good.

Since the sample group is small, the results of the present normal range study must be interpreted with some caution. In order that one might obtain true normal range limits, a non-parametric method for calculating the range should be used. This would remove any problems involving a non-Gaussian distribution (94). The non-parametric method could not be applied to the data from this study since 120 subjects is a minimum number required for 90 percent confidence intervals.

4. Conclusions

The preceding experimental work produced the following data necessary for genetic studies:

(1) Agarose gel electrophoresis is capable of providing quantitative plasma lipoprotein levels which agree

TABLE VII

A COMPARISON OF β AND PRE-β LIPOPROTEIN-LIPID LEVELS OF NORMAL HUMANS OBTAINED BY ANALYTICAL ULTRACENTRIFUGATION (96) AND QUANTITATIVE LIPOPROTEIN ELECTROPHORESIS

Method of Analysis	Average Age (Years)	Average β Lp-Lipids (mg/100 ml)	Range of β lp-Lipids (mg/100 ml)	Average Pre-β Lp-Lipids (mg/100 ml)	Range of Pre-β Lp-Lipids (mg/100 ml)
Analytical Ultracentrifugation	44.4	375	130-520	142	38-246
Quantitative Electrophoresis	45.5	390	280-520	121	35-330

with those obtained by the referee method of analytical ultracentrifugation.

(2) Using a quantitative agarose gel electrophoretic method, normal ranges were obtained for β, pre-β, and α lipoprotein-lipid levels. Also obtained were normal ranges for plasma triglycerides, cholesterol, and total lipids.

B. Genetic Studies of Some Hyperlipoproteinemias

It was proposed to carry out a study of the possible mode of inheritance in some hyperlipoproteinemias by using quantitative serum lipoprotein agarose gel electrophoresis as a means of detecting affected individuals. It was also hoped that such a study might provide a suitable "pool" of Type IV and Type III individuals whose serum could be used as a source of lipoproteins for physico-chemical studies. As Type I or Type V individuals were not found, the study was restricted to Fredrickson Types II, III, and IV hyperlipoproteinemic families.

1. Type II hyperlipoproteinemia

Possible Type II patients were detected from blood submitted to the Department of Clinical Pathology, University Hospital, Edmonton, Alberta for lipid studies. The patient's physician was contacted to determine if the hyperlipoproteinemia was a defect secondary to disease, i.e., diabetes, hypothyroidism, and if this was the case the individual was not further studied. If the lipidemia appeared primary in nature, a letter was written to the patient explaining the purpose of the investigation and

requesting names and addresses of blood relatives over a period of three to four generations. Deceased members of the family were included and the cause of death was stated when known. Letters were then written to each of the family members explaining the nature of the study and requesting their family doctor's name and address. Upon receiving the physician's consent, fasting blood specimens were collected as previously described. Where possible, blood was taken personally, however out-of-town specimens were taken by the physician and shipped to this laboratory. Whenever transportation of the specimen was over a period of two to three days, it was shipped on ice. Lipid analysis was carried out as soon as possible or stored at 4° Centigrade.

Three families were investigated. The propositus in each case exhibited plasma β lipoprotein-lipid values from 615 to 990 mg/100 ml (normal <570 mg/100 ml) while plasma cholesterol levels were over 300 mg/100 milliliters (normal <250 mg/100 ml).

(a) Family I.R.

The propositus (female, aged 45 years) presented β lipoprotein-lipid levels of 990 mg/100 ml and a cholesterol level of 300 mg/100 milliliters. Both parents of the propositus were living, but blood specimens were not available. However, the mother suffers from coronary insufficiency. Three of the propositus' five brothers and sisters had β lipoprotein-lipid values over 600 mg/100 ml coupled with cholesterol levels over 290 mg/100 milliliters. A brother

who had a stroke at age 45 years had a β lipoprotein-lipid level of 850 mg/100 millliliters and a cholesterol level of 520 mg/100 millliliters. One of three children borne by the propositus exhibited a Type II pattern, having a β lipoprotein-lipid of 810 mg/100 ml and a cholesterol of 370 mg/100 millliliters. The only child (4 years of age) of this individual exhibited β lipoprotein-lipid level of 1,040 mg/100 ml and a cholesterol of 430 mg/100 millliliters. One of the affected brothers of the propositus had an 18-year-old daughter who presented a β lipoprotein-lipid level of 730 mg/100 ml and a cholesterol of 270 mg/100 millliliters.

Summarizing the findings, seven of the thirteen family members representing three generations presented Type II pattern of hyperlipoproteinemia. Plasma β lipoprotein-lipid values ranged from 600 to 1,040 mg/100 ml and cholesterol levels ranged from 290 to 520 mg/100 millliliters. The highest triglyceride level found was 210 mg/100 ml, however, quantitative lipoprotein electrophoresis revealed that the bulk of the triglycerides were present in the β lipoprotein fraction since the pre-β lipoprotein-lipid value was 175 mg/100 millliliters.

In summary, the genetic transmission of Type II hyperlipoproteinemia in this family resembles an autosomal dominant inheritance.

(b) Family K.D.

The propositus (female, aged 46 years) exhibited a β lipoprotein-lipid level of 650 mg/100 ml (normal to 520

mg/100 ml) and a cholesterol of 340 mg/100 ml (normal to 215 mg/100 milliliters). Unfortunately, her relatives are living in Holland and were not investigated. The propositus stated that her father (68 years of age) had suffered from heart problems and her only brother had died at 12 years of age from cancer of the liver. Of the propositus' four children, one daughter (23 years of age) exhibited a Type II pattern, having a beta lipoprotein-lipid level of 975 mg/100 ml and a plasma cholesterol of 470 mg/100 milliliters. Her triglycerides were slightly elevated (225 mg/100 ml), but were mainly attributed to the beta lipoprotein fraction since her pre-beta lipoprotein-lipid level was only 180 mg/100 ml (normal less than 280 mg/100 ml). No other form of hyperlipoproteinemia was detected within the family.

Although no conclusions can be stated for such a small family investigation, it would appear that an autosomal dominant gene might explain transmission of the disease.

(c) Family J.H.

The propositus (male, age 35 years) had been troubled for some years with coronary artery disease. At the time of investigation, he had been brought to Edmonton for a Vineberg operation. His plasma cholesterol level on two occasions was 340 and 300 mg/100 ml (normal to 250 mg/100 ml). On the last occasion, his beta lipoprotein-lipid level was 800 mg/100 ml (normal to 570 mg/100 milliliters).

The history of heart disease in his family was

formidable. All three of the propositus' uncles had died from heart disease at early ages (34, 35, and 52 years). A brother (aged 35 years) and a cousin (aged 32 years) had also died from heart disease. One hundred and three individuals constituted the family tree. Of these:

1. Six were deceased, five of these by premature heart disease.

2. Twenty-one were related to the propositus by marriage only.

3. Seventy-six were blood relatives.

Of the 76 blood relatives, 50 were available for biochemical study. The majority of the remaining 26 were under five years of age. The kinship study, covering four generations, is illustrated in Figure X. It is evident that the disease followed an autosomal dominant course of inheritance which clinically originated from the uncles and aunts of the propositus. Eleven of the fifty members investigated exhibited the characteristic Type II lipid pattern. Plasma β lipoprotein-lipid levels ranged from 610 mg/100 ml to 970 mg/100 ml, the majority being over 700 mg/100 milliliters, while cholesterol levels ranged from 225 mg/100 ml to 335 mg/100 milliliters. Although a cholesterol of 225 mg/100 ml is usually considered normal or borderline abnormal, this level was obtained from three children aged approximately five years. The incidence of Type II hyperlipoproteinemia within this family would be increased if it was assumed that five of the six deceased members would have presented a Type II

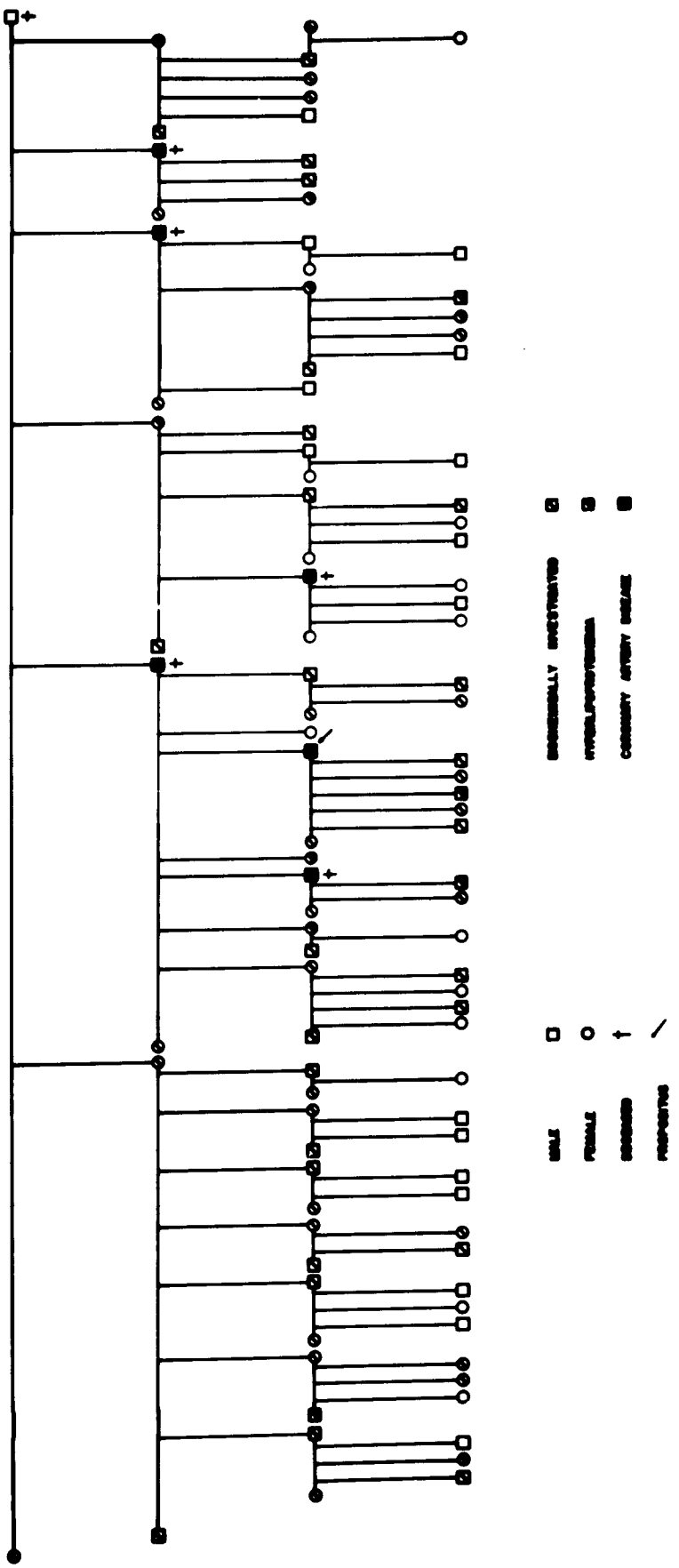

FIGURE X. Pedigree of the J. H. family.

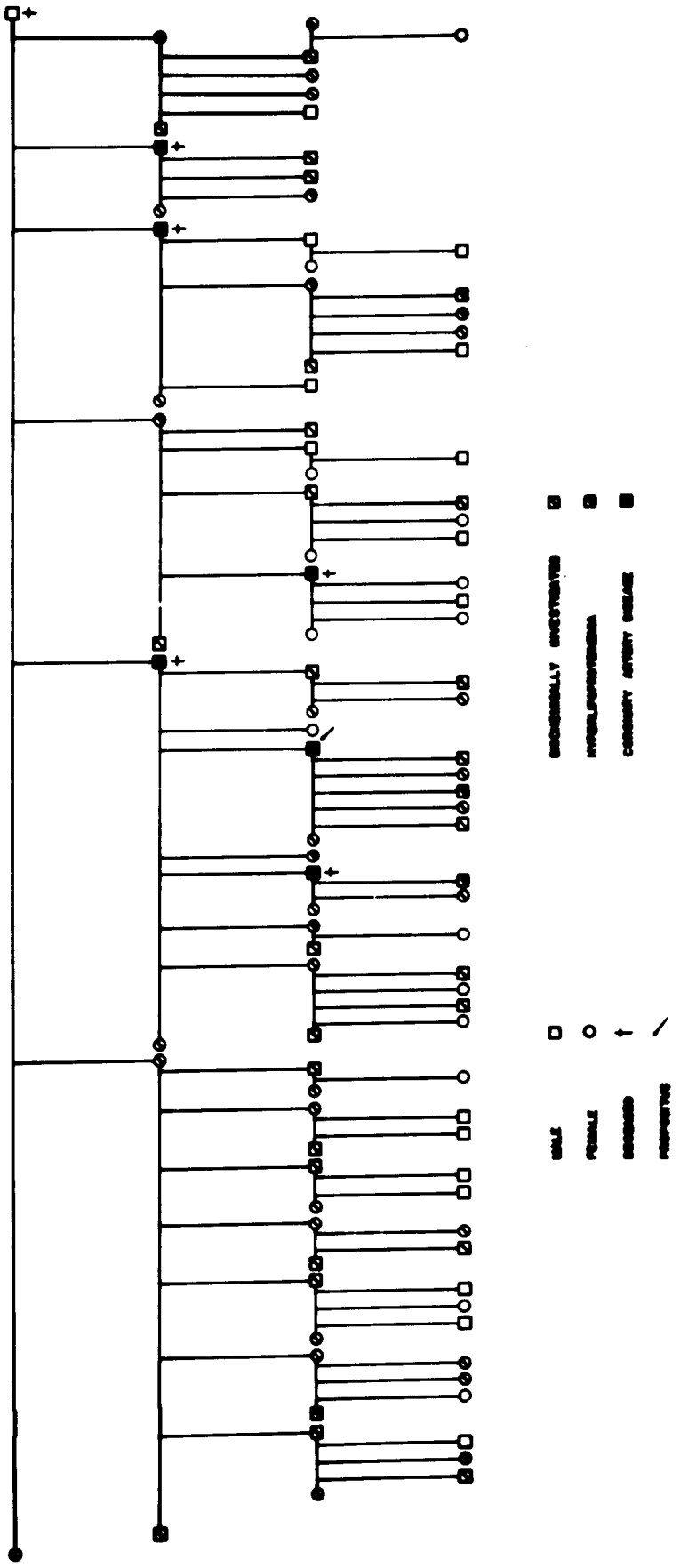

FIGURE X. Pedigree of the J. H. family.

lipid pattern.

None of the marriage-related members of the family (n=18) displayed abnormal lipid patterns. In the total of 68 people investigated, no one presented as a Fredrickson Type III or IV hyperlipoproteinemia. The highest level of plasma triglycerides observed was 193 mg/100 millliliters.

As in family K.D., the disease appeared to be transmitted from the first generation by a mutant gene which had very limited penetrance in either of the great-grandparents since neither one expressed the disease. The great-grandfather died at the age of 77 years from cancer and the great-grandmother was alive at 82 years of age, having a β lipoprotein-lipid level of 650 mg/100 ml and a cholesterol of 220 mg/100 milliliters.

The results of the above three family studies tend to agree with those of Polano, et al (95) who conclude that Type II hyperlipoproteinemia has a dominant mode of heredity with incomplete penetrance and a variable expressivity.

2. Type III hyperlipoproteinemia

Only one individual could be positively identified as having Fredrickson Type III hyperlipoproteinemia. Biochemical diagnosis was made by demonstrating the presence of a "broad-β" electrophoretically migrating lipoprotein which possessed the flotation characteristics of a pre-β lipoprotein. The family study was conducted as previously described. Unfortunately, this was limited to two sisters and one son of the propositus, all of whom had normal lipid

values. There had been no history of heart disease in the family except for the propositus, who had suffered from a coronary occlusion at the age of 44 years. The propositus (male, aged 52 years) repeatedly demonstrated plasma cholesterol levels exceeding 300 mg/100 ml (normal <215 mg/100 ml) and triglycerides ranging from 500 to 1,200 mg/100 ml (normal <230 mg/100 ml), depending on the degree of chylomicronemia. On each occasion a "broad-β" lipoprotein band was observed by agarose gel electrophoresis and confirmed by preparative ultracentrifugation.

Certainly nothing was learned about the inheritance pattern of Type III hyperlipoproteinemia from the study of this family. The absence of a history of heart disease might suggest that the propositus had either inherited a double dose of a mutant recessive gene or a dominant mutant gene of variable penetrance. Fredrickson and Levy (96) have observed vertical transmission of Type III hyperlipoproteinemia in 5 of 36 families affected with this disorder of lipid metabolism. They suggest that an incompletely penetrant dominant mode seems somewhat more likely than an autosomal recessive nature.

3. Type IV hyperlipoproteinemia

Six patients affected by Type IV hyperlipoproteinemia and their respective families were investigated. In five families the propositus was the only individual affected by the disease. In the remaining family there was a history of heart disease. The propositus of this family (male, aged

42 years) was diagnosed as having coronary artery disease. At the time of this investigation his plasma pre-β lipoprotein-lipid level was 375 mg/100 ml (normal <330 mg/100 ml), while the triglyceride level was 365 mg/100 ml (normal <230 mg/100 milliliters). His father had died at the age of 52 years from a heart attack. Investigation of his two children, mother, and brother revealed normal plasma lipid and lipoprotein-lipid levels.

In the other five families studied, the propositi exhibited pre-β lipoprotein-lipid levels of 1,325, 740, 325*, and 95* mg/100 ml and plasma triglyceride levels of 950, 525, 600, 265*, and 180* mg/100 milliliters. As mentioned previously, none of these family members demonstrated any plasma lipid or lipoprotein abnormalities.

Figure XI illustrates one of the above family studies in which the propositus (male, aged 46 years) had suffered angina for two years. There had been no history of heart disease in his predecessors. At the time of the family investigation, his pre-β lipoprotein-lipid level was only 265 mg/100 ml (normal <330 mg/100 ml) while his plasma triglycerides were 325 mg/100 ml (normal <235 mg/100 ml) due to drug and diet therapy. Prior to therapy, his fasting triglycerides had reached 1,000 mg/100 ml at one point. As evident from Figure XI, his hyperlipoproteinemic condition was not found in any family members. Both his parents were

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*These values were obtained after the propositus had been on drug and/or diet therapy.

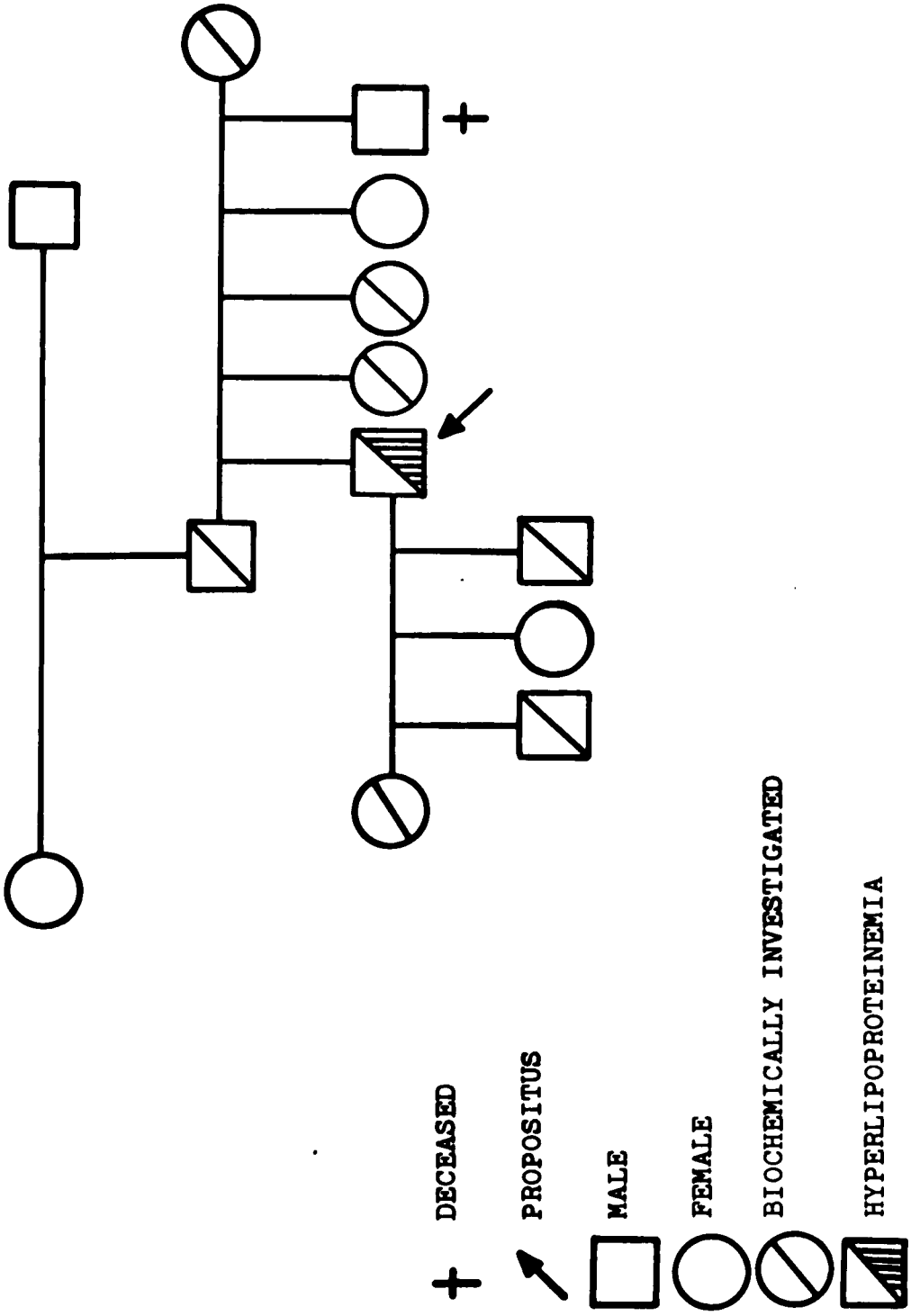


FIGURE XI. Pedigree of the R. H. family.

alive and well at ages of 71 and 73 years.

From these family studies, it appears that a recessive mutant gene is responsible for the disease. However, it is difficult to rule out a dominant mutant gene which has incomplete penetrance.

In summary, Type II hyperlipoproteinemia appears to follow an autosomal dominant mode of inheritance but with variable penetrance. Types III and IV hyperlipoproteinemia appear to be recessive in nature but incomplete penetrance of a dominant gene cannot be ruled out.

The present interest in studying the inheritance patterns of the hyperlipoproteinemias in Types II or IV was kindled by a report of Matthews (15) in which both Type III and Type IV hyperlipoproteinemias were detected within a single family. From his study of eighteen members over a period of five generations, he stated that the two patterns appeared to be transmitted through an autosomal dominant mode of inheritance. His findings were not convincing in that preparative ultracentrifugation was not employed in the diagnosis of Type III individuals. Blankenhorn, et al (97) recently reported a study of Type II individuals affected by varying degrees of endogenous hypertriglyceridemia, producing elevated pre-β as well as β lipoprotein levels. However, preparative ultracentrifugation and lipoprotein electrophoresis demonstrated discrete β and pre-β lipoproteins, rather than broad-β lipoproteins. It is therefore quite possible that Matthew's study may have included Type

II individuals with endogenous hypertriglyceridemia mistaken as Type III. The possibility of different hyperlipoproteinemia phenotypes within a family, however, does exist. Very recently Strunge and Trostman (98) reported the finding of Type II, Type III, and Type IV individuals within one family. The experimental details of their study are slight, thus no comment can be made on this preliminary report.

C. Studies of Very Low Density Lipoproteins (VLDL) in Plasma of Type III and Type IV Hyperlipoproteinemia

Whether lipid or protein defects are present in the various types of hyperlipoproteinemias is unknown. Two obvious means by which a hyperlipoproteinemic condition could arise are increased anabolism or decreased catabolism. Langer, et al (99) have demonstrated that the difference in β lipoprotein levels between normal and Type II individuals lies in a decreased rate of catabolism of β lipoprotein and is not due to increased synthesis of body compartmental distribution changes of the β lipoproteins. Hypocatabolism could involve β lipoprotein, its apoprotein, cholesterol, or other components. A study by Pinot and Laudat (100) indicates that the amino acid composition of β lipoprotein from Type II and normal individuals is identical. This suggests that hypocatabolism of Type II β lipoproteins is not due to a structural abnormality of the β apolipoprotein.

Regarding Type IV hyperlipoproteinemia, Quarfordt, et al (101) have presented evidence that Type IV individuals are affected by a relative defect in mechanisms for the

removal of fatty acids in triglycerides of plasma S_f 20-400 lipoproteins. This suggests that the metabolic error of Type IV hyperlipoproteinemia is due to hypocatabolism rather than hyperanabolism of the lipoprotein species. Also, Brown, et al (13) have shown the apoprotein of pre-β lipoproteins obtained from normal and Type IV individuals to be identical.

It appears that the apolipoprotein of Type III (broad-β) VLDL has not been similarly studied. Thus, an investigation of the protein components of the "broad-β" lipoprotein was undertaken. The experimental approach to this biochemical and physical study was based on that used by Brown (13) for studying the apolipoprotein of VLDL from Type IV and normal patients.

1. Methodology

(a) Separation of Plasma Lipoprotein Fractions by Preparative Ultracentrifugation

Patients were instructed to fast for 12 to 16 hours prior to blood-letting. By use of an EDTA Platelet Pack (Fenwal Laboratories, Morton Grove, Illinois) approximately 400 ml of blood was collected by venipuncture from a Type III and a Type IV patient. The blood was immediately centrifuged and the cells and plasma separated. The plasma was stored at 4^{o} Centigrade. An additional source of plasma was outdated plasma obtained from the blood bank of the University of Alberta Hospital.

Several types of rotors were employed in the Beckman L2-65B ultracentrifuge. When only a small volume of plasma

was available for phenotyping a possible Type III individual, isolation of the S_f 0-20 and S_f 20-400 lipoproteins was carried out in the Type 30.2 rotor for which 6 ml per tube are required. When isolation of large quantities of plasma lipoprotein fractions was required, as in the apolipoprotein studies, a Type 60 Ti (titanium) rotor was used. Here rotor volumes of 24 ml per tube and a capacity of 8 tubes facilitated the isolation of lipoproteins from as much as 192 ml of plasma per ultracentrifugal run. When the isolated lipoprotein fractions were required in a more concentrated form, use of the Type 65 rotor allowed rapid concentration of the particular lipoprotein fraction because of high "g-forces" developed by the rotor.

Solutions required for ultracentrifugal isolation of the plasma lipoproteins were prepared according to Hatch and Lees (86). The salts used in the solution were dessicated prior to solution preparation.

Solution density 1.006 g/ml; 11.40 g of sodium chloride (Fisher cat. #S-311) were dissolved in 1 L of 0.001 N sodium hydroxide (Fisher cat. #SO-S-26). Three additional ml of deionized water were added and mixed.

Solution density 1.182 g/ml; to 500 ml of the 1.006 g/ml solution, 124.90 g of sodium bromide (Fisher cat. # S-255) were added and mixed.

Solution density 1.478 g/ml; to 100 ml of the 1.182 g/ml solution, 78.32 g of sodium bromide was added and mixed.

Two additional solutions, not described by Hatch

and Lees were necessary for further purification of the lipoprotein fractions.

Solution density 1.064 g/ml; this was prepared by mixing 100 ml of 1.182 g/ml solution with 213.3 ml of the 1.006 g/ml solution.

Solution density 1.21 g/ml; mix 100 ml of 1.478 g/ml solution and 131.4 ml of the 1.006 solution.

The S_f >400 lipoprotein fraction was separated as follows: Twenty-four ml of plasma was carefully overlaid with 12.0 ml of the 1.006 g/ml solution. Ultracentrifugation was carried out at 10,000 r.p.m. for twenty minutes (1.42×10^5 "g-min") at room temperature. The top 5 ml containing the chylomicrons (S_f >400) were removed by tube slicing with a Beckman tube slicer (Beckman cat. #30381). Since chylomicrons were not required for further studies, this fraction was discarded.

Isolation of S_f 20-400 lipoprotein fraction was done as follows: The bottom 24.0 ml of the infranatant from the chylomicron isolation were carefully overlaid with 12.0 ml of 1.006 g/ml solution. Ultracentrifugation was performed for 7.5 hours at 60,000 r.p.m. (11.44×10^7 "g-min") at 17° Centigrade. The supernatant containing the S_f 20-400 lipoproteins (approximately 5 ml) was removed by tube slicing. The isolated S_f 20-400 lipoproteins were suspended in the 1.006 g/ml solution to give a total volume of 36.0 ml and ultracentrifugation was repeated in the same manner as described above. This procedure was repeated. The purpose

of the two additional ultracentrifugal spins is to wash the S_f 20-400 lipoproteins free of traces of albumin and other contaminating lipoproteins.

When isolation of S_f 20-400 lipoproteins from a large volume of plasma was being performed, the supernatants obtained from two or three tubes during the initial S_f 20-400 flotation run were pooled and then run through the wash procedure.

To isolate the S_f 0-20 fraction, the lower 24.0 ml of the infranatant from the S_f 20-400 isolation was mixed with 12.0 ml of the 1.182 g/ml solution resulting in 36 ml of 1.064 g/ml density solution. Ultracentrifugation was performed for 9.0 hours at 60,000 r.p.m. (13.73×10^7 "g-min") at 17° Centigrade. The supernatant containing S_f 0-20 lipoproteins was removed by tube slicing and resuspended in the 1.064 g/ml solution to give a total volume of 36.0 milliliters. Ultracentrifugation was repeated in an identical manner as described above. The wash procedure was repeated.

The α lipoprotein fraction (S_f <0) was isolated as follows: The bottom 24.0 ml of the infranatant obtained from the S_f 0-20 isolation was mixed with 12.0 ml of the 1.478 g/ml solution, giving 36 ml of a solution of density 1.20 g/milliliters. Ultracentrifugation was performed for 18 hours at 60,000 r.p.m. (27.46×10^7 "g-min" at 7° Centigrade. The isolated α lipoproteins contained in the supernatant were twice washed in a solution of density

1.21 g/ml in the manner described above.

The isolated α and β lipoprotein fractions contained high concentrations of ions which would distort agarose gel electropherograms. Thus, the concentration of ions was reduced by dialysis as follows: The lipoprotein fraction was placed in a previously water-soaked dialysis tubing (Visking #8/10) and dialyzed against 10L of 0.15M sodium chloride solution containing 1mM EDTA, pH 8.0 for 24 hours. The perfusate was discarded and replaced with fresh solution, whereupon dialysis was repeated for an additional 24 hours.

Cholesterol and triglyceride analysis was performed on the isolated lipoprotein fractions in the manner previously described. Also, lipid phosphorus content of the lipoprotein fractions was measured by the method of Bartlett (102) after an extraction of the phospholipids had been performed by the method of Youngberg and Youngberg (103).

It was necessary to determine the protein content of the lipoprotein fractions for chemical composition and further apolipoprotein studies. The method used for this measurement was that of Lowry, et al (104). Treatment of apoprotein solution with an alkaline copper reagent forms a copper-protein complex, which in turn reduces a phosphomolybdic-phosphotungstic reagent. The resulting greenish-blue colour is measured photometrically at 620 nanometers.

The following reagents are required for the Lowry protein method: Reagent A consists of 2g of sodium carbonate (Fisher cat. #S-263) dissolved in 100 ml of 0.10N sodium

hydroxide (Fisher cat. #S-266). Reagent B is made up of 1.0 g of potassium tartrate (Fisher cat. #P-313) and 0.5 g of cupric sulfate (Fisher cat. #C-495) dissolved in 100 ml of deionized water. Reagent C consists of 0.5 ml of Reagent B mixed with 25 ml of Reagent A just prior to each protein analysis. Reagent D consists of Folin and Ciocalteu Phenol Reagent (Harleco cat. #9315P) which had been diluted with deionized water to 0.1 Normality.

To 1 ml of protein solution, 5.0 ml of Reagent C was added and thoroughly mixed. After incubating for ten minutes at room temperature, 0.5 ml of Reagent D was quickly added and the solutions vigorously mixed. After a 30-minute incubation period, the absorbance of the greenish-blue complex is measured at 620 nanometers.

Several adjustments of the method were made to suit the particular lipoprotein preparation being measured. If the protein concentration of a pre-β or β lipoprotein specimen was being measured, turbidity due to the lipids prevented accurate absorbance measurements. Extraction of the final colour-developed mixture with 5.0 ml of ether with subsequent centrifugation resulted in a clear solution. However, a small volume change due to the ether extraction usually resulted. This possible source of error was remedied by performing a similar extraction on the blank and standard solutions.

When protein measurements of the apolipoproteins were desired, a blank consisting of the buffer solution used

to dissolve the apolipoprotein was needed. It is likely that the TRIS buffer resulted in a slight degree of colour development and thus had to be corrected in the protein measurements.

A problem of protein analysis by the Lowry method involves different colour yields between identical amounts of protein and the standard, bovine albumin. Accurate protein measurements would be obtainable by deriving appropriate compensating colour factors for the apolipoprotein and polypeptides, however, since most studies reported here involved comparisons of protein content, no correction factors were required.

The reproducibility of the Lowry method was investigated and found to exhibit a four percent coefficient of variation.

(b) Analytical Ultracentrifugation of Serum Lipoprotein Fractions.

As a further means of positive identification of the lipoprotein fractions isolated by preparative ultracentrifugation, S_f values were obtained by analytical ultracentrifugation.

The solution containing the lipoprotein fraction was adjusted to a density of 1.063 g/ml at 26°C by an appropriate dilution with 1.182 g/ml solution. The S_f 20-400 lipoproteins were spun in a Beckman Model E Analytical Ultracentrifuge at 36,000 r.p.m. and the Schlieren pattern recorded at four-minute intervals over a period of sixteen to twenty

minutes. The S_f 0-20 lipoproteins were spun at 52,000 r.p.m. and the Schlieren patterns recorded at eight-minute intervals over a period of forty minutes. All analytical ultracentrifugal runs were made at 26° Centigrade.

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

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

where F = the flotation constant

x = the distance in cm from the axis of rotation

w = the angular velocity in radians per second

t = the time in minutes.

(c) Acrylamide Gel Disc Electrophoresis of Plasma Lipoproteins.

Disc gel electrophoresis was used to compare Type III and Type IV plasma lipoproteins. Of particular interest was the electrophoretic behaviour of Type III and Type IV VLDL in acrylamide gel. The method of acrylamide gel disc electrophoresis used was that of Narayan, et al (105) employing a 3.75 percent acrylamide monomer separating gel and a 2.5 percent acrylamide spacer gel. The electrophoretic cell, power supply, and other necessary equipment for disc electrophoresis was manufactured by Shandon Scientific Co. Ltd., London, England. The following reagents were required:

Reagent A: TRIS (36.6 g) was dissolved in approximately 40 ml of deionized water after which 48 ml of 1.0N hydrochloric acid (Fisher cat. #SO-A-48) and 0.23 ml of

N, N, N', N'-tetramethylethylenediamine (TEMED) (Fisher cat. #8178) were added and the final volume adjusted to 100.0 ml with deionized water. The pH of the solution was 8.9.

Reagent B: TRIS (5.989 g) was dissolved in approximately 40 ml of deionized water after which 48.0 ml of 1.0N hydrochloric acid and 0.23 ml of TEMED were added. The final volume was adjusted to 100.0 ml with deionized water. The resulting pH of the solution was 6.7.

Reagent C: Acrylamide (28.0 g) (Fisher cat. #5521-x) and N, N'-methylenebisacrylamide (0.735 g) (Fisher cat. #8383) were dissolved in deionized water to give a final volume of 100 milliliters.

Reagent D: Acrylamide (10.0 g) and N, N'-methylenebisacrylamide (2.5 g) were dissolved in deionized water to give a total volume of 100 milliliters.

Reagent E: Riboflavin (4.0 mg) (Sigma cat. #R-4500) was dissolved in 100.0 ml of deionized water.

Reagent F: Sucrose (40 g) (Fisher cat. #S-5) was dissolved in 100.0 ml of deionized water.

Reagent G: Ammonium persulfate (0.14 g) (Fisher cat. #A-682) was dissolved in 100 ml of deionized water. This solution must be prepared daily.

Stock buffer solution: TRIS (6.0 g) and glycine (28.2 g) (Fisher cat. #G-46) were dissolved in water to give a final volume of 1 liter. The resulting pH of this solution was 8.3. The working buffer solution was prepared by diluting the stock buffer solution with an equal volume of

deionized water.

Sudan Black B staining solution: Sudan Black B (1.0 g) was added to 100.0 ml of ethylene glycol (Fisher cat. #E-178) and heated to a boil. The mixture was cooled and then filtered.

Disc gel electrophoresis basically consists of two gel matrices which differ in pore size. The lower gel layer consists of the separating gel (also known as the main gel) while the upper gel layer consists of the stacking gel (also known as the spacer gel). The separating gel used in this method was prepared by mixing two parts of Reagent A, two parts of Reagent C, four parts of deionized water, and eight parts of Reagent G. The running tubes, capped at one end, were filled to 2/3 capacity with the separating gel solution and then carefully overlaid to a 4-5 mm depth with deionized water. Polymerization of the gel matrix was complete after a one hour period.

The spacer gel was prepared by mixing one part of Reagent B, two parts of Reagent D, one part of Reagent E, and four parts of Reagent F. The layer of water covering the separating gel was removed by a Pasteur pipette and the inside of the tube rinsed with the spacer gel solution. The spacer gel was then poured to a height of about 6-7 mm from the top of the running tube. The gel was again overlaid with deionized water as previously described. Photopolymerization of the spacer gel was accomplished by exposing the poured spacer gels to ultraviolet light.

Twenty-four hours prior to the electrophoretic analysis, 0.100 ml of the plasma specimen or lipoprotein fraction was mixed with 0.050 ml of the Sudan Black B staining solution. When specific lipoprotein fractions were to be electrophoresed, an additional appropriate dilution was made with lipid-poor plasma.

The pre-stained sample solution was mixed with a 15 percent sucrose solution of Reagent B in equal parts. The water layer over the spacer gel was removed, the running tubes mounted in the electrophoretic chamber and the sample solution (0.030 ml) then layered onto the spacer gel. The working buffer solution was carefully layered on top of the sample solution and the anode and cathode reservoirs filled with working buffer solution. Electrophoresis was carried out at a constant current of 2.5 ma/tube for 45 minutes.

The results obtained from disc gel electrophoresis of the plasma specimens and lipoproteins are represented diagramatically.

(d) Delipidation of the Plasma Lipoprotein Fractions

This was necessary in order to characterize the apolipoproteins. The presence of lipids results in a macromolecule which is not fractionated by relatively simple physical processes such as gel filtration chromatography. It is also difficult to obtain a suitable protein concentration when a solution of the lipoprotein is used. The relatively large lipid:protein ratio results in gel-like solutions when the lipoprotein preparation is suitably

concentrated for adequate protein concentrations.

Total removal of the lipids of β, pre-β, and α lipoproteins was accomplished by the method of Brown, et al (13). A volume of the dialyzed lipoprotein fraction containing approximately 15 units of protein was shell-frozen and lyophilized in a 50 ml round-bottom centrifuge tube. Forty ml of an ethanol:ether (3:1) mixture was added to the tube and the resulting large particles of lipoprotein were dispersed by use of a glass rod. The tube was stoppered and mounted on a multi-purpose rotator (Scientific Industries Inc., Springfield, Massachusetts). The initial lipid extraction was carried out for 16 hours at 12-15 r.p.m. and at a temperature of 4° Centigrade.

Following the initial extraction, the tube containing the extraction mixture was removed from the rotator and centrifuged at 2,000 r.p.m. for 15 minutes. The ethanol: ether mixture containing the extracted lipids was carefully removed by use of a Pasteur pipette and the white protein precipitate resuspended in 40 ml of the ethanol:ether extraction solution. Large clumps of the protein were again dispersed by use of a glass rod. The protein preparation was extracted under previously described conditions for a further eight hours. The mixture was then centrifuged and the supernatant removed. The protein was suspended and dispersed in another 40 ml of the extraction solution and extraction carried out for an additional sixteen hours. The protein was spun down by centrifugation, the supernatant

removed and the protein resuspended in 40 ml of ether. The mixture was shaken 6 to 8 times by hand and then centrifuged. After removal of the ether, the ether extraction was repeated. The ether treatment is necessary to remove traces of water. Upon removal of the ether, the protein precipitate was dried under a stream of nitrogen. When the protein appeared dry, 2.0 ml of a 0.2M TRIS solution pH 8.2, containing 0.1 M sodium decyl sulfate (Swartz/Mann cat. #8519) was added. Complete solubilization of the protein was accomplished by an incubation of the solution at 37°C overnight. The apoprotein of α lipoprotein did not require the presence of sodium decyl sulfate and was therefore omitted from the 0.2M TRIS solution.

Analysis of the apoprotein preparation for phosphorus revealed only a trace quantity present. The protein recovered after delipidation and solubilization constituted approximately 90 percent of the original concentration of protein in the lipoprotein preparation.

The solubilized apoprotein mixture was stored at 4°C, or if a considerable delay was anticipated before using the apoprotein, it was stored as a dried powder (after nitrogen treatment) in a test tube at 4° Centigrade.

(e) Gel Filtration Chromatographic Separation and Molecular Weight Estimations of VLDL Apolipoproteins.

Gel filtration chromatography has been successfully used as a physical means of resolving apoVLDL into further protein components. Brown, et al (13) reported

Sephadex G-100 fractionation of Type IV and normal VLDL into three protein components as mentioned previously. However, resolution of the α apoprotein component from either the β or pre-β components was poor. In this investigation, Sephadex G-200 (Pharmacia, Uppsala, Sweden) was used to separate the protein components of pre-β apolipoprotein in hopes to achieving better resolution. Recently, Brown, et al (14) have demonstrated that Sephadex G-150 satisfactorily resolved the pre-β apolipoprotein into three components which were designated as Sephadex fraction (SF1) which is β apoprotein, SF2 which is α apoprotein, and SF3 which is unique to pre-β apolipoprotein and is probably apoprotein C as designated by Gustafson (80).

Sephadex G-200 was swollen over a 72 hour period in a 0.2M TRIS buffer solution, pH 8.2, which contained 2 mM sodium decyl sulfate. The prepared Sephadex was packed by gravity into a 2.5 x 110 cm glass column (Metalloglass Inc., Boston, Massachusetts). The column was equilibrated for 72 hours at room temperature with the eluting buffer, 0.2M TRIS, pH 8.2 containing 2 mM sodium decyl sulfate. The buffer was pumped through the column by a reverse flow technique using a peristaltic pump (Technicon Corp., Chauncey, New York) at a flow rate of approximately 10 ml/hour.

In order that different chromatographic elution profiles could be compared and molecular weight (M.W.) estimations of the SF3 component made, it was necessary that several column calibration standards be employed. These

standards consisted of:

(1) Blue Dextran 2000 (Pharmacia). M.W. of 2×10^6 and used for determining the column void volume (V_o)

(2) Aldolase (Pharmacia). M.W. of 158,000

(3) Bovine serum albumin (Sigma cat. #A-4503). M.W. of 67,000

(4) Ovalbumin (Pharmacia). M.W. of 45,000

(5) Chymotrypsin A (Pharmacia). M.W. of 25,000

(6) Potassium Iodide (Fisher cat. #P-256). M.W. of 166 and used for determining the column total bed volume (V_t).

The standards (approximately 2 mg) were pumped onto the column in a volume of 1 milliliter. The column eluant was collected in an LKB Model 7000 Ultrarac Fraction Collector (LKB Produkter, Sweden) at 12 minute intervals (approximately 2 ml/tube). The absorbance at 230 nm of each fraction was obtained by use of a Beckman Model DU Spectrophotometer equipped with a Gilford optical density converter. The elution volume (V_e) of each standard was determined by the tube number x, the volume of each tube at which the maximum $A_{230\ nm}$ was obtained. All chromatographic runs were performed at room temperature.

The apolipoprotein samples containing up to 20 mg in 5 ml of buffer were pumped onto the column, the fractions collected, and the $A_{230\ nm}$ measured as described above. The resulting fractions were appropriately pooled and dialyzed against deionized water. The resulting salt-free solutions

were then shell-frozen and lyophilized.

Molecular weights of the SF3 fraction of pre-β apolipoprotein were obtained by two methods. By plotting the partition coefficient (Kav) of each standard against the log M.W., a standard graph was obtained (106). The Kav values of the SF3 component was then applied to the graph and the M.W. obtained. The Kav was calculated according to the formula:

$$Kav = \frac{V_t - V_e}{V_t - V_o}$$

where: V_t = total bed volume (ml)

V_e = elution volume of the standard of SF3 fraction

V_o = void volume of the column.

The second method of calculating M.W. values was that of Ackers (107). The method involves calculating the Stokes radius of the SF3 component from column calibration factors (obtained from the standards) and then calculating the M.W. by the formula:

$$M.W. = \frac{4\ Na^3}{3\ \bar{v}\ (1.46)}$$

where: N = Avogadro's Number 6.02×10^{23}

a = Stokes radius in cm

$\bar{v}$ = partial specific volume of the protein.

The method by which the Stokes radius was calculated is outlined in Appendix 1.

(f) DEAE Cellulose Chromatography of SF3 Fractions.

Ion-exchange chromatographic analysis of the SF3 component from VLDL obtained by Sephadex G-200

chromatography was performed in order that the polypeptides of Type III and Type IV SF3 components could be isolated and compared for possible differences in polypeptide composition.

Whatman microgranular, pre-swollen DE52 DEAE cellulose (Scientifica Division, Reeve Angel, Clifton, New Jersey) was used as the anion exchange column packing.

The DEAE cellulose was pre-cycled by adding a suitable amount of DEAE cellulose to a 0.5N hydrochloric acid solution containing 1.0M sodium chloride. After allowing a suitable equilibration period (1-2 hr), the hydrochloric acid-sodium chloride solution was decanted and the DEAE cellulose washed with deionized water until the supernatant reached a neutral pH. A solution of 0.5N sodium hydroxide was then added and the mixture allowed to equilibrate for one hour. The pH was then adjusted to neutrality by successive washes of the DEAE cellulose. Degassing was accomplished by suspending the pre-cycled DEAE cellulose in a 0.1M TRIS buffer solution, pH approximately 4.0, and applying a strong vacuum to the suspension for one hour. Solid TRIS was added to the mixture until the pH reached 8.2 and the DEAE cellulose allowed to settle. If the pH of the supernatant was not 8.2, the DEAE cellulose was removed by filtration and resuspended in 0.1M TRIS, pH 8.2. This step was repeated until the supernatant reached a pH of 8.2.

A 0.9 x 30 cm glass column (Metalloglass Inc., Boston, Massachusetts) was packed, under pressure, with the above

prepared DEAE cellulose using 0.1M TRIS buffer, pH 8.2. The column was then equilibrated at 4°C with the starting buffer (.005M TRIS, 6M urea; pH 8.2) at a flow rate of 20 ml/hr for 24-48 hours. A peristaltic pump (Technicon Corp., Chauncey, New York) was employed.

The starting buffer solution was prepared by dissolving TRIS-hydrochloride (0.532 g) and TRIS-base (.197g) in 6M urea to a final volume of 1 liter. The correct preparation of 6M urea was important. An 8M urea solution was prepared by dissolving 480.48 g of urea (Fisher cat. #U-15) in deionized water to a total volume of 1 liter. When made up in this form, the urea solution contained ion impurities which resulted in a high specific conductivity (30 umho/cm) and $A_{230\ nm}$ (approximately 2.0). When the 8M urea solution was passed through a 2.5 x 25 cm column containing Rexyn I-300 (Fisher cat. #R-208), the resulting 8M urea solution had a specific conductivity of 1.0 umho/cm and an $A_{230\ nm}$ of approximately .085. The 8M urea solution was then appropriately diluted with deionized water to give a 6m urea solution.

The solution used for producing the sodium chloride gradient was the starting buffer which contained 0.08M sodium chloride. The gradient was produced by use of a Varigrad (Buchler Instr., Fort Lee, New Jersey) in which one tank contained 300 ml of the starting buffer (0.005M TRIS, 6M urea, pH 8.2) and the second tank contained 300 ml of a 0.005M TRIS, 0.08 sodium chloride, 6M urea, pH 8.2 solution.

The protein sample to be chromatographed was dissolved in 4.0 ml or less of the starting buffer and then dialyzed against the starting buffer overnight at $4^{o}C$ to insure the proper pH and specific conductivity. The sample was pumped onto the column and when complete, was followed by a wash of approximately 18 ml of the starting buffer. Following this, a sodium chloride gradient was applied to the column. Eluant fractions were collected in a fraction collector at 12-minute intervals giving an approximate volume of 4 ml per tube. The presence of protein in the eluant fractions was determined by the absorbance at 230 nm using the previously described spectrophotometer. The specific conductivity of each fraction was determined by use of a Y.S.I. Model 31 conductivity bridge (Yellow Springs Instruments Co. Inc., Yellow Springs, Ohio).

(g) Acrylamide Gel Disc Electrophoresis of the Apolipoproteins.

This was used to seek differences between Type III and Type IV VLDL apolipoproteins and their constituent polypeptides. The method differs greatly from the disc gel method previously described for electrophoretic analysis of lipoproteins in that the gel pore size of the separating gel was much smaller and 6M urea was necessary throughout the system to completely dissociate the protein components into their respective polypeptides.

Acrylamide separating gels of 7.5 and 15 percent acrylamide monomer were used while a 2 percent acrylamide

stacking gel was used. The buffer system of Reisfeld and Small (108) was employed. The disc electrophoresis apparatus used was a Canalco Model 12 equipped with running and destaining tubes and miscellaneous equipment (Canalco Inc., Rockville, Maryland). The power supply used was a Shandon Vokam SAE 2761 (Shandon Scientific Co. Ltd., Shandon, England).

The buffers and gel system required the presence of 6M urea having a low specific conductivity in order to avoid distortion of the electropherograms. It was therefore necessary to pre-treat the urea solution with an ion-exchange resin as previously described. It was also necessary to degas the urea solution, as polymerization of the gels might be inhibited by the presence of oxygen in the aqueous solutions. The following reagents were also required:

Reagent A: Acrylamide (6.0 g) and N, N'-methylene-bisacrylamide (0.15g) was dissolved in 6M urea to a total volume of 10 milliliters.

Reagent B: TRIS (4.5375 g) was dissolved in approximately 15 ml of 6M urea. To this solution, 1N hydrochloric acid (6.0 ml) and TEMED (0.06 ml) was added and the final volume brought to 25 milliliters. The resulting pH of the buffer was 9.4.

Reagent C: Ammonium persulfate (0.035 g) was dissolved in 25.0 ml of 6M urea.

Reagent D: Acrylamide (2.0 g) and N, N'-methylene-bisacrylamide (0.2 g) were dissolved in 6M urea to a final

volume of 25.0 milliliters.

Reagent E: TRIS (0.5575 g) was dissolved in approximately 20 ml of 6M urea. To this solution, 1M phosphoric acid (3.2 ml) and TEMED (0.025 ml) were added and the final volume brought up to 25.0 milliliters.

Reagent F: Riboflavin (approximately 0.5 mg) and ammonium persulfate (0.04 g) were dissolved in a final volume of 50.0 ml of 6M urea.

Upper reservoir buffer: TRIS (2.58 g) and glycine (1.743 g) were dissolved in a total volume of 500 ml of 6M urea. The resulting pH of the buffer was 8.9.

Lower reservoir buffer: TRIS (7.257) was dissolved in approximately 400 ml of deionized water. To this solution, 35.0 ml of 0.1N hydrochloric acid was added and the final volume brought to 500 ml with deionized water. The resulting pH of the buffer solution was 6.7.

Staining solution: Buffalo Black NBR (0.7 g) (Fisher cat. #NA-484) was dissolved in 100 ml of 7 percent acetic acid and filtered prior to each electrophoretic run.

Destaining solution: Glacial acetic acid (70 ml) (Fisher cat. #A-38) was mixed with 930 ml of deionized water to give a 7 percent acetic acid solution.

Preparation of gels: The separating gel was prepared by mixing 1 part of Reagent A, 1 part of Reagent B, and 2 parts of Reagent C. The solution was pipetted into the capped running tubes (5 x 75 mm) to a height of 45 mm after which degassed deionized water was carefully layered onto the

top of the gel. Polymerization was complete within 45 minutes. The spacer gel was prepared by mixing 1 part of Reagent D, 1 part of Reagent E, and 2 parts of Reagent F. The water overlay on the separating gel was removed by a Pasteur pipette, the tube rinsed with spacer gel solution, and then spacer gel solution pipetted onto the separating gel to a height of 20 millimeters. This was overlaid with water as previously described. Photopolymerization of the gel matrix was complete within 1 hour.

Sample preparation and electrophoresis: The running tubes containing the separating and spacer gels were mounted in the electrophoretic cell and the lower and upper buffer reserviors filled with their respective buffers. The protein sample solutions were adjusted to 0.2M TRIS, 6M urea, and 0.6M sucrose, the pH of the solution being 8.2. The sample solution was drawn into a microsyringe and 0.100 ml of the solution was carefully placed on the surface of the spacer gel. The dense sample solution displaced the much lighter buffer solution from the gel surface. Electrophoresis was performed for 2 hours at 2.5 ma/tube. The electrophoretic cell was cooled by circulating tap water at a temperature of approximately 10^{o} Centigrade.

Staining and destaining: The acrylamide gels were removed from the running tubes and placed in a 15 x 100 mm test tube containing approximately 8 ml of the staining solution. Staining was complete in 1 hour. The gels were

then placed in the destaining tubes, mounted in the electrophoretic cell and electrophoretically destained for 2 hours at 7 ma/tube using 7 percent acetic acid solution in both reservoirs.

The acrylamide gels were stored in 7 percent acetic acid. When permanent records of the electropherograms were required, photographs were taken of the separated polypeptide fractions using either a Polaroid or 35 mm camera.

(h) Amino Acid Analysis of the SF1 Component.

Due to the different elution patterns of SF1 from Type III VLDL obtained by G-200 chromatography, amino acid analysis was performed on the SF1 component. If significant differences between the Type III and Type IV SF1 components exist, amino acid analysis should reflect these differences.

Samples of the proteins (1-3 mg) were weighed into 15 x 125 mm test tubes containing 1 ml of 6N hydrochloric acid. The tubes were evacuated, degassed, and sealed. After hydrolysis at 110°C for timed periods (20-70 hr), the hydrolyzates were flash evaporated and redissolved in 0.5 to 1.5 ml of 0.2M lithium citrate buffer, pH 2.8. Aliquots were analyzed in a Technicon single column Amino Acid Analyzer (Technicon Corp., Chauncey, New York) with Chromobeads B resin and the lithium citrate buffer system of Perry, Stedman, and Hansen (109). Correction for loss of serine and threonine was made by extrapolation to zero time (110). Cystine, cysteine, tryptophan, and amide ammonia were not

determined.

2. Results

The data obtained from plasma lipid analysis, native lipoprotein, and apolipoprotein studies of Type III and Type IV VLDL are now presented.

The plasma lipid analysis of the Type III and IV patients selected for intensive study of their VLDL are given in Table VIII.

Agarose gel electrophoretic analysis of whole plasma of the two patients revealed distinct differences in the lipoprotein distribution. From Figure XII it can be seen that in the Type IV plasma, a distinct separation of the β and pre-β lipoproteins was achieved. However, electrophoretic analysis of the Type III plasma revealed a single but broad migrating band of which the trailing edge possessed β lipoprotein mobility. Also noteworthy was the presence of a large number of chylomicrons in the Type III plasma specimen which remained in the sample application slot.

Disc gel electrophoretic analysis of the two plasmas revealed slight differences. Observation of Figure XIII reveals that the pre-β lipoprotein band normally found in Type IV individuals is more diffuse in the Type III plasma. However, unlike the findings in agarose gel electrophoresis, there is a distinction between the β and pre-β lipoproteins in both plasmas. Thus, the electrophoretic results obtained by agarose gel and acrylamide gel indicate a different behaviour of the lipoprotein molecules in the two media.

TABLE VIII

PLASMA LIPID ANALYSIS OF A TYPE III AND A TYPE IV HYPERLIPOPROTEINEMIC PATIENT

Plasma Phenotype	Appearance of Plasma	Triglycerides (mg/100 ml)	Cholesterol (mg/100 ml)	Total Lipids (mg/100 ml)
Type III	Cloudy with creamy layer	1,300	375	2,100
Type IV	Cloudy	560	193	1,240

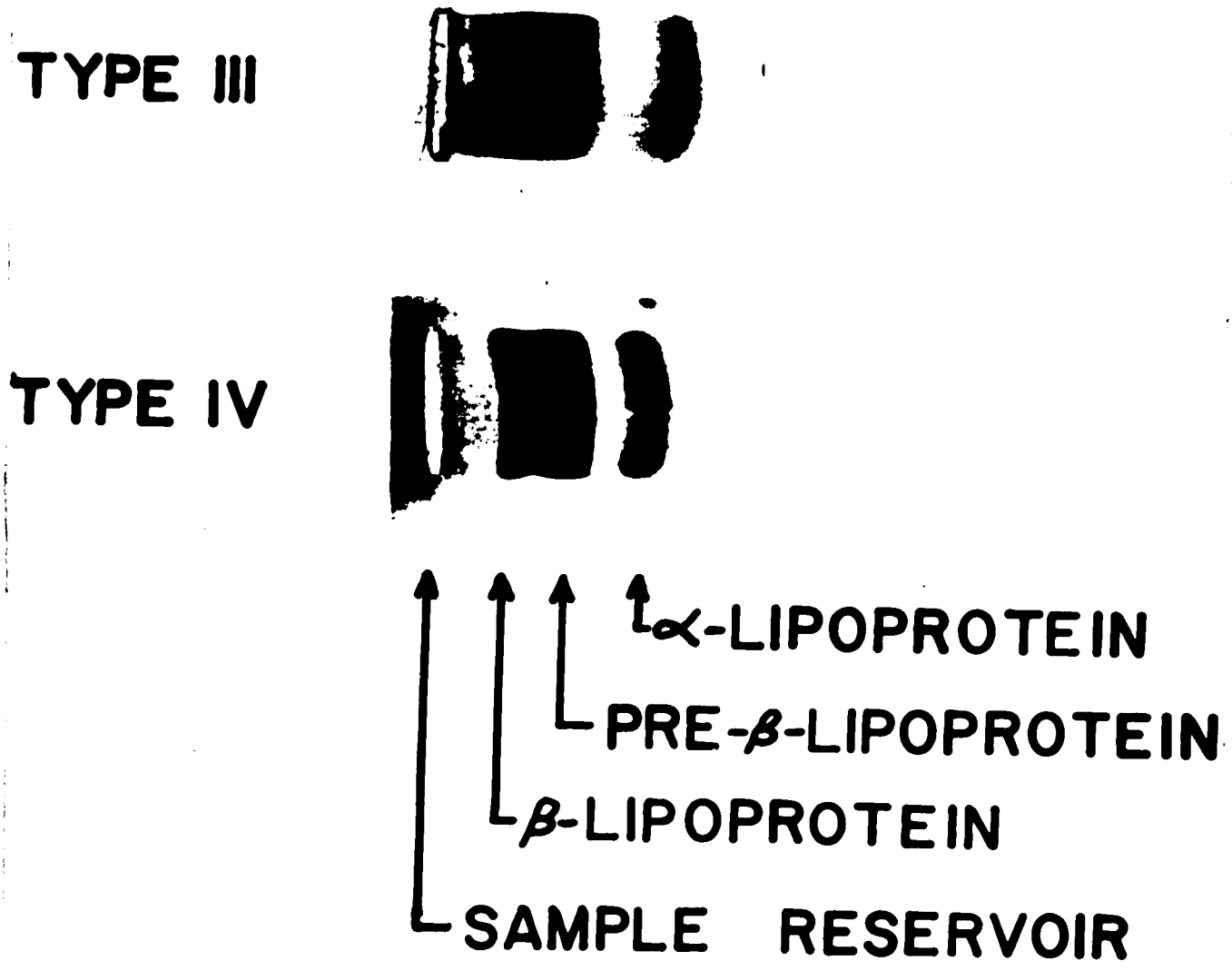


FIGURE XII. Agarose gel electrophoretic separation of plasma lipoproteins of Type III and Type IV hyperlipoproteinemias.

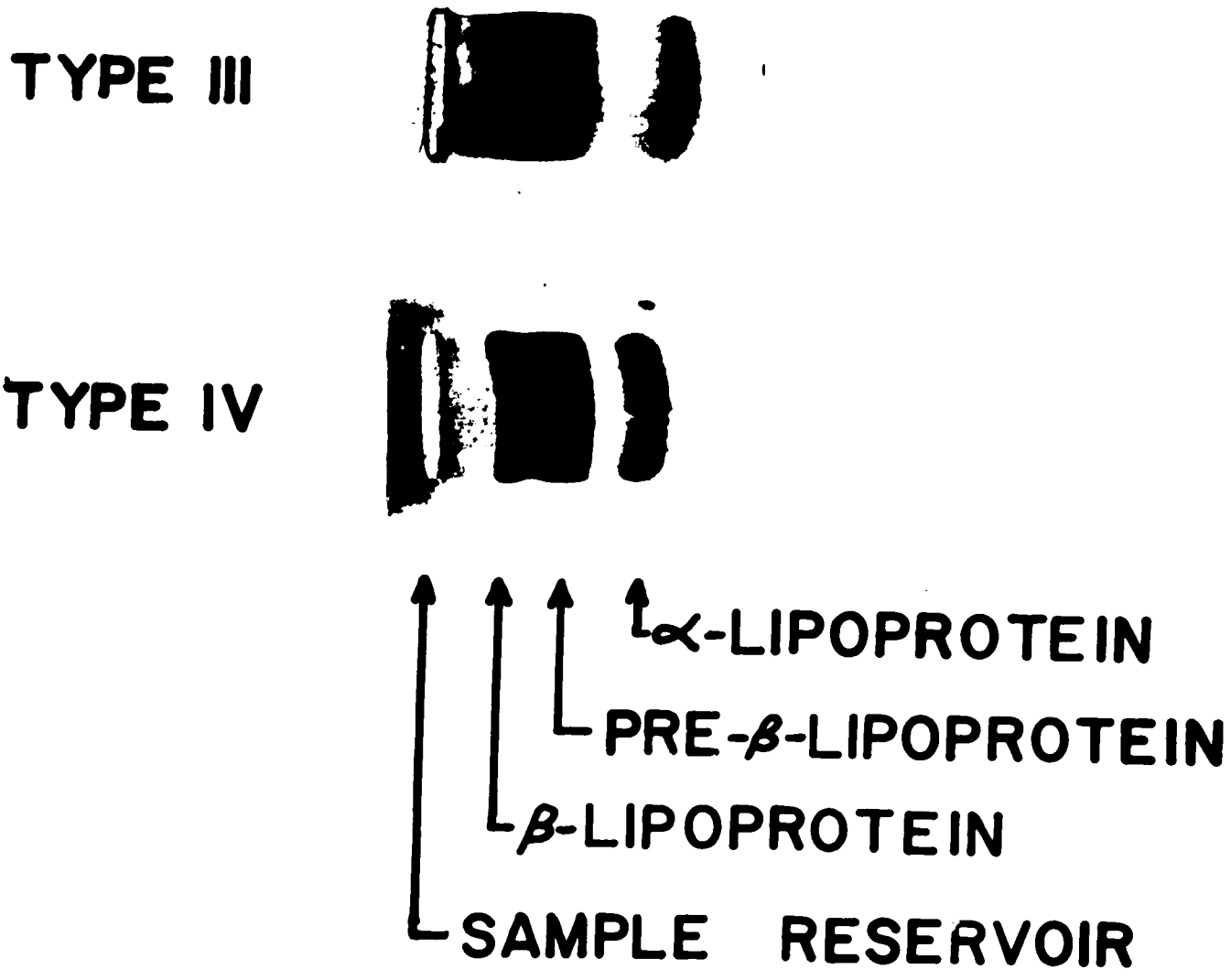


FIGURE XII. Agarose gel electrophoretic separation of plasma lipoproteins of Type III and Type IV hyperlipoproteinemias.

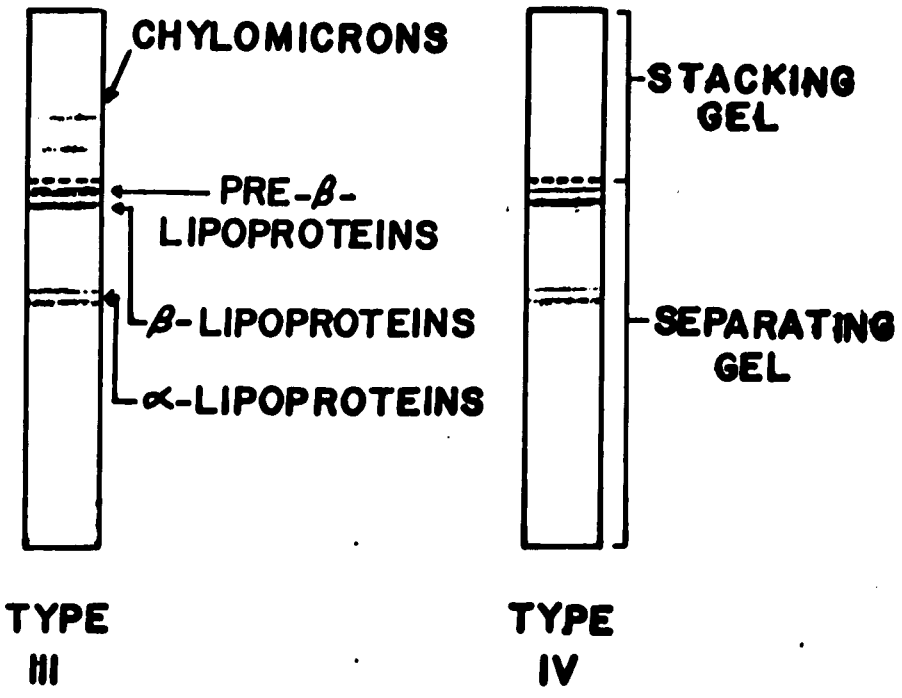


FIGURE XIII. Acrylamide gel disc electrophoretic separation of plasma lipoproteins of Type III and Type IV hyperlipoproteinemias.

This is likely due to the molecular sieving effecting of the acrylamide gel matrix which would tend to retain the larger lipoprotein molecules, such as the S_f 20-400 species. Thus, in acrylamide gel, the "broad-β" lipoprotein electrophoretic migration characteristics resemble the normal pre-β lipoprotein.

From the above findings, it appears that we are dealing with Type III and Type IV hyperlipoproteinemic plasmas. However, as stated by Fredrickson and Lees (6), for Type III phenotyping it is necessary to demonstrate a β-migrating lipoprotein which floats as a S_f 20-400 lipoprotein. Preparative ultracentrifugation, as previously described, was therefore performed on the Type III and Type IV plasma. In addition to studying the electrophoretic properties of the isolated lipoprotein fractions, other physical and chemical characteristics were investigated. These included disc gel electrophoresis, chemical composition, and analytical ultracentrifugation studies.

Agarose gel electrophoresis of the Type III and Type IV ultracentrifuge isolated lipoprotein fractions confirmed the Type III diagnosis. Note in Figure XIV that the Type III S_f 20-400 lipoproteins migrate as a β lipoprotein, but smear forward into the pre-β position. In contrast, the Type IV S_f 20-400 lipoprotein fraction migrates as distinct pre-β lipoproteins. In both cases, the S_f 0-20 lipoprotein migrate as β lipoproteins.

When acrylamide gel disc electrophoresis was done on

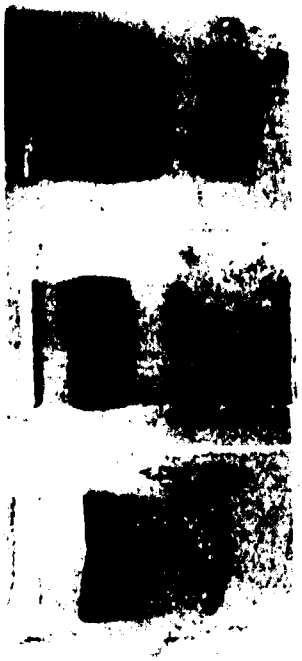


FIGURE XIV. Agarose gel electrophoresis of Type III and Type IV S_f 20-400 and S_f 0-20 lipoprotein fractions.

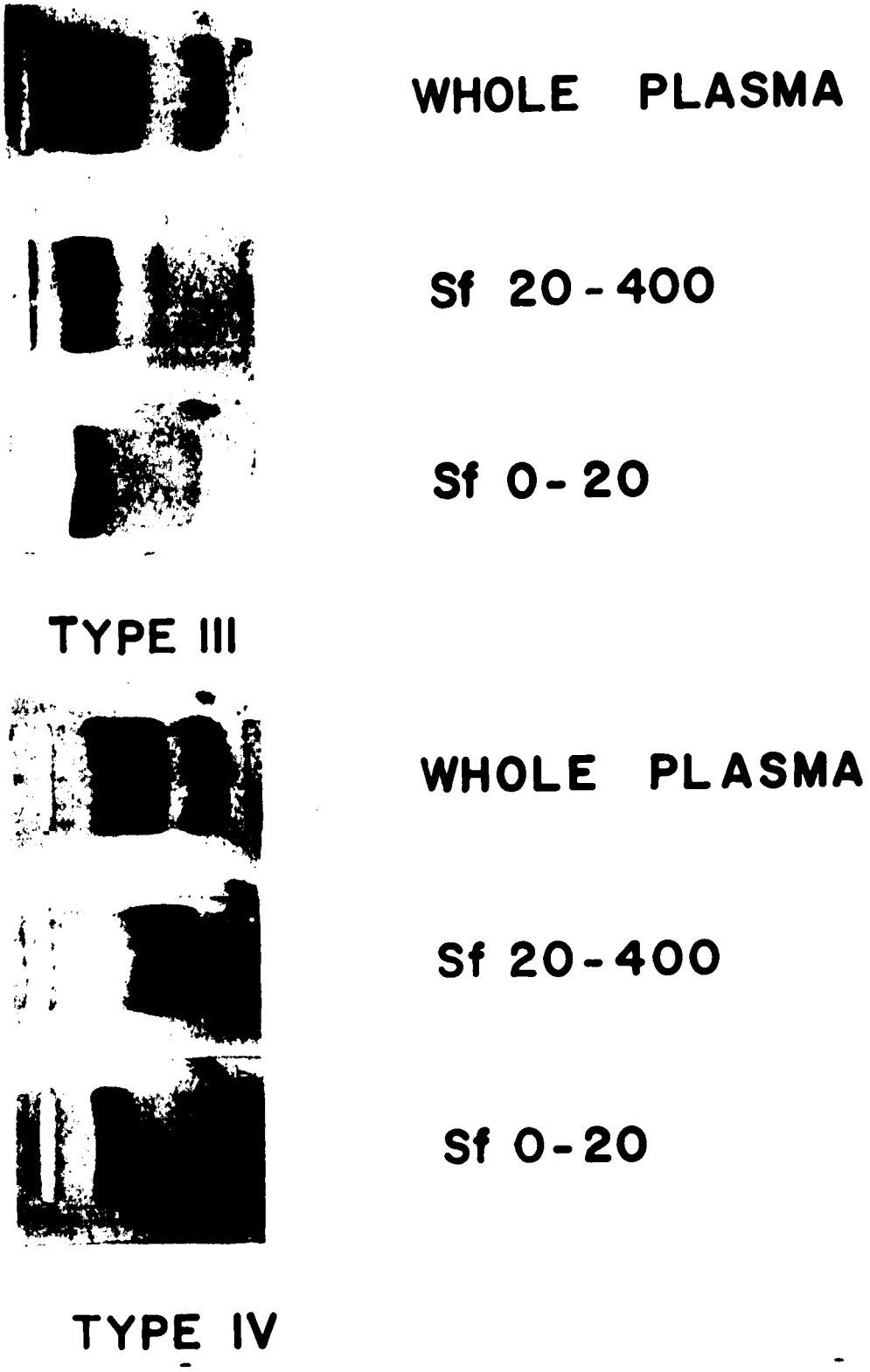


FIGURE XIV. Agarose gel electrophoresis of Type III and Type IV S_f 20-400 and S_f 0-20 lipoprotein fractions.

these fractions, little difference in migration characteristics was observed, as evident from Figure XV. It cannot be stated that the diffuse appearance of the Type III S_f 20-400 lipoprotein band is characteristic of Type III hyperlipoproteinemia, however it appears that the use of acrylamide gel disc electrophoresis for phenotyping Type III plasmas and their lipoprotein fractions is of little use.

To further demonstrate differences between Type III and Type IV VLDL, the chemical composition of the ultracentrifugally isolated lipoprotein fractions was performed. The results of this study (Table IX) reveal a much higher cholesterol content of the Type III S_f 20-400 fraction relative to that found in Type IV and normal S_f 20-400 lipoproteins. It is also evident that a much lower triglyceride content exists in the Type III S_f 20-400 lipoproteins. These results agree with those of Hazzard, et al (111).

The lipid composition of S_f 0-20 lipoproteins of both Type III and Type IV plasmas is similar except for an increased amount of triglycerides in the Type III fraction. There have been no reports on the lipid composition of the S_f 0-20 lipoproteins found in Type III hyperlipoproteinemia. Whether or not the finding of an unusually elevated proportion of triglycerides in the Type III S_f 0-20 lipoproteins is significant in the pathogenesis of the hyperlipoproteinemic condition cannot be stated at the present time.

In order to compare the flotation behaviour of the Type III and Type IV lipoprotein fractions, analytical

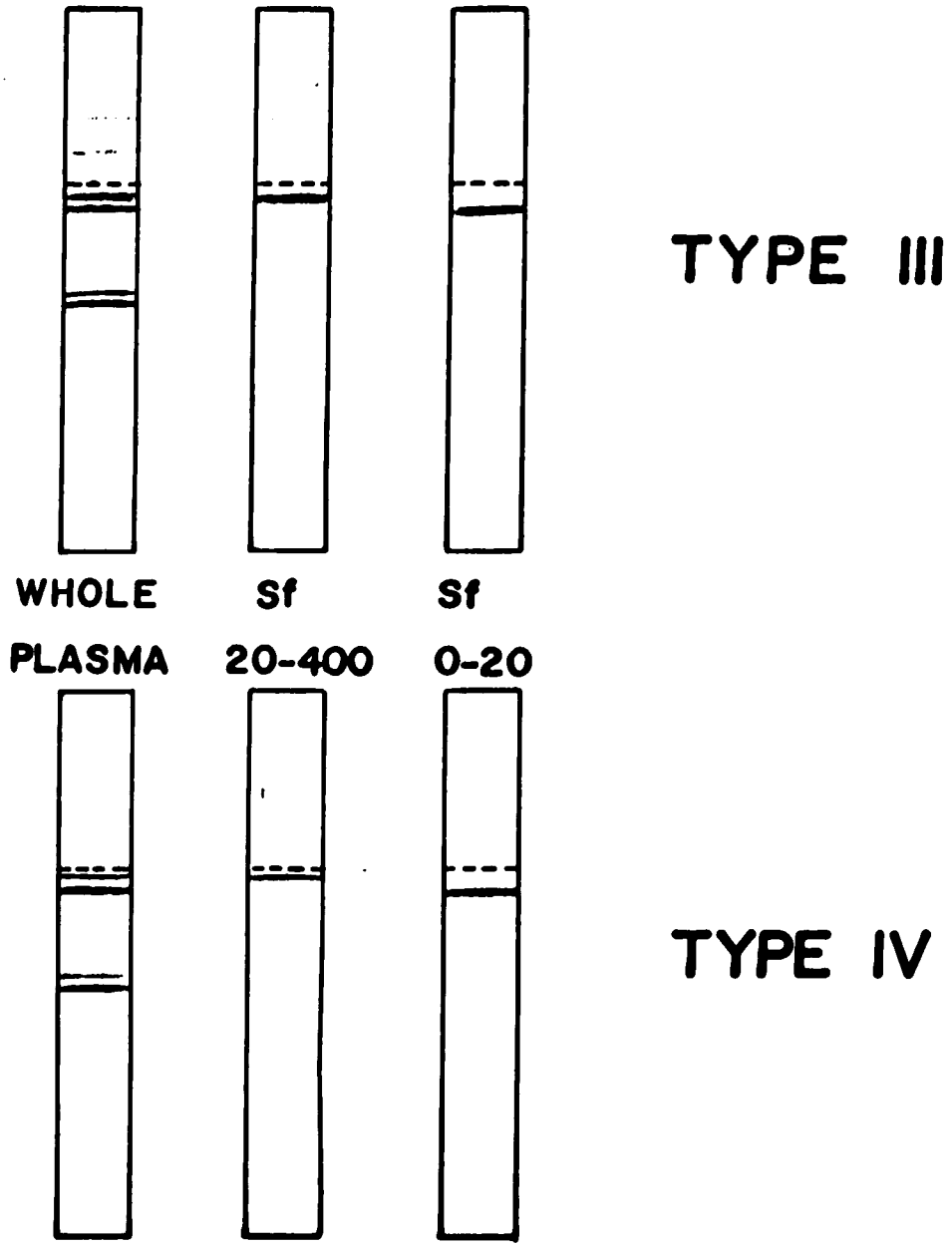


FIGURE XV. Acrylamide gel disc electrophoresis of Type III and Type IV S_f 20-400 and S_f 0-20 lipoprotein fractions.

TABLE IX

THE CHEMICAL COMPOSITION OF S_f 20-400 AND S_f 0-20 LIPOPROTEIN FRACTIONS ISOLATED FROM TYPE III AND TYPE IV PLASMA

	Cholesterol (mg/100 ml)	Triglycerides (mg/100 ml)	Phospholipids (mg/100 ml)	Protein (mg/100 ml)
Normal S_f 20-400 (86)	13-18	50-60	13-20	5-12
Type III S_f 20-400	27	47	17	9
Type IV S_f 20-400	14	64	12	10
Normal S_f 0-20 (86)	46	12	20	22
Type III S_f 0-20	35	21	21	23
Type IV S_f 0-20	36	13	18	33

ultracentrifugation was carried out as previously described. The obtained S_f values were not corrected for concentration effects in that low concentrations were used throughout the flotation runs and the correction factor for concentration would not significantly alter the S_f values.

The results of the average S_f values obtained from S_f 0-20 and S_f 20-400 lipoproteins of Type III and Type IV plasmas are summarized in Table X. It is apparent that little difference in the S_f 20-400 main peak existed between Type III and Type IV plasmas. The presence of an S_f 19.5 peak in the S_f 0-20 fraction of Type IV plasma is in agreement with the observation of Fisher (112) in that an S_f 20 lipoprotein frequently occurs in abundance in the plasma of patients affected with hyper pre-β lipoproteinemia.

To investigate the protein moiety of the Type III and Type IV lipoprotein fractions, delipidation of the lipoprotein fractions was performed as previously described. A preliminary study of the resulting apolipoproteins was accomplished through acrylamide gel disc electrophoresis employing the 6M urea system.

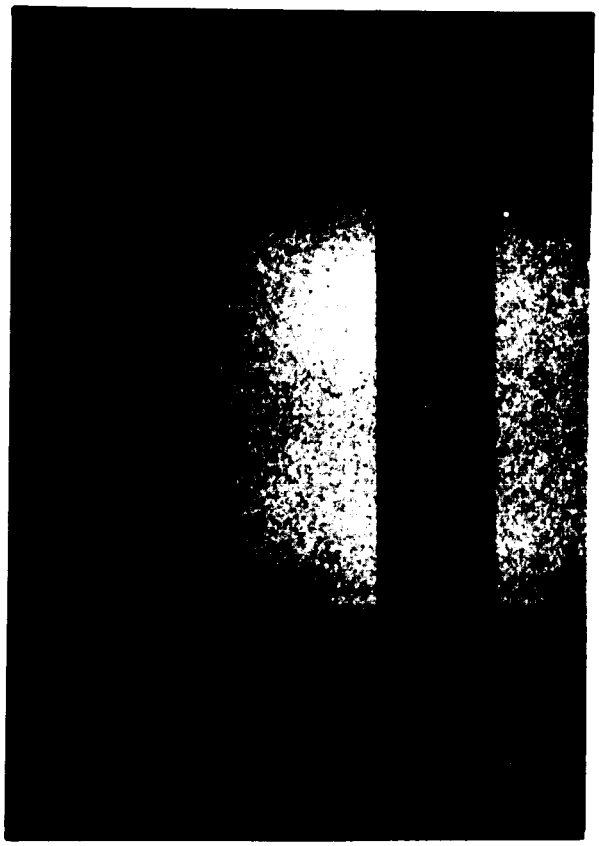
It can be seen from Figure XVI that the apolipoprotein of the Type III S_f 20-400 lipoproteins differs considerably from the corresponding Type IV apolipoprotein. The presence of several additional protein bands in the upper portion of the separating gel is observed in the Type III apolipoprotein.

In light of the above findings, fractionation of the apolipoproteins found in Type III S_f 20-400 lipoproteins by

TABLE X

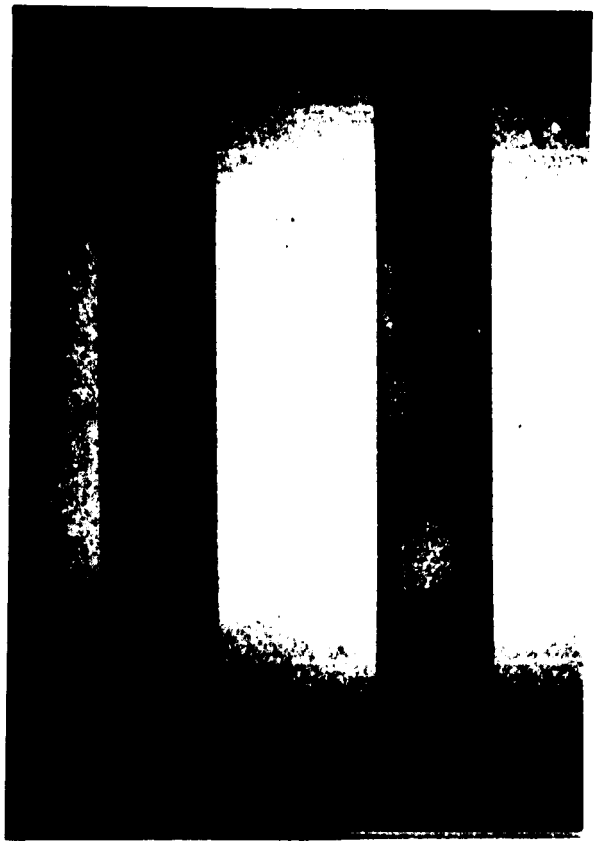
THE S_f VALUES OBTAINED BY ANALYTICAL ULTRACENTRIFUGATION OF TYPE III AND TYPE IV S_f 20-400 AND S_f 0-20 LIPOPROTEINS

	S_f (main peak)	S_f (additional peaks)
S_f 0-20 Type III Plasma	6	-
S_f 0-20 Type IV Plasma	4	19.5
S_f 20-400 Type III Plasma	54	-
S_f 20-400 Type IV Plasma	40	-



A B

FIGURE XVI. Acrylamide gel disc electrophoresis of the apolipoproteins isolated from Type III (A) and Type IV (B) S_f 20-400 lipoproteins.



A B

FIGURE XVI. Acrylamide gel disc electrophoresis of the apolipoproteins isolated from Type III (A) and Type IV (B) S_f 20-400 lipoproteins.

gel filtration chromatography was pursued in order that these unusual protein bands could be characterized. This was performed on the following apolipoprotein preparations:

1. S_f 20-400 lipoproteins from Type III plasma
2. S_f 20-400 lipoproteins from Type IV plasma
3. S_f 0-20 lipoproteins from Type III plasma
4. S_f 0-20 lipoproteins from Type IV plasma
5. S_f <0 lipoproteins isolated from normal plasma

In order that a reasonable comparison could be made between the elution profiles of the apolipoproteins of Type III and Type IV S_f 20-400 lipoproteins, equal amounts of the apolipoproteins were applied to the Sephadex G-200 column. Prior to each chromatographic run, a solution containing approximately 2 mg of Dextran Blue 2000 in a volume of 1.0 ml was applied to the column followed two hours later by an application of 2 mg of potassium iodide in a volume of 1.0 ml to the column. By determining the elution volumes of Dextran Blue 2000 and potassium iodide, the V_o and V_t of the column could be calculated. This was necessary to insure that the two chromatographic runs could be directly comparable with respect to the elution volumes (V_e) of the protein peaks.

The elution profile of the apolipoprotein of S_f 20-400 lipoproteins from Type IV plasma is illustrated in Figure XVII. It can be seen that two major and one minor protein peaks result from gel filtration. The first peak, SF1, is eluted at the V_o of the column. The second peak, SF2, appears as a shoulder on the third peak, SF3. When the apolipoprotein

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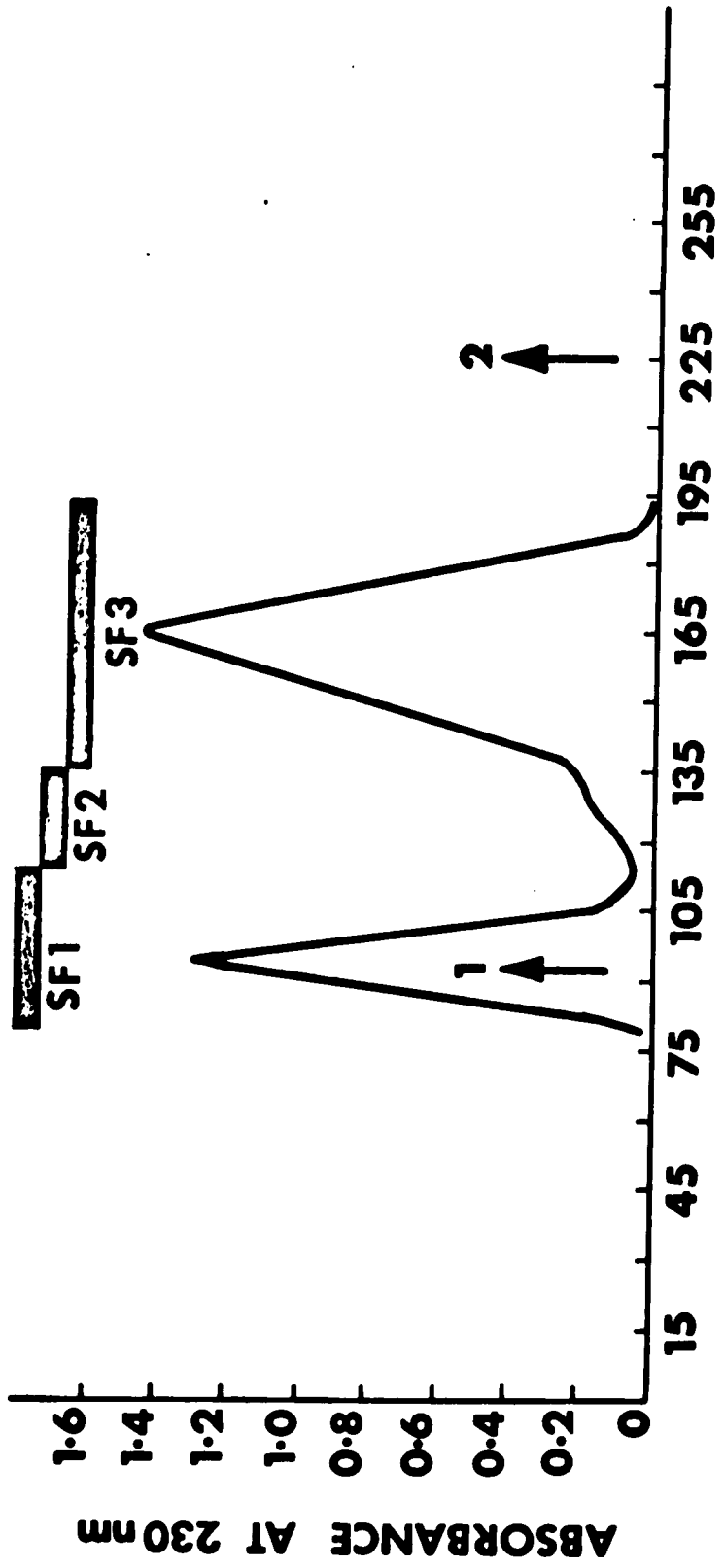


FIGURE XVII. Sephadex G-200 fractionation of the apolipoprotein obtained from the S$_f$ 20-400 lipoproteins of a Type IV hyperlipoproteinemic patient. The numbered arrows, 1 and 2, indicate the void volume and total bed volume of the column. Approximately 15 Units of protein were applied to the column.

of the S_f 0-20 lipoprotein fraction from Type IV plasma was applied to the column, a single peak, elution at the V_o of the column, resulted. Thus, it appears that the SF1 protein component of the Type IV delipidated S_f 20-400 lipoproteins consists of the only protein found in the S_f 0-20 lipoprotein, commonly known as β apoprotein which consists of the polypeptide apoLp-Serine. Further evidence of the apoβ and SF1 similarity will be presented in later studies of this investigation.

When the apolipoprotein of the S_f <0 lipoproteins was applied to the column, the main peak was eluted in a volume similar to the SF2 peak; however, it did spread into the SF3 peak. It would thus appear that the SF2 peak of the apolipoprotein of S_f 20-400 lipoproteins isolated from Type IV plasma would correspond to the major protein found in the S_f <0 lipoproteins, commonly known as α apoproteins, and consisting of the polypeptides apoLp-Threo and apoLp-Glutamine.

The three protein peaks obtained by gel filtration of the apolipoprotein of S_f 20-400 lipoproteins from Type IV plasma are consistent with the findings of Brown, et al (14) who used Sephadex G-150.

After the SF1, SF2, and SF3 components were separately pooled and dialyzed against deionized water as previously described, they were redissolved in 0.2M TRIS, pH 8.2 solution containing 0.1M sodium decyl sulfate. Protein analysis of the fractions, as previously described, revealed

that the apolipoprotein consisted of approximately 35 percent SF1, 4 percent SF2, and 62 percent SF3 by weight. It is quite likely that the SF2 component may be slightly underestimated and SF3 overestimated in that clear resolution of the two peaks was not accomplished. Despite this, the percent composition values of this study agree well with those reported by Brown, et al (14) in which the relative proportions of the three protein fractions were: SF1, 40 percent; SF2, 10 percent; and SF3, 50 percent.

The apolipoprotein of the S_f 20-400 lipoproteins isolated from Type III plasma was then applied to the G-200 column in the same manner as previously described. The elution profile of the chromatographic run is illustrated in Figure XVIII. Note the three protein components similar to those obtained from the Type IV apolipoprotein are present. However, two distinct differences are apparent:

1. The SF1, SF2, and SF3 components of Type III are different from the Type IV apolipoprotein in their relative amounts. The Type III SF1 component is present in a much larger proportion of the total apolipoprotein.

2. A distinct shoulder on the SF1 component peak is present. Of extreme interest is a possibility of an unusual polypeptide composition of the Type III apolipoprotein.

To demonstrate the differences in relative amounts of protein in each column fraction, the SF1 (containing the shoulder peak), SF2 and SF3 components were prepared for protein analysis by appropriate pooling of tubes dialysis

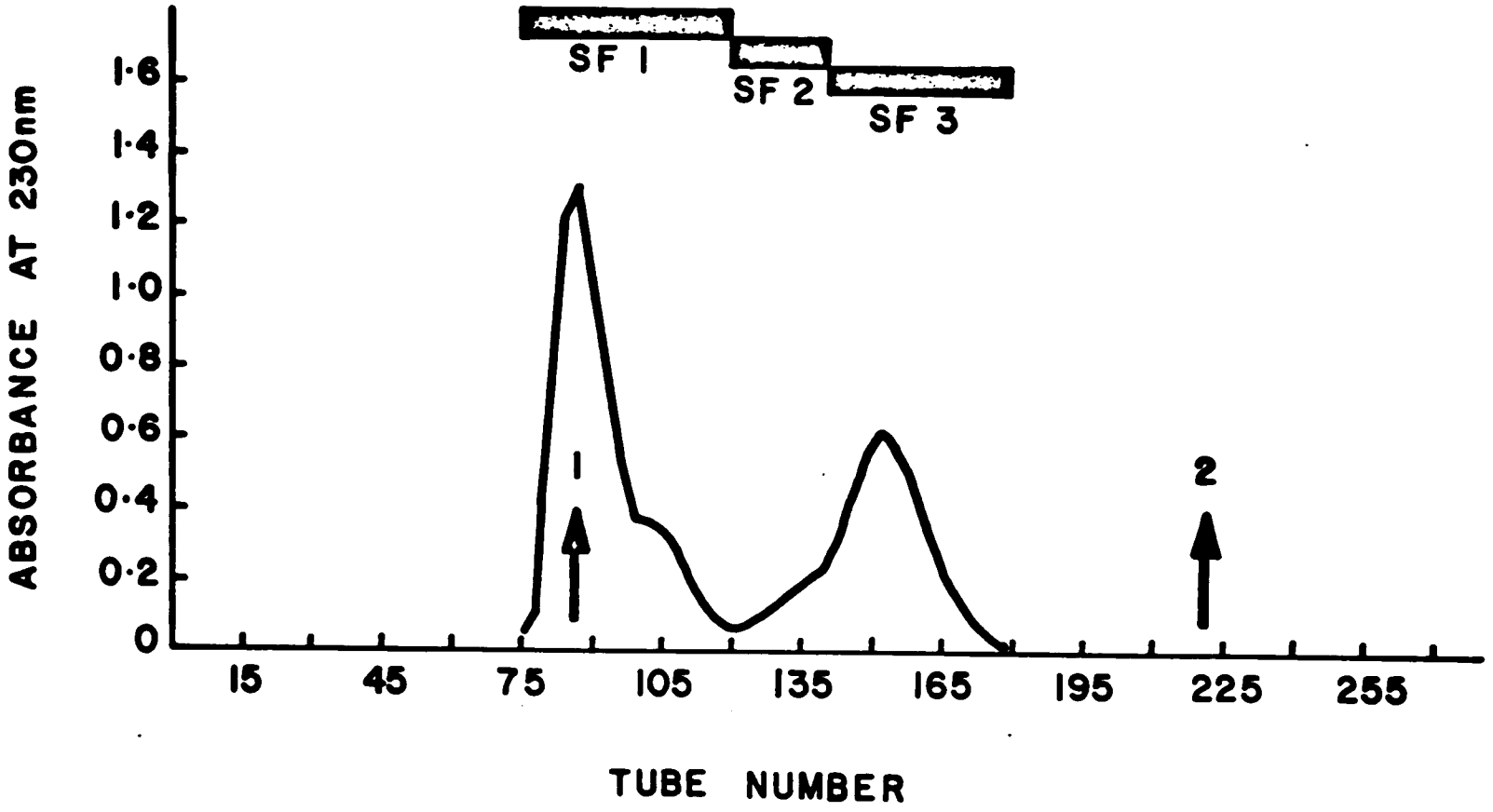


FIGURE XVIII. Sephadex G-200 fractionation of the apolipoprotein obtained from the S_f 20-400 lipoproteins of a Type III hyperlipoproteinemic patient. The numbered arrows, 1 and 2, indicate the void volume and total bed volume of the column. Approximately 15 Units of protein were applied to the column.

against deionized water and freeze drying as previously described. The protein residues were redissolved in a 0.2M TRIS, pH 8.2 solution containing 0.1M sodium decyl sulfate. Protein analysis on the resulting protein solution revealed that the apolipoprotein of Type III VLDL contained 62 percent SF1, 2 percent SF2, and 36 percent SF3 components. When these results are compared to those obtained on the Type IV apolipoprotein (Table XI), it can be seen that the relative amount of SF1 in Type III apolipoprotein is much higher than that found in Type IV apolipoprotein. The data in Table XI represents duplicate chromatographic analyses of two separate runs each of Type IV and Type III apolipoproteins of S_f 20-400 lipoproteins. Also included are the results of chromatographic analyses of apolipoproteins isolated from six different S_f 20-400 lipoproteins obtained from outdated plasma specimens.

It is apparent that when the SF1 and SF3 components are expressed as a ratio, the Type III and Type IV apolipoproteins differ considerably in that the Type III exhibits a greater proportion of the SF1 component.

This finding of an increased proportion of SF1 (β apoprotein) in the Type III apolipoprotein of S_f 20-400 lipoproteins provides an explanation for the β-migrating properties of the Type III S_f 20-400 lipoproteins. Also, the increased cholesterol composition of Type III S_f 20-400 lipoproteins may be explained by the increased amounts of the cholesterol-carrying β apoprotein.

TABLE XI

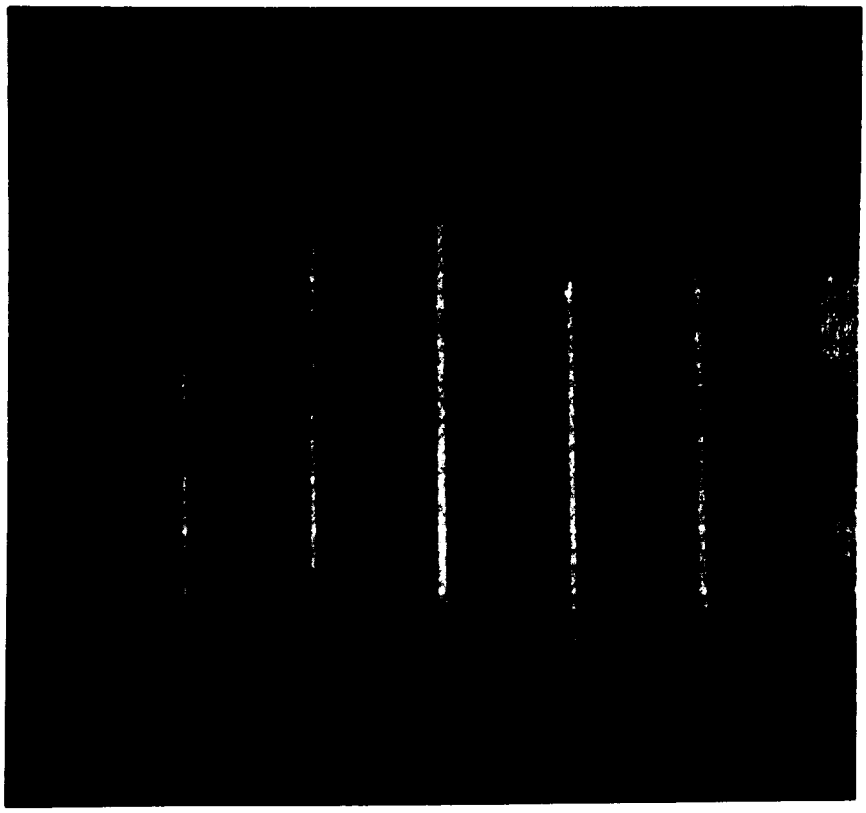
THE DISTRIBUTION OF SF1, SF2, AND SF3 PROTEIN COMPONENTS FOUND IN THE APOLIPOPROTEINS ISOLATED FROM S_f 20-400 LIPOPROTEINS OF TYPE III AND TYPE IV PLASMAS

	SF1 (% wt)	SF2 (% wt)	SF3 (% wt)	SF1:SF3 Ratio
Type III	62	2	36	1.7
Type IV	36	3	61	0.6
Outdated Plasmas (n=6)	30-39	3-5	58-68	0.5-0.7

With this information in hand, it was thought that a study of the polypeptide composition of the Type III SF1 component might be of value. Acrylamide gel disc electrophoretic analysis of the SF1 component would reveal any additional polypeptides in the Type III SF1 component. When acrylamide gel disc electrophoresis of the Type III and Type IV SF1 components was performed, at least three protein bands were observed in the Type III SF1 component which were not apparent in the Type IV SF1 component (Figure XIX). The SF1 fraction isolated from several other G-200 chromatographic runs of Type IV apolipoproteins of S_f 20-400 lipoproteins failed to exhibit the peculiar protein bands observed in the Type III SF1 component. Common to the Type III SF1 and Type IV SF1 components was the presence of β apoprotein, which was retained at the interface between the separating and stacking gels.

When the apolipoproteins of S_f 0-20 lipoproteins from Type III and Type IV plasmas were subjected to disc electrophoresis, only the β apoprotein band was observed in both cases.

In order that the additional protein bands found in the Type III SF1 component could be more clearly resolved, the separating gel was diluted to give a 7.5 percent acrylamide monomer concentration. When the SF1 protein component of the Type III and IV apolipoproteins (S_f 20-400 lipoproteins) was subjected to disc gel electrophoresis in a 7.5 percent gel, four additional protein bands were readily



A B C D E F

FIGURE XIX. Acrylamide gel disc electrophoresis of Type III and Type IV SF1 components;

A. β apolipoprotein
B. Type IV SF1 component
C. Type III SF1 component
D. Combined Type III SF1 component and α apolipoprotein
E. Combined Type IV SF1 component and α apolipoprotein
F. α apolipoprotein

A B C D E F

FIGURE XIX. Acrylamide gel disc electrophoresis of Type III and Type IV SF1 components;

A. β apolipoprotein
B. Type IV SF1 component
C. Type III SF1 component
D. Combined Type III SF1 component and α apolipoprotein
E. Combined Type IV SF1 component and α apolipoprotein
F. α apolipoprotein

observed in the Type III SF1 component, as seen in Figure XIX. The possibility of these additional bands being the result of SF2 contamination was investigated. The addition of a suitable amount of α apolipoprotein to Type III SF1 with subsequent disc gel electrophoretic analysis in 7.5 percent gel, revealed the protein bands of Type III SF1 component to be completely different from those characteristic of α apolipoprotein. The electropherograms shown in Figure XIX reveal the four bands of Type III SF1 to be distinct from the three bands of α apolipoprotein.

It is evident that the SF1 (β apoprotein) component of the apolipoprotein of Type III and IV S_f 20-400 lipoproteins is different. If these differences represent additional peptides, amino acid analysis of the Type III SF1 component should reveal the differences. To investigate this possibility, amino acid analysis was carried out on β apoprotein, Type III SF1, and Type IV SF1.

The results are summarized in Table XII. From this table it can be seen that the differences between SF1 Type IV and β apoprotein amino acid composition were non-significant. The slight differences in amino acid composition can probably be attributed to the heterogeneity of β apoprotein. A comparison of the amino acid analysis of SF1 Type III and SF1 Type IV or β apoprotein reveals significant differences in composition for most of the amino acids. The most notable differences exist between the relative amounts of aspartic acid, glutamic acid, isoleucine, phenylalanine, and histidine.

TABLE XII

AMINO ACID COMPOSITION OF APOLDL AND THE CORRESPONDING APOPROTEIN COMPONENTS OF VLDL FROM PATIENTS WITH TYPE IV AND TYPE III HYPERLIPOPROTEINEMIA

Mean ± standard error of the mean in moles/100 moles of amino acids.

	apoLDL[a]	P*	SF1 Type IV[b]	P**	SF1 Type III[c]
Asp	11.38±0.06	NS	11.42±0.10	<<0.0005	9.62±0.07
Thr	6.40±0.04	NS	6.19±0.09	<0.0005	5.55±0.11
Ser	8.75±0.22	NS	7.57±0.16	<<0.025	7.65±0.30
Glu	12.73±0.06	NS	13.00±0.22	<<0.0005	16.36±0.09
Pro	3.25±0.13	NS	3.30±0.21	NS	2.93±0.19
Gly	4.85±0.48	<0.01	5.06±0.18	<0.0005	5.45±0.03
Ala	6.34±0.15	NS	6.16±0.10	<0.0005	7.63±0.21
Val	5.76±0.09	NS	5.76±0.11	<0.05	6.06±0.10
Met	1.52±0.04	NS	1.41±0.06	<0.005	1.67±0.02
Ile	6.16±0.10	NS	6.12±0.08	<<0.0005	4.47±0.10
Leu	11.65±0.08	NS	11.56±0.08	NS	11.68±0.25
Tyr	3.10±0.03	NS	3.03±0.06	<0.0005	2.60±0.05
Phe	5.02±0.06	<0.01	4.75±0.07	<<0.0005	3.62±0.01
Lys	8.20±0.31	NS	8.26±0.28	<0.025	7.46±0.20
His	2.28±0.07	NS	2.29±0.07	<0.0005	1.92±0.02
Arg	3.78±0.09	NS	3.89±0.19	<<0.0005	6.12±0.04

a: Duplicate hydrolyses at each of 20, 40, and 70 hours.

b: Duplicate hydrolyses at 20, 40, and 70 hours of samples from two different patients.

c: Hydrolyses at 20 and 40 hours.

* Probability that the means of the populations of apoLDL and SF1 Type IV do not differ ("Student's" t-test).

** Probability that the means of the populations of SF1 Type IV and SF1 Type III do not differ ("Student's" t-test).

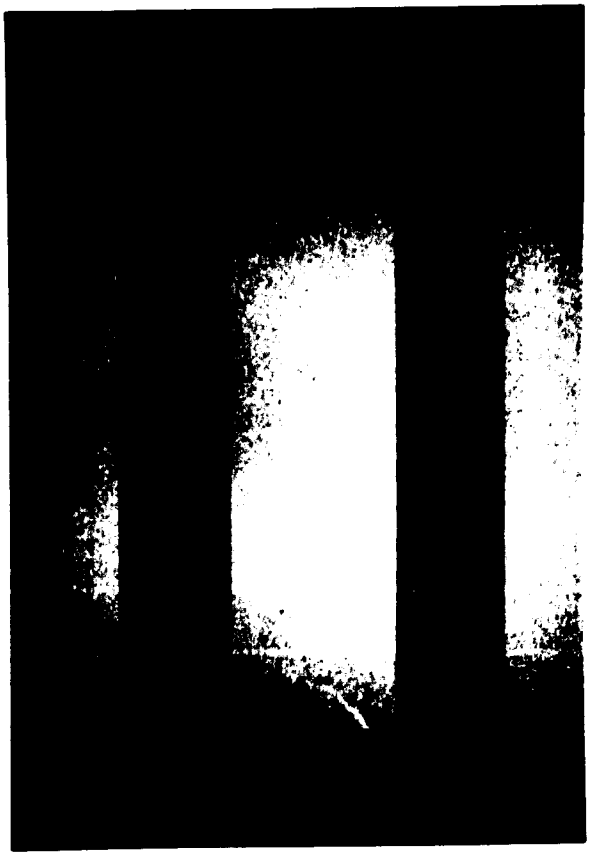
It therefore appears that the β apoprotein component of the apolipoproteins of Type III S_f 20-400 lipoproteins contains additional proteins or polypeptides not normally found in the SF1 component of normals or Type IV patients. Evidence for the presence of these additional proteins or polypeptides has been obtained from gel filtration, acrylamide gel disc electrophoretic and amino acid composition studies. Unfortunately, only one Type III hyperlipoproteinemic patient was available at this time. Thus, it cannot be stated that these peculiar proteins are characteristic of the "broad-β" lipoprotein. Future investigations will be dependent on the detection of other Type III individuals with subsequent biochemical and physical studies as reported on the present investigation.

It was previously noted that approximately 3 percent of the Type III and Type IV apolipoprotein of S_f 20-400 lipoproteins contained α apoprotein (SF2 component). Due to the limited amounts of SF2 available for further studies, very limited and inconclusive statements can be made about this protein component. It has been demonstrated by Gustafson (81), Brown, et al (13), and Levy, et al (113) that α apoprotein is found in small amounts in the pre-β apolipoprotein. The statement made in this investigation that the SF2 component is α apoprotein is based on a comparison of the elution profile of the apolipoprotein of S_f 20-400 lipoproteins to that shown by Brown, et al (14).

When a solution of α apoprotein was subjected to

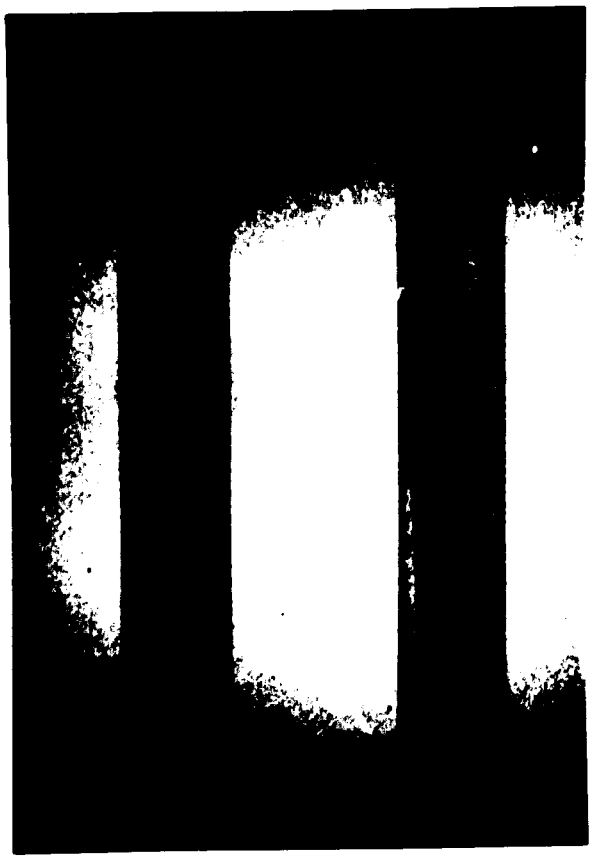
Sephadex G-200 column chromatography, its main protein peak appeared to resemble the elution characteristics of the SF3 component rather than the SF2 component peak. Acrylamide gel disc electrophoresis, using the 6M urea system, was performed on the SF2 fraction and α apoprotein; there was some similarity between the two apoproteins. From Figure XX it can be seen that the most prominant band of α apoprotein corresponds to the only band seen in the SF2 component. None of the minor bands seen in α apoprotein was visible in the SF2 component. Future investigations in this area will require adequate amounts of the SF2 component and a more suitable system for resolving the SF2 and SF3 components of the apolipoprotein of S_f 20-400 lipoproteins.

The finding of a different SF1 component of the apolipoprotein of Type III S_f 20-400 lipoproteins provided explanations for the unusual physical and chemical properties of the lipoprotein species. However, the possibility that the Type III SF3 component also differs from the normal or Type IV SF3 component exists, and therefore several physical characteristics of the SF3 component were investigated. These included M.W. estimations by G-200 gel filtration chromatography and DEAE cellulose chromatographic resolution of the polypeptides found in the SF3 component. Acrylamide gel disc electrophoresis was also performed on the SF3 component and its respective polypeptides. Insufficient amounts of the Type III SF3 component were available for



A B

FIGURE XX. Acrylamide gel disc electrophoresis of α apolipoprotein (A) and SF2 component (B) in 15 percent gel.



A B

FIGURE XX. Acrylamide gel disc electrophoresis of α apolipoprotein (A) and SF2 component (B) in 15 percent gel.

amino acid analysis.

As mentioned previously, the two approaches to M.W. estimations used were those of Andrews (106) and Ackers (107). The method of Andrews is based on the relationship of the V_e of a protein being determined by the M.W. of the protein. The method gives a fairly accurate estimation of the M.W. provided the protein is spherical. However, the degree of asymmetry and hydration of a molecule do affect the V_e of a protein (107) and thus can lead to erroneous results.

As mentioned previously, four protein standards were used for obtaining the standard graph by the Andrew technique. The Kav of each standard was calculated from the V_o and V_t data as previously described and the Kav of each standard plotted on linear x log graph paper against the molecular weight. The standard graph obtained for Sephadex G-200 is illustrated in Figure XXI. The Kav of the SF3 component of the apolipoprotein of Type III, Type IV, and outdated plasma S_f 20-400 lipoproteins was applied to the standard graph and M.W. estimations obtained. The results of these estimations are summarized in Table XIII and included the average Kav and M.W. calculated for two chromatographic runs of Type III SF3, two runs of Type IV SF3, and three runs of each of the protein standards. The apolipoproteins of S_f 20-400 lipoproteins isolated from thirteen suitable outdated plasmas were chromatographed and Kav and M.W. estimations obtained. The standard deviation and coefficient of variation of the SF3 M.W. estimations were

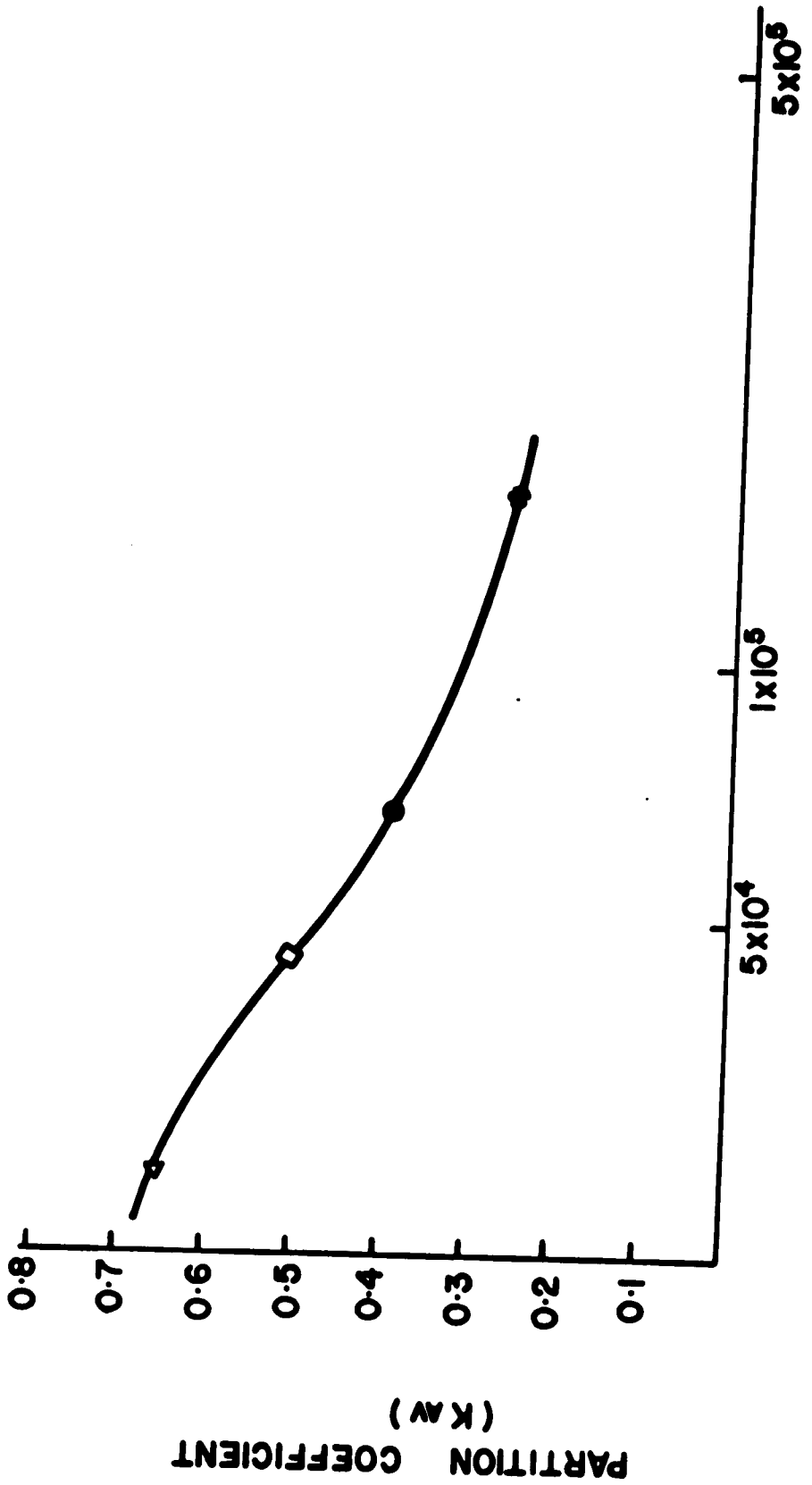


FIGURE XXI. The molecular weight standard graph obtained from gel filtration chromatography of Chymotrypsin A (▲), Ovalbumin (■), Bovine serum albumin (●) and Aldolase (◆) on Sephadex G-200.

TABLE XIII

THE AVERAGE KAV AND MOLECULAR WEIGHT ESTIMATIONS OF SF3 COMPONENTS OBTAINED FROM THE SEPHADEX G-200 STANDARD GRAPH

	Kav (average)	M.W. (average)
Type III SF3	.515	44,000
Type IV SF3	.508	44,500
Type IV SF3 (outdated plasma)	.499	46,000
Aldolase	.259	158,000
Albumin	.390	67,000
Ovalbumin	.504	45,000
Chymotrypsin A	.653	25,000

then calculated by statistical methods previously described. The average M.W. obtained was 46,000, having a standard deviation (1 sigma unit) of 3,000 which resulted in a 6 percent coefficient of variation. From this it can be seen that the Type III and Type IV SF3 components are quite similar in M.W. and no apparent difference exists between the two SF3 components.

A second method by which M.W. estimates were obtained was that proposed by Ackers (107). In this method, the Stokes radius of a protein is calculated from column calibration coefficients with subsequent M.W. calculations of the SF3 component as outlined in Appendix I. Chymotrypsin A and ovalbumin were used as standards of known Stokes radii for calculating the column calibration coefficients.

The results of the Stokes radii and M.W. calculations for Type III, Type IV, and thirteen outdated Type IV SF3 components are summarized in Table XIV. Statistical analysis of the thirteen outdated plasma Type IV SF3 components revealed an average M.W. of 49,500 and a standard deviation of 5,000, resulting in a 11.5 percent coefficient of variation. From the data presented in Table XIV, it is apparent that no significant difference exists between the Type III and Type IV component when the Stokes radii and M.W. estimations are compared.

The M.W. data obtained by the two methods of M.W. estimations agree remarkably well in that insignificant

TABLE XIV

THE AVERAGE STOKES RADII AND MOLECULAR WEIGHT ESTIMATIONS OF TYPE III AND TYPE IV SF3 COMPONENTS OBTAINED BY THE METHOD OF ACKER (107)

	Average Stokes Radius ($\times 10^{-8}$ cm)	Average M.W.
Type III SF3	27.6	46,000
Type IV SF3	27.9	47,500
Type IV SF3 (outdated plasma)	28.3	49,500
Chymotrypsin A	20.9	25,000
Ovalbumin	27.3	45,000

differences existed between the average SF3 component M.W. of an apolipoprotein calculated by both methods. Brown, et al (13) reported the M.W. of the SF3 component of Type IV and normals to be slightly greater than 25,000; however, this was obtained by a simple comparison of the V_e of chymotrypsinogen to the V_e of the SF3 component obtained on a Sephadex G-100 column.

Brown, et al (14) have reported the polypeptide composition of the SF3 fraction of Type IV and normal pre-β apolipoproteins. In view of the finding of a different polypeptide composition of the Type III SF1 component and because the Type III SF3 component had not been studied before, it was decided to similarly study the polypeptide composition of the Type III SF3 component in anticipation of detecting differences in its polypeptide composition.

When DEAE cellulose chromatographic analysis of the Type IV SF3 component was performed, four major protein peaks were resolved as evident from Figure XXII. These results were in agreement with the results obtained by Brown, et al (13) in which four polypeptides were isolated from the SF3 component and designated as D_1, D_2, D_3, and D_4. It was further demonstrated by Brown, et al (13,14) that D_1 consisted of apoLp-Val, D_2 of apoLp-Glu, and D_3 and D_4 consisted of apoLp-Alanine. The reason for the resolution of two apoLp-Ala polypeptides was attributed to D_3 containing 1 mole of sialic acid per mole of protein, while D_4 contained 2 moles of sialic acid per mole of protein.

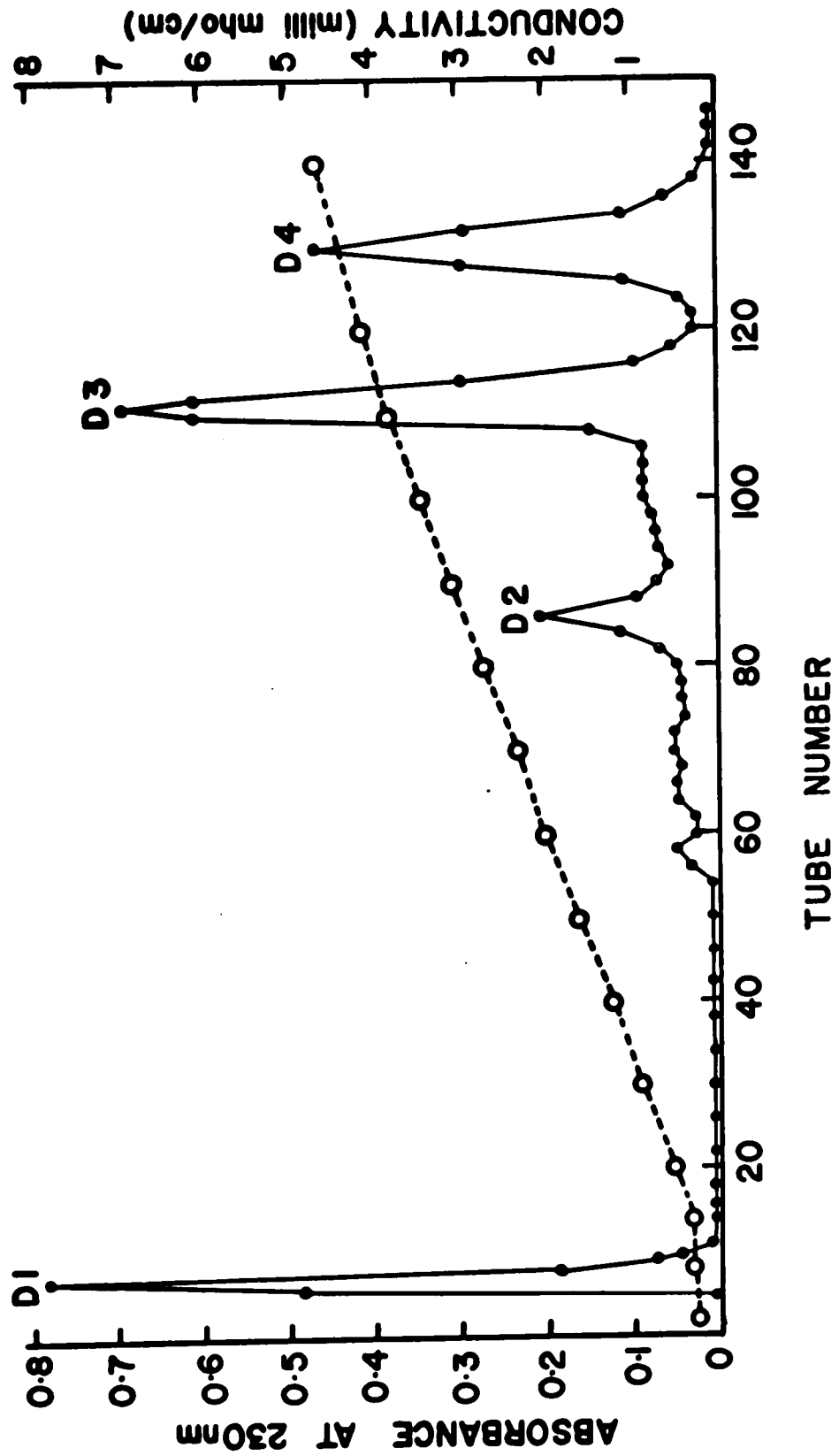


FIGURE XXII. DEAE cellulose chromatographic separation of the polypeptides found in the Type IV SF3 component. The column eluants were monitored at A_{230} nm (●) for the presence of protein and the conductivity (●) measured.

When the Type III SF3 component was subjected to DEAE cellulose chromatographic analysis, four protein peaks were also observed, as evident from Figure XXIII. Comparison of the protein peaks isolated from Type III and Type IV SF3 component with respect to their elution characteristics reveals a probable identity between the respective polypeptide fractions. It can be seen that the specific conductivity at which a particular polypeptide is eluted from the column is similar for the corresponding D fractions of both chromatographic runs. It is also apparent from a comparison of Figures XXII and XXIII that the proportion of the D fractions of the total SF3 component is roughly similar in Type III and Type IV. Two unexplained differences exist between the DEAE cellulose chromatographic elution profiles of Type III and Type IV SF3 components. From Figure XXIII, it appears that a shoulder exists on the D_1 peak and also a shoulder on the D_4 peak. Whether these shoulders are due to small amounts of additional polypeptides similar in elution characteristics to D_1 and D_4 or due to analytical errors cannot be determined at this time. A more suitable quantity of the Type III SF3 component used for DEAE cellulose chromatography would have yielded a more suitable elution profile for comparison to the Type IV SF3 chromatographic results.

From DEAE cellulose chromatographic analysis, it appears that the polypeptide composition of Type III SF3 component is similar to the Type IV SF3 component. However, acrylamide gel disc electrophoresis might reveal slight

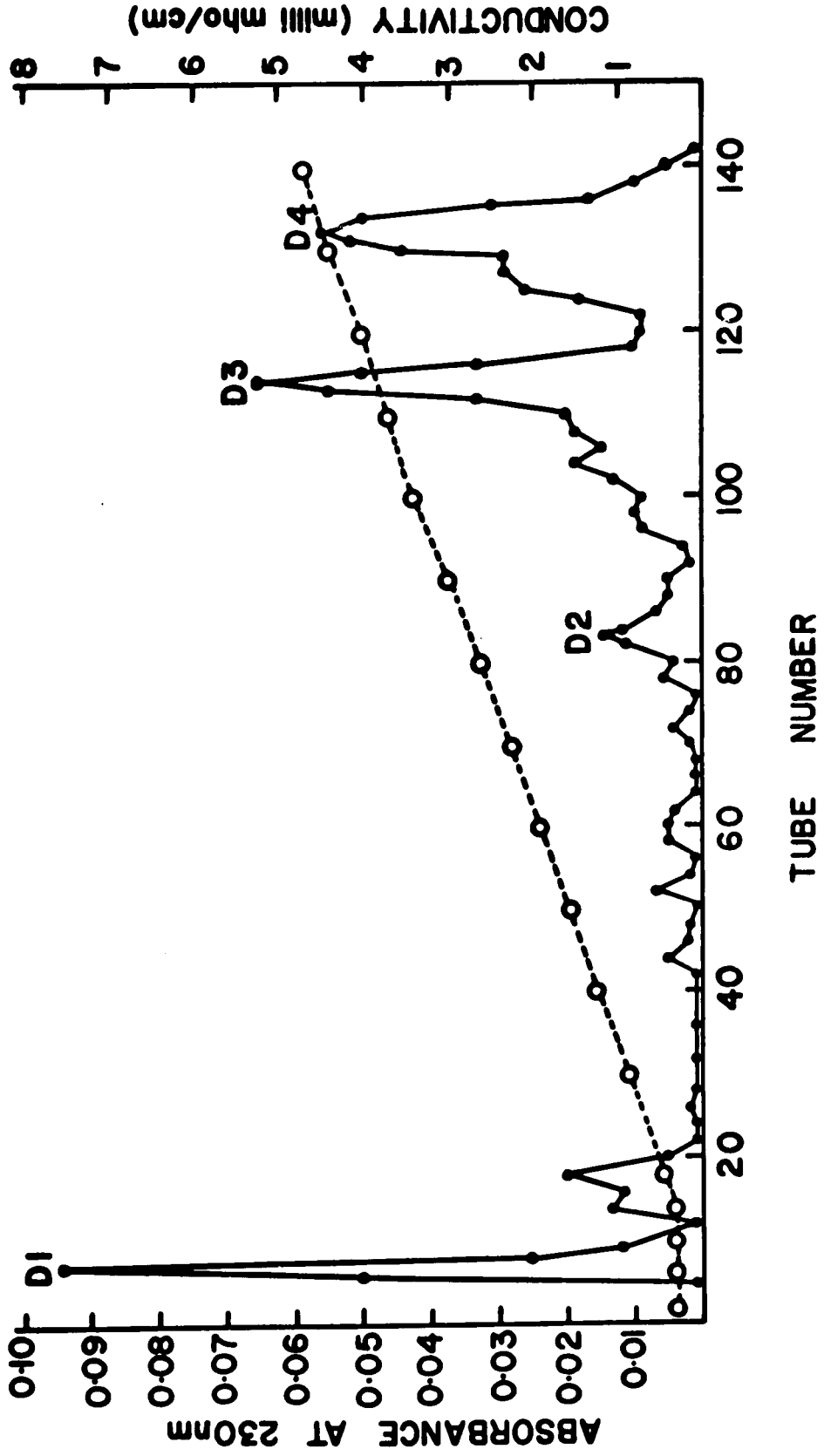
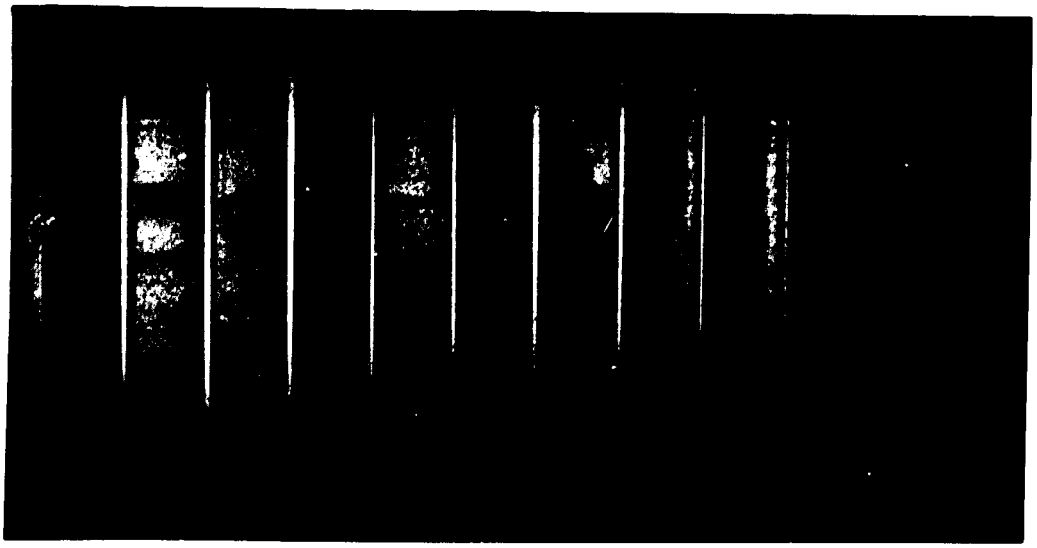


FIGURE XXIII. DEAE cellulose chromatographic separation of the polypeptides found in the Type III SF3 component. The column eluants were monitored at A_{230nm} (●) for the presence of protein and the conductivity (○) also measured.

differences in polypeptide composition.

The DEAE cellulose column eluants containing the four polypeptides were separately pooled and dialyzed against deionized water. The eluant pools were then shell-frozen and lyophilized. The resulting protein residues were redissolved in 0.2M TRIS, pH 8.2 containing 0.1M sodium decyl sulfate. Sample preparation for disc electrophoretic analysis was carried out as previously described.

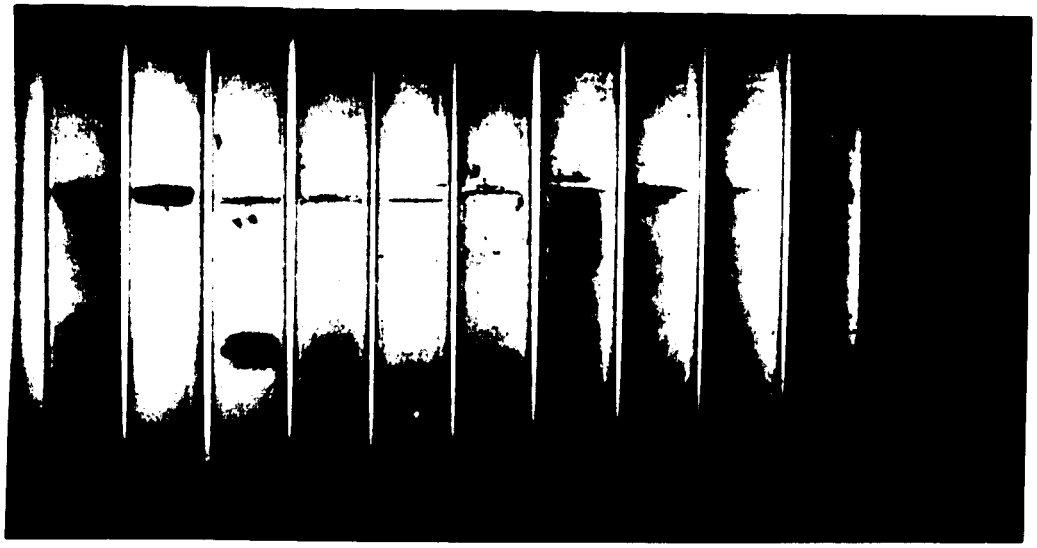
Figure XXIV illustrates the resulting acrylamide electropherograms of the Type IV SF3 component and the four polypeptides isolated by DEAE cellulose chromatography. The migration rates of D_3 and D_4 appeared similar; however, when a mixture of the two polypeptides was electrophoresed two distinct bands were evident. Examination of Figure XXIV also reveals that the D_2 polypeptide (apoLp-Glu) as first reported by Brown, et al (13) was also obtained in an impure form. A later study by Brown (14) involving modifications of the DEAE cellulose chromatographic conditions resulted in the isolation of pure apoLp-Glutamic acid. Although D_2 was obtained in a pure form, it appears that his purification is not consistently obtained in that Gotto, et al (114) very recently reported the D_2 fraction to contain two to three additional polypeptides. One possible contaminating polypeptide is that of apoLp-Ala, which differs from D_3 and D_4 in that sialic acid is completely absent. Examination of a recent paper by Albers, et al (83) reveals that the sialic acid deficient apoLp-Ala migrates in acrylamide gel to a



A B C D E F G H I J K L

FIGURE XXIV. Acrylamide gel disc electrophoretic analysis of Type IV and Type III SF3 components and their respective polypeptides previously isolated by DEAE cellulose chromatography;

A. Type IV SF3
B. D_1 Type IV SF3
C. D_2 Type IV SF3
D. D_3 Type IV SF3
E. D_4 Type IV SF3
F. D_3 + D_4 Type IV SF3
H.- L. are the D fractions of Type III SF3 (G).



A B C D E F G H I J K L

FIGURE XXIV. Acrylamide gel disc electrophoretic analysis of Type IV and Type III SF3 components and their respective polypeptides previously isolated by DEAE cellulose chromatography;

A. Type IV SF3
B. D_1 Type IV SF3
C. D_2 Type IV SF3
D. D_3 Type IV SF3
E. D_4 Type IV SF3
F. D_3 + D_4 Type IV SF3
H.- L. are the D fractions of Type III SF3 (G).

position between the D_3 and D_4 polypeptides.

The relative electrophoretic migration rates of the polypeptide fractions of Type IV SF3 component were consistent with those obtained by Brown, et al (13,14) and recently by Gotto, et al (114). The D_1 fraction was often difficult to visualize in the electropherograms despite relatively large amounts of protein in the D_1 fraction. However, when unusually large amounts of D_1 were subjected to disc electrophoresis, an adequate amount of stain was bound by the polypeptide to permit visualization of the band.

When the DEAE chromatographic fractions of Type III SF3 component were subjected to disc electrophoresis, protein bands resembling those found in Type IV SF3 were obtained. From Figure XXIV it can be seen that the D fractions of Type III and Type IV SF3 components closely correspond in electrophoretic mobility. It was difficult to visualize the D_1 fraction due to its poor staining characteristics.

From the gel filtration chromatographic, DEAE cellulose chromatographic and acrylamide gel disc electrophoretic studies, it appears that the SF3 component of Type III and IV apolipoproteins (of the S_f 20-400 lipoproteins) have identical polypeptide composition. Conclusive evidence for identity would have been obtained by total and end-group amino acid analysis of the polypeptides; however, the very limited amounts of the polypeptides obtained from the Type III SF3 component prevented such a study.

3. Conclusions

Based on the physical and chemical studies carried out during this investigation of the apolipoproteins obtained from Type III and Type IV S_f 20-400 lipoproteins, several conclusions can be made:

(1) The main flotation peak value of the Type III and Type IV VLDL is similar. The Type III VLDL do not exhibit the characteristic S_f 20 flotation peak of Type IV low density lipoproteins.

(2) Type III and Type IV VLDL differ in electrophoretic migration properties in that Type III migrates as a "broad-β" lipoprotein instead of a pre-β lipoprotein in agarose gel. In acrylamide gel, VLDL from both have similar electrophoretic migration characteristics. However, Type III VLDL presents as a more diffuse band.

(3) The SF1 component of the apolipoprotein of Type III VLDL is greatly increased when compared to Type IV. This may account for the high proportion of cholesterol in the Type III VLDL and its β-migrating characteristics.

(4) The SF1 component of the apolipoprotein of Type III VLDL contains at least four polypeptides not previously reported in Type IV very low density lipoproteins.

(5) The SF3 component of the apolipoprotein of Type III and Type IV VLDL appear to be identical.

In a recent paper by Quarfordt, et al (115) on the lipoprotein abnormality in Type III hyperlipoproteinemia,

it was demonstrated by starch block electrophoresis that the S_f 20-400 lipoprotein in Type III individuals consisted of two species, one termed a β-migrating and the other an α_2-migrating lipoprotein. The β-migrating lipoprotein was consistently found in only Type III individuals. When this lipoprotein was isolated by starch block electrophoresis, he demonstrated that it was immunologically identical to β apoprotein, and did not cross-react with anti-α lipoprotein nor an antibody to the protein component (SF3) of S_f 20-400 lipoproteins. It may only be assumed that his β-migrating lipoprotein species in Type III VLDL is reflecting the increased SF1 protein component reported in the research described here.

Quarfordt also stated that β-migrating VLDL contained a high amount of triglyceride compared to normal β lipoprotein. This finding is difficult to explain if both lipoprotein species contain the same protein component. A likely explanation is that the additional peptides reported here might have a marked affinity for triglycerides.

What remains to be done is to find out if other patients with Type III hyperlipoproteinemia also display the additional peptides found in the VLDL fraction of the patient studied here. If this is the case, these should be rigorously investigated, as their lipid-binding properties might be related to the severe vascular disease found in Type III hyperlipoproteinemia.

IV. CONCLUSIONS

Agarose gel electrophoretic separation of human plasma lipoproteins has been further developed to include quantitation of lipoprotein fractions. This was accomplished by expressing lipoprotein levels in terms of lipoprotein-lipids as derived from a combination of the plasma total lipid level and the percent of total dye uptake by the lipoprotein fraction. Validation of this approach to lipoprotein quantitation was demonstrated by dye uptake correlation studies. Using this method, normal values of plasma lipoprotein levels, as well as plasma lipid levels, in normal male individuals of varying ages was obtained. As expected, the average lipid or lipoprotein level increased with age; however, the range of values in each age group was similar.

Using the established normal plasma lipoprotein and lipid levels, an investigation of the inheritance pattern of Fredrickson Types II, III, and IV hyperlipoproteinemias was carried out. It was concluded that three families of Type II disease displayed an autosomal dominant mode of genetic transmission, although the possibility of a recessive mutant gene of variable penetrance could not be eliminated. A study of the Type III inheritance pattern was done, but only one family was available and only the propositus showed the lipoprotein abnormality. Five Type IV families were investigated and the mode of inheritance of the gene defect was autosomal recessive in nature. In all family studies performed, the hoped-for finding of mixed Types of

hyperlipoproteinemia was not accomplished.

A detailed physico-chemical study of the very low density lipoproteins of a Type IV and Type III patient was performed in hopes of elucidating the "broad-beta" migrating nature of this lipoprotein species in the Type III individual. Biochemical and physical methods of analysis by which a comparison of the Type III and Type IV VLDL was made included chemical, chromatographic, electrophoretic, and ultracentrifugal techniques.

The results are as follows:

1. Type III plasma is unusually rich in cholesterol when compared to Type IV plasma.

2. Type III plasma reveals a "broad-beta" lipoprotein band upon agarose gel electrophoretic separation of plasma lipoproteins.

3. The "broad-beta" lipoprotein of Type III plasma remains in the S_f 20-400 lipoprotein fraction when isolated by preparative ultracentrifugation. In contrast, the S_f 20-400 lipoprotein fraction of Type IV plasma electrophoretically migrates as a distinct pre-beta band.

4. The Type III S_f 20-400 lipoproteins are rich in cholesterol, thus explaining the elevated plasma levels of cholesterol in Type IV patients.

5. Acrylamide gel disc electrophoretic analysis of delipidated Type III VLDL reveals additional peptide bands not observed in the corresponding Type IV apolipoprotein.

6. Sephadex G-200 gel filtration of Type III apoVLDL

revealed three distinct protein components, designated as SF1, SF2 and SF3, as also obtained from the corresponding normal or Type IV apolipoprotein. However, an increased amount of SF1 component with a shoulder peak was observed in Type III. As SF1 is the major peptide component of beta apolipoprotein, it is suggested that the beta-migrating and cholesterol-carrying characteristics of "broad-beta" lipoprotein are due to the presence of increased amounts of SF1 component in Type III very low density lipoproteins.

7. Acrylamide gel disc electrophoretic analysis of Type III and Type IV SF1 component revealed the presence of previously unreported peptides in Type III.

8. The SF3 fraction of Type III apoVLDL appeared identical to the corresponding Type IV SF3 component when compared by electrophoretic and chromatographic techniques.

It remains to be seen if the above are characteristic of all Type III individuals. If confirmed, isolation and characterization of the reported additional peptides of Type III S_f 20-400 lipoprotein may contribute to understanding the pathogenesis of Fredrickson Type III hyperlipoproteinemia.

In conclusion, a protein abnormality has been found in a serum lipoprotein associated with severe peripheral vascular disease. It is believed that this is the first report of such a finding.

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VI. APPENDIX I

Stokes Radii Calculations

The method of column calibration used involves only one adjustable parameter, the effective gel pore radius. This parameter is calculated from the effluent peak position of a macromolecule of known Stokes radius. A column calibrated by this method is then used for the determination of Stokes radii of other macromolecules and an upper limit for the hydrated molecular weight can be obtained. A Stokes radius calculated is outlined as follows:

(1) Calculation of x values for standards and SF3 components

from $\text{erfc}(x) = 1-\sigma$

where σ = Kav of standard

and x is determined from error function tables.

(2) Calculation of column calibration factors, a_o and b_o

from $b_o = \dfrac{a_1 - a_2}{x_1 - x_2}$

$$a_o = \tfrac{1}{2}\left[a_1 + a_2 - b_o(x_1 + x_2) \right]$$

where a_1 = Stokes radius of standard 1

a_2 = Stokes radius of standard 2

x_1 = x factor of standard 1

x_2 = x factor of standard 2.

(3) Calculation of Stokes radius for an SF3 component

from $a_x = a_o + b_o x$

where a_2 = Stokes radius of an SF3 component

a_o and b_o = column calibration coefficients

x = x factor of the SF3 components as calculated in (1).