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# PHYSICAL, CHEMICAL AND IMMUNOCHEMICAL CHARACTERIZATION OF HUMAN PLASMA LOW DENSITY LIPOPROTEINS

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DIANA M. LEE

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PHYSICAL, CHEMICAL AND IMMUNOCHEMICAL CHARACTERIZATION

OF HUMAN PLASMA LOW DENSITY LIPOPROTEINS

APPROVED BY

Peter Alaupovic

A. C. Kurtz

Leon S. Lewis

Earl G. Larsen

Paul B. McCay

DISSERTATION COMMITTEE

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# PHYSICAL, CHEMICAL AND IMMUNOCHEMICAL CHARACTERIZATION OF HUMAN PLASMA LOW DENSITY LIPOPROTEINS

## CHAPTER I

## INTRODUCTION

It has long been recognized that the blood lipids are combined with other substances rather than existing in the free state. These complexes serve as the vehicles for maintenance and transport of lipids in the aqueous plasma. Numerous early investigators, including Nerking (1), Hardy (2), Haslam (3), Chick (4), Theorell (5) and Sorensen (6) studied the distribution of various lipids during investigations of the precipitation of serum protein fractions and noted the presence of cholesterol and phospholipid in the water-insoluble euglobulin subfractions. However, it was not until 1929 that a lipoprotein which displayed a relatively constant chemical composition was isolated from horse plasma by Macheboeuf (7). Lipoprotein research has developed rapidly since 1940 and some eighteen times as much work on lipoproteins has been published in the five year period 1957-1962 as compared to the 1947-1952 period (8).

### Classification of Lipoproteins

The most striking aspect of plasma lipoprotein preparations is

their heterogeneity. They differ in density and composition (9, 10), electrophoretic mobility (11), protein moiety (12, 13, 14, 15), particle size (16), sedimentation coefficient, S, and flotation coefficient, $S_f$ (17, 18). This heterogeneity renders the exact definition of lipoprotein classes difficult. At the present time there are five recognized classes, based on ultracentrifugal isolation techniques: (1) chylomicrons ($\bar{d}$ = 0.94, (2) very low-density lipoproteins (VLDL, $\bar{d}$ = 0.98), (3) low-density lipoproteins (LDL, $\bar{d}$ = 1.03), (4) high-density lipoproteins (HDL, $\bar{d}$ = 1.12), and (5) very high-density lipoproteins (VHDL, d > 1.210). Each class, however, is still heterogeneous with respect to protein moiety, composition, density, particle size, sedimentation, and flotation rate. The usual criteria of purity and homogeneity are not applicable. Chylomicrons are described as the lipoproteins of $S_f$ 10,000 $\pm$ 5,000 by Oncley (16) and as lipoproteins of $S_f$ > 400 by others (19). Chylomicrons with $S_f$ > 5,000 have particle sizes of 2500 Å or more in diameter and molecular weights greater than $5 \times 10^9$. Very low-density lipoproteins are those of $S_f$ 20-400 or of $S_f$ 20-5,000 (20). This group contains three different protein moieties: apolipoprotein A, apolipoprotein B, and apolipoprotein C (14). However, the high lipid content of this range (> 90%) renders the isolation of lipoproteins with specific protein moieties very difficult. Low-density lipoproteins are generally subdivided into two fractions: (1) LDL isolated at solvent density range 1.006-1.019 g/ml, corresponding to $S_f$ 12-20, and (2) LDL isolated at solvent density range 1.019-1.063 g/ml, corresponding to $S_f$ 0-12 (19). Both fractions contain apolipoprotein B as the major protein moiety. This class of lipoproteins is also designated as β-lipoproteins due to their electrophoretic mobil-

ity, which is similar to that of β-globulin (21, 22). However, the presence of high-density lipoproteins (HDL) in the upper density range of LDL was recognized by Freeman et al. (23). DeLalla, Levin and Brown (24) found HDL in the LDL of density less than 1.063 g/ml from a patient with biliary cirrhosis. For this reason, only the purified LDL containing a single protein moiety, apolipoprotein B is designated as ApoB-LP in this study. High-density lipoproteins ($HDL_2$ + $HDL_3$) are lipoproteins isolated at the solvent density 1.063-1.125 g/ml and 1.125-1.20 g/ml (18, 23). This class of lipoproteins contains mainly α-LP, the name having originated with the discovery that some serum lipids move with the $\alpha_1$-globulin in free electrophoresis (21, 22). The presence of β-LP was found in the density range 1.063-1.100 g/ml by Ayrault-Jarrier, et al. (25, 26), and was also noticed by Levy and Fredrickson (27). Very high-density lipoprotein is the name given to the α-LP in the density range higher than 1.21 g/ml. DeLalla et al. (24) first noticed the presence of α-LP of density greater than 1.240 g/ml. Alaupovic et al. (28) isolated and characterized the $VHDL_1$ at density 1.210-1.250 g/ml and $VHDL_2$ of density greater than 1.250 g/ml. Their results suggested that VHDL consists of lipid-protein complexes separable into two major groups characterized by the presence of apolipoprotein A and albumin, respectively, as lipid-carrying proteins.

## Distribution of β-Lipoproteins

The distribution of lipoproteins can best be illustrated by a typical pattern taken from a study by Freeman et al. (23) and reproduced in Figure 1. As mentioned in the previous section, lipoproteins in the density range 1.006-1.063 g/ml are mainly β-LP. However, the presence of

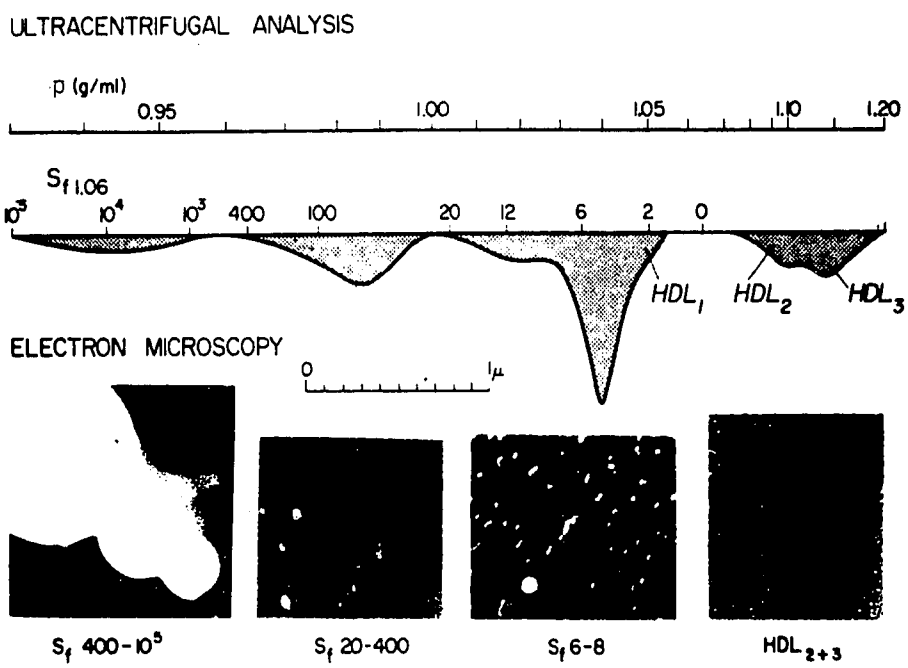


Figure 1. The ultracentrifugal spectrum of human serum lipoproteins.

Physical sizes of each lipoprotein class are illustrated by the electron micrographs. Lipoprotein hydrated density is indicated for each region of the serum lipoprotein spectrum (23).

β-LP is not restricted to this range. They are found in lipoprotein segments up to the density of 1.10 g/ml (25, 26, 27) as well as in the VLDL and chylomicrons. Subfractionating VLDL at various centrifugal forces and times ("g min"), Gustafson et al. (14) found that only the subfraction of $S_f$ 20-50 contained a single protein moiety, apolipoprotein B. Maximum concentration of β-LP lies in the hydrated density range 1.030-1.045 g/ml for normal human serum or plasma. The distribution pattern varies considerably with sex and age and varies slightly from individual to individual (29). For hypercholesterolemic plasma the pattern shifts its maximum to the left, i.e., lower density. For normal plasma drawn after an extended fast (20 hours), the maximum concentration shifts to the right, i.e., higher density, as noticed in the present study. A two-dimensional distribution function of the β-lipoproteins of various density ranges was studied by Numa and Oncley (16). Their plot of relative amounts of protein against lipoprotein density and size indicates an almost continuous distribution of lipoproteins from density 0.947 to 1.041, with diameters (assuming spherical shape) from about 1150 Å to about 160 Å.

## Isolation of β-Lipoproteins

The first fractionation of lipoproteins was performed by Macheboeuf (7) using the method of salt fractionation and manipulating both salt concentration and pH. Cohn et al. (30) used successfully ethanol at low ionic strength, low temperature, and in conjunction with pH manipulation to fractionate lipoproteins. Beta-lipoproteins were present in fractions I, II and III and α-LP in fractions IV, V and VI. The use of metal ions to decrease the solubility of protein salts in the water-organic solvent mixture resulted in a modified, faster method of

fractionation (31, 32). However, Mills et al. (33) observed that treatment of Cohn's fractions I + II + III with concentrated salt solutions brought about the loss of some lipid components. They suggested that this was not a good method for the preparation of intact lipoproteins, but may be a useful route for the lipid-deficient preparations which are required for the investigation of lipoprotein structure.

Electrophoresis has also been used as a means of isolating lipoproteins. Blix, Tiselius, and Svensson (22) used free electrophoresis to separate β-LP from γ-globulin. Kramer and Tiselius (34), Turba and Enenkel (35), and Durrum (36) introduced paper electrophoresis which has been widely applied in clinical work. However, it is not a preparative method. Starch block electrophoresis has been used by Kunkel and Slater (11), but the difficulties of eluting the lipoproteins have limited the use of this technique. The newly developed polyacrylamide gel electrophoresis is now available also for work on a preparative scale (37). Reissell et al. (38) developed the use of starch granules as a matrix for thin layer electrophoresis and coupled this with quantitative thin layer chromatography for the lipids extracted from lipid-containing bands in the starch electrophoretic patterns.

Lipoprotein research has been greatly stimulated since 1940 by the development of ultracentrifugation. An important ultracentrifugal technique was introduced by Gofman, Lindgren, and Elliott (17). These investigators showed that the anomalous schlieren patterns which had been obtained with whole serum by McFarlane (39) and Pedersen (40) could be explained by the Johnston-Ogston effect (41) involving lipoproteins. Preparative ultracentrifugal flotation of the serum in solutions of in-

creased density eliminated the anomalous schlieren patterns in the infranatant fraction. The flotation technique has now been standardized by DeLalla and Gofman (18) and DelGatto, Lindgren, and Nichols (42). Essentially it consists of adjusting the density of serum (excluding the density contributed by the protein) to the desired point by the addition of salt or a salt solution of known density, and centrifuging the adjusted solution in a preparative rotor at 40,000-50,000 rpm. Different salts have been used, including sodium chloride, potassium bromide, and sodium bromide. The latter has been recommended by most workers because of its high solubility and its non-interference with nitrogen determinations.

The precipitation of lipoproteins by polyanions has been reported by several authors: Bernfeld (43); Burstein and Samaille (44, 45); Burstein (46); Oncley, Walton, and Cornwell (47). Polyanions such as sulfated amylopectin, dextran sulfate, and heparin have been used to precipitate ApoB-LP in the presence of bivalent ions such as calcium chloride, magnesium chloride and manganese chloride. Oncley *et al.* (48), using a combination of dextran sulfate precipitation and ultracentrifugation, found that the precipitated lipoproteins had an $S_f$ range of 10-100 with a preponderance in the $S_f$ 20-60 range. Cornwell and Kruger (49) studied the precipitation by sulfated polysaccharide and found that this polyanion precipitated the whole spectrum of low-density lipoproteins. Bernfeld *et al.* (50) examined the precipitated β-lipoprotein complex and found marked changes in the electrophoretic mobilities. Granda (51) also observed that the electrophoretic mobility of dextran sulfate precipitated β-LP was three times as high as that of β-LP in whole serum. This indicated that the dextran sulfate could not be removed completely from β-LP.

Gel filtration has been applied recently to the separation of β-LP from α-LP (52). Separation is based on diffusional partition of solutes between a stationary phase within the bed material and an external mobile phase. Solute molecules which are small enough to penetrate the porous gel beads are retarded relative to larger molecules which are unable to enter the pores. Among the bed materials available at present (e.g., latex rubber, starch, dextran, polyacrylamide), agar offers the largest pore size. Agarose, a neutral constituent of agar, has been chosen by many workers to avoid specific adsorption of solutes to the bed matrix.

Separation of the plasma lipoproteins by column chromatography has also been performed by several workers (53, 54, 55). Cramér (53) studied the isolation of β-LP by hydroxyapatite column chromatography and compared the results with those obtained by other methods.

## Characterization of LDL or β-Lipoproteins

Although β-lipoproteins isolated by any technique are heterogeneous in density, size, chemical composition, sedimentation and flotation coefficients, they do possess some unique properties different from α-LP and from other proteins. The methods of characterization can be classified as (1) physical-chemical, (2) immunological, and (3) chemical.

### Physical-Chemical Methods

Electrophoresis. Beta-lipoproteins possess the same electrophoretic mobility as β-globulin in whole serum. Free boundary electrophoresis is a precise but expensive method, and therefore has not been used widely. Zone electrophoresis techniques are more frequently used.

Paper electrophoresis is the least expensive and most widely applied in clinical work; however, the resolution is poor and the sensitivity is low. Cellulose acetate has gradually replaced paper. Starch gel, agar gel, and polyacrylamide gel all show higher resolving power than paper.

Ultracentrifugation. The variations in density of β-LP render them well suited for characterization in the analytical ultracentrifuge. Lipoproteins of different densities have correspondingly different flotation coefficients ($S_f$), sedimentation coefficients (S) and diffusion coefficients (D). Diameter of the particles of a given density range can be calculated from the $S_f$ value, assuming the particle to be a sphere. Molecular weight for a density range can be determined either by the Archibald method or calculated from the S/D value. The physical chemical data available for LDL fractions are shown in Table 1. Molecular weights of LDL fractions including light scattering results are shown in Table 2.

## Immunological Methods

Immunology is the simplest but most sensitive method for characterization of lipoproteins. It is also the most sensitive method of determining the degree of purity of the protein moiety in a given preparation, since the antigen-antibody interaction is protein specific. Both ApoA-LP and ApoB-LP contain similar lipid components and both are heterogeneous with respect to chemical composition and densities. A contamination of the low-density lipoprotein range by ApoA-LP can hardly be noticed by physical-chemical characterization or by determination of gross chemical composition. Electrophoresis has been used more frequently than immunochemistry as the method for determining the purity of lipoproteins. However, the sensitivity of immunochemical methods is far superior to

TABLE 1

PHYSICAL CHEMICAL CHARACTERIZATION OF HUMAN PLASMA OR SERUM LDL

| Fraction | $\bar{d}$ g/ml | $S_{20,w}$ | $S_f^o$ | $D_{20,w}$ x$10^7$ $cm^2$/sec | Diameter Å | Axial ratio | Reference |
|---|---|---|---|---|---|---|---|
| $S_f$ 13 | 1.015 | - | - | - | - | - | (19) |
| $S_f$ 12.1 | 1.016 | - | - | - | - | - | (19) |
| $S_f$ 9.7 | 1.022 | - | - | - | - | - | (19) |
| $S_f$ 4-10 | - | 5.9 | - | 1.7 | - | - | (56) |
| $S_f^o$ 8 | - | - | 8.1 | - | 212 | 2.42 | (57) |
| $S_f^o$ 6-8 | 1.029 | - | - | - | - | - | (19) |
| $S_f^o$ 6.4 | - | - | 6.4 | - | 205 | 2.16 | (57) |
| $S_f^o$ 6.1 | 1.035 | - | - | - | - | - | (19) |
| $S_f^o$ 5.9 | - | - | 5.9 | - | - | - | (57) |
| $S_f$ 4.1 | 1.041 | - | - | - | - | - | (19) |
| 1.007-1.063 | - | - | - | - | 350 ± 25[a] | - | (58) |
| 1.019-1.063 | - | - | - | - | 185 | - | (58) |
| 1.006-1.022 | - | - | 14.9 | - | - | - | (59) |
| 1.031-1.035 | - | - | 6.9 | - | - | - | (59) |

[a]Electron microscopy.

TABLE 2

MOLECULAR WEIGHT OF HUMAN PLASMA OR SERUM LDL

| Eraction | S/D $x10^{-6}$ g/mole | Light Scattering $x10^{-6}$ g/mole | Stokes Sphere minimum M. W. $x\ 10^{-6}$ g/mole | Reference |
|---|---|---|---|---|
| $S_f$ 13 | - | - | 3.4 | (19) |
| $S_f$ 4-10 | 2.6 | - | - | (56) |
| $S_f^o$ 8.1 | - | 3.08 (pH 6.7) | - | (57) |
| $S_f^o$ 7.9 | - | 3.20 | - | (60) |
| $S_f^o$ 6.4 | - | 2.80 (pH 6.7) | - | (57) |
| $S_f$ 6 | - | - | 2.5 | (19) |
| $S_f^o$ 5.9 | - | 2.77 (pH 6.7) | - | (57) |
| $S_f$ 4 | - | - | 1.7 | (19) |

those of electrophoretic techniques (see section of Results).

A number of investigators (12, 24, 61-67) have been able to produce antisera which will react with the α-LP but not with β-LP and, likewise, antisera which are specific to β-LP but not to α-LP. Gitlin et al. (68) found antigenic differences in separated narrow bands of LDL. Aladjem et al. (12, 69) have obtained similar results by absorption, precipitation, and agar diffusion techniques. Lawrence and Shean (70) found two antigens more frequently than one in the LDL. However, Korngold et al. (65) and Levine (67) showed antigenic homogeneity of this lipoprotein class. Scanu et al. (71) using agar immunoelectrophoresis and the agar double diffusion technique of Ouchterlony (72) found that lipoproteins with $S_f$ 0-20 were antigenically homogeneous. Blumberg et al. (73) also showed that all LDL possessed the same antigenic form.

## Chemical Methods

Havel et al. (10) and Cramér (74) showed that the chemical composition of β-LP varied considerably with age and sex. However, the weight ratio of total cholesterol (expressed as free cholesterol) to phospholipid was found to be nearly a constant (1.25-1.41) for lipoproteins isolated from normal human serum at density range 1.019-1.063 g/ml. Bragdon et al. (75) also obtained a similar value. Data on the chemical composition of β-LP available in current literature are summarized in Table 3. The results of phospholipid composition of LDL are shown in Table 4. The bound fatty acid components include palmitic, palmitoleic, stearic, oleic, linoleic, and arachidonic acid (23). In LDL with $S_f$ 0-20 linoleic (50.7%) and oleic (18.2%) are the major cholesterol ester fatty acids; palmitic (30.1%) and linoleic (19.3%) are the major acids in the

TABLE 3

THE PERCENT CHEMICAL COMPOSITION OF HUMAN PLASMA OR SERUM LDL PREPARATIONS

| Fraction | Cholesterol Esters | Free Cholesterol | Triglycerides | Free Fatty Acids | Phospholipids | Protein | Reference |
|---|---|---|---|---|---|---|---|
| 1.006-1.019 g/ml | 26 | 6.5 | 35 | - | 18 | 15 | (76) |
| 1.019-1.063 g/ml | 39.4 | 7.5 | 9.3 | - | 23.1 | 20.7 | (75) |
| 1.019-1.063 g/ml | 38.0 | 8.1 | - | - | 25.0 | 26.5 | (77) |
| Preparation with Cornstarch | 36.8 | 10.4 | 8.8 | - | 18.8 | 25.2 | (78) |
| Chromatography on hydroxylapatite | 38.5 | 7.5 | 9.0 | - | 22.0 | 23.0 | (74) |
| Preparation with organic solvent | 39.1 | 8.3 | - | - | 29.3 | 23.0 | (79) |
| 1.031-1.035 | 38 | 8 | 10 | 1 | 22 | 21 | (80) |
| 1.045-1.065 | 25.5 | 16 | 14 | 4 | 15 | 25 | (81) |
| $S_f$ 3-9 | 39.4 | 7.4 | 8.9 | - | 22.4 | 21.9 | (49) |
| $S_f$ 0-12 (♀) | 48.4 | 10.2 | 8.0 | - | 17.1 | 16.3 | (83) |
| $S_f$ 0-12 (♂) | 50.8 | 9.9 | 6.4 | - | 17.1 | 15.8 | (83) |

TABLE 4

THE PERCENT COMPOSITION OF PHOSPHOLIPID OF HUMAN PLASMA OR SERUM LDL FRACTIONS

| Fraction g/ml | Lecithin | Lysolecithin | Sphingomyelin | Phosphatidyl ethanolamine | Reference |
|---|---|---|---|---|---|
| 1.006-1.063 | 68.8 | Not determined | 25.6 | 5.5 | (84) |
| 1.019-1.063 | 66.3 | 4.1 | 25.3 | 4.4 | (85) |
| 1.019-1.063 (♂) | 37.5 | 12.0 | 45 | 5.5 | (86) |
| 1.019-1.063 (♀) | 58.5 | 8.5 | 29 | 4 | (86) |
| 1.019-1.063 | 62.8 | 6.3 | 30.8 | Not determined | (87) |

phospholipids.

Carbohydrates in β-LP have been studied by several workers. Rimington (88) determined the hexosamine content. Kirby (89) found that carbohydrate content was high in Cohn fractions III and IV-1. Epstein and Block (90) observed that the amount of glycoprotein, expressed in terms of hexosamine concentration, was less than 3 mg/100 ml of serum in lipoproteins of density less than 1.063 g/ml. Marshall and Kummerow (91) reported the percent carbohydrate composition of LDL of the same density range: hexose 3.23%, hexosamine 1.2%, and sialic acid 0.35%. Abraham et al. (92) isolated LDL and higher lipoprotein classes from chick sera and egg yolk lipoproteins and found that they contained bound galactose, mannose, glucosamine and sialic acid. Sialic acid and other carbohydrates were also studied by Schultze and Heide (93), Ayrault-Jarrier et al. (94) and Margolis and Langdon (95). The latter authors reported that sialic acid accounted for about 0.5% of the weight of delipidized β-LP.

Terminal amino acid of β-LP has been reported by several authors (96, 97) to be distinct from that of α-LP. These authors reported that the N-terminal amino acid for lipoproteins of density 1.019-1.063 g/ml was glutamic acid, and that of lipoproteins of density 1.063-1.210 g/ml was aspartic acid. Shore (60) confirmed these results but found a small amount of serine in addition to glutamic acid as N-terminal amino acids for LDL. He also found that the C-terminal amino acids for LDL and HDL were serine (with traces of alanine) and threonine, respectively. Rodbell's results (98) showed more heterogeneity in that glutamic acid, serine and threonine were all found as N-terminal amino acids for lipoproteins of density 1.019-1.063, and aspartic acid, serine and threonine

for HDL (1.063-1.21 g/ml).

Amino acid composition has been studied by Shore and Shore (99) who measured the fluorescence of amino acid-sugar complexes developed by spraying chromatograms of protein hydrolysates with a xylose sodium bisulfite spray. They also measured the DNP derivatives of the hydrolysate amino acids. The authors concluded "the proteins of the lipoproteins of hydrated density 1.006-0.96 g/ml ($S_f$ 20-400), 1.040-1.006 g/ml ($S_f$ 4-20), 1.015 g/ml ($S_f$ 13) and 1.035 g/ml ($S_f$ 6) are of quantitatively identical amino acid composition and very similar, if not identical to that of high density lipoproteins of hydrated density 1.075 and 1.145 g/ml". However, some differences in amino acid composition between LDL and HDL were reported by Brown et al. (97). If the elegant results reported by Margolis and Langdon (95) on β-LP isolated by dextran sulfate precipitation are compared to those of Scanu and Hughes on α-LP (100), differences between the amino acid composition of α-LP and β-LP are definitely indicated. There is a striking difference in the values for isoleucine, aspartic acid, and phenylalanine. Granda and Scanu (101) concluded recently that the amino acid composition of LDL of the density ranges 1.006-1.019 g/ml and 1.019-1.063 g/ml showed no difference.

## Polymorphism of Human LDL

Since the discovery in 1961 by Allison and Blumbery (102) of isoprecipitins against LDL in the serum of a multitransfused patient, it has become obvious that there exists an immunological heterogeneity among LDL of human subjects. Immunological types are genetically determined and display group specificity (102). A considerable number of polymorphic LDL-variants has been described (103-106).

## Study of β-Lipoprotein Structure

It is not within the scope of this study to discuss the normal or deranged lipoprotein metabolism with all its possible consequences on the development of atherosclerosis. However, the correlation of the elevated blood cholesterol concentration with the elevation of β-LP in atherosclerotic patients has brought to our attention the importance of β-LP and, correspondingly, the importance of the structures of β-LP and apolipoprotein B.

In order to study the protein structure, the lipids must first be removed from a lipoprotein. Avigan (109) used peroxide-free ether to delipidize solutions of β-LP and found that nearly all of the cholesterol, most of the triglycerides, but little of the phospholipids were removed into the ether phase. Scanu et al. (110), using ethanol-ether (3:1 v/v), delipidized α-LP at -20° C and obtained a soluble protein moiety practically free of lipid. However, the same procedure applied to β-LP failed to yield a soluble protein. Grundy et al. (111, 112) used the modified Avigan's two-phase procedure with lyophilization of the aqueous phase and found that the partially delipidized LDL of a broad density range showed two peaks in the analytical ultracentrifuge. If a narrow density range of LDL was used, however, the delipidized material showed only a single peak. Granda and Scanu (101) obtained solubilized apolipoprotein B by delipidizing β-LP either partially or totally in the presence of excess sodium dodecyl sulfate (SDS). The delipidized material was then dialyzed against Tris buffer extensively (10-12 days). They were unable to remove the SDS completely. Although the totally and partially delipidized LDL possessed two ultracentrifugal components, the succinylated apolipoprotein

B showed only a single peak of 7.2S. The totally delipidized β-LP exhibited affinity for micellar phospholipids, and the partially delipidized β-LP would remove and bind cholesterol from a heptane solution (51).

The currently available model of an LDL is that set forth by Vandenheuvel (113) who calculated it from the data of Oncley et al. (114). Vandenheuvel suggested that a water containing sphere with diameter of 126 Å was covered by lipids with an annular space of 22 Å. The external shell, formed by hydrated protein was 7 Å thick, resulting in a final LDL sphere with a diameter of 185 Å. However, this calculation was based on a molecular weight of an anhydrous LDL of $1.3 \times 10^6$ g/mole with a water content of 60% of its dry weight. This percentage of water was considered later to be too high (16). Therefore this model would be invalid. So far, no other models of LDL structure have appeared in the literature.

The structure of β-LP is still far from clear. The elucidation of structure in a complex molecule such as a lipoprotein requires detailed information regarding the molecular weight, particle size, chemical composition and physical or chemical properties. For β-LP such information must cover a wide range of different particles. A comprehensive study on the chemical composition and physical properties of LDL isolated at narrow density ranges has not been reported in the literature; such studies have been confined either to the broad range or only to a few segments of LDL.

Therefore, in order to provide such information the purpose of the present study is to isolate β-LP subfractions, to determine their chemical composition and physical-chemical properties and to characterize their protein moieties. It is hoped that such information would contrib-

ute to a better understanding of the relationship between subfractions differing in the hydrated density and lipid composition, establish possibly the concept of a single apolipoprotein as the most characteristic feature of this class of lipoproteins, and offer thus a new insight into the structural aspects of LDL.

# CHAPTER II

# MATERIALS AND METHODS

## Materials

### Human Plasma

Venous blood was obtained from healthy males and females of 20 to 35 years of age after an overnight fast. Blood from each individual was collected in a 470 ml plastic vacuum container (Abbott) which contained 2115 U.S.P. units sodium-heparin in saline with sodium citrate as buffer. The blood was cooled at 4°C for two hours before centrifugation to remove red cells and white cells. Analyses of cholesterol, triglyceride and phospholipid were made on each plasma sample, and only the samples with normal lipid levels were pooled and utilized.

### Antibodies

Rabbit serums containing antibodies to human whole serum, human ApoA-LP, human ApoB-LP, human albumin, and human γ-globulin were obtained from Hoechst Pharmaceutical Company (Lloyd Brothers, Cincinnati, Ohio). Goat serums containing antibodies to rabbit whole serum, rabbit γ-globulin and rabbit whole globulin were purchased from Mann Laboratory, New York. Rabbit serum containing antibodies to human VLDL was prepared in our laboratory.

## Chemicals

Disodium salt of EDTA, sodium bromide, sodium barbital, and boric acid were obtained from Mallinckrodt Chemical Works, New York; Noble special agar from Difco, Detroit, Michigan; Amido Black 10B and 99% caesium chloride from K and K Laboratories, Plainview, New York; sodium chloride from Baker, Phillipsburg, N. J.; Ponceau Red and Oil Red O from Allied Chemical, New York; nigrosin and sodium acetate from Fisher, Fair Lawn, N. J.; amino acid calibration mixture, type 1, for amino acid analyzer from Beckman, Palo Alto, California; glucosamine · HCl and Tris from Mann, New York.

## Methods

### Isolation and Fractionation of LDL

The isolation procedure, a modification of the method of Lindgren, Elliot and Gofman (19), is outlined in Figure 2. Fresh plasma was subjected to ultracentrifugation by layering under NaCl solution of density 1.006 g/ml (8:3 by volume) in Beckman polyallomer tubes (5/8" dia. x 3") in the No. 50 rotors of the Spinco Model L or Model L-2 preparative ultracentrifuges at 4°. Lipoproteins of density less than 1.006 g/ml were floated at 105,000 g for 22 hours, and the top layer was removed by a tube-slicing technique. The bottom portion was collected. A sample was dialyzed against a NaCl solution of known density at 4°C until equilibrium was reached. The difference in density at equilibrium between dialysate and the sample was considered as the density contributed to the salt solution by proteins and lipoproteins. This $\Delta d$ was taken into consideration each time during actual measuring of the density of a protein

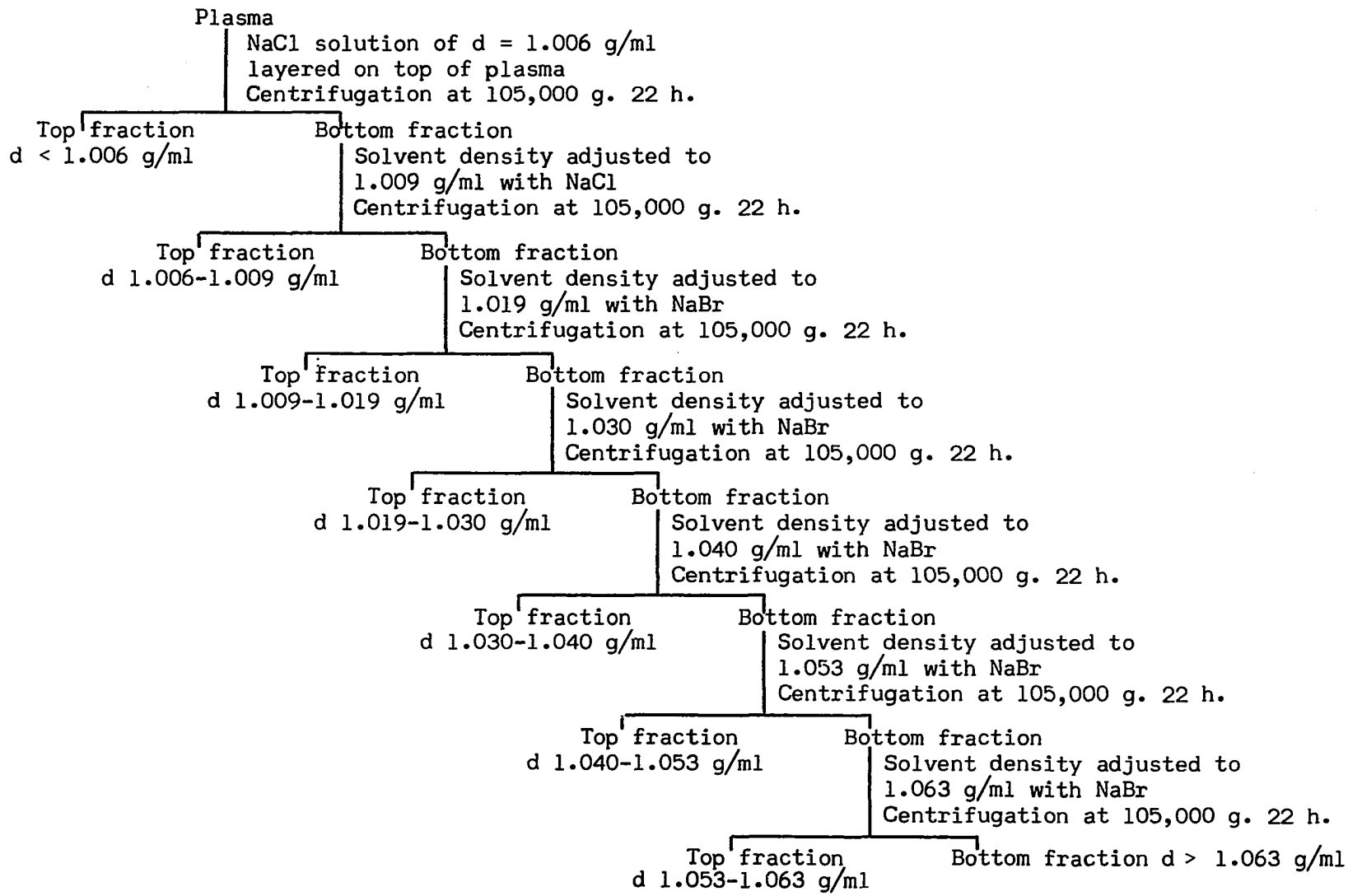


Figure 2. Ultracentrifugal isolation and fractionation of human plasma low-density lipoproteins.

or lipoprotein solution for a true solvent density. After adjusting the solvent density to 1.009 g/ml with NaCl, the bottom fraction was centrifuged at 105,000 x g for 22 hours. Lipoproteins collected on the top of the tubes were designated as d 1.006-1.009 g/ml. Solvent densities of the bottom fractions were increased to 1.019, 1.030, 1.040, 1.053 and 1.063 g/ml successively with NaBr and each fraction was centrifuged at 105,000 x g for 22 hours. The density ranges of the lipoproteins collected from the top of the tubes were designated as 1.009-1.019, 1.019-1.030, 1.030-1.040, 1.040-1.053 and 1.053-1.063 g/ml, respectively. Each solvent density was determined from infranatant sample by dialysis against a salt solution till equilibrium, as described previously. Each subfraction was washed with NaBr at its upper density for 22 or 48 hours once or twice to remove the traces of albumin. The washing procedure yielded subfractions free of albumin, as demonstrated by immunochemical analyses.

## Purification of LDL

Ultracentrifugally isolated low density lipoproteins frequently contained ApoA-LP, especially at the density range 1.030-1.063 g/ml. Washing and repeated centrifugations did not remove completely the ApoA-LP. In order to remove the traces of ApoA-LP a procedure for purifying LDL with antibodies to human ApoA-LP was developed. Fifteen to twenty-five ml of rabbit serum containing antibodies to human ApoA-LP were pooled, solution density was adjusted to 1.250 g/ml with caesium chloride, and the mixture was centrifuged at 165,000 g for 44 hours. Rabbit serum lipoproteins were removed from the top of the tubes. In the lower portion of the tubes, γ-globulin and other lipid-free proteins were collected and tested for the presence of rabbit γ-globulin with goat serum con-

taining antibodies to rabbit whole serum and/or rabbit total globulin or rabbit γ-globulin. Subsequently, a series of test tubes containing different ratios of antigen and antibody, that is, different ratios of LDL subfractions containing traces of ApoA-LP and anti-ApoA-LP of density greater than 1.25 g/ml were set up. After gently mixing and setting for 24 hours, they were centrifuged at 840 x g to remove the antigen-antibody precipitate. The supernate was tested for the possible presence of ApoA-LP with anti-ApoA-LP by double diffusion technique. The traces of ApoA-LP were removed by utilizing the optimal ratio of antigen-antibody. Then, the density of the mixture was adjusted to the upper density of the original fraction and centrifuged at 165,000 g for 44 hours to remove the rabbit proteins. The purified ApoB-LP subfractions were collected from the top of the centrifuge tubes and used for characterization studies.

### Delipidization of ApoB-LP Subfractions

The ApoB-LP subfractions isolated and purified by ultracentrifugation were dialyzed at 4°C against 0.05% EDTA, pH adjusted to 7.0-7.3 with NaOH, with changes of EDTA solution three times daily until the dialysate was free of chloride and bromide ion. The dialyzed ApoB-LP solution was subsequently concentrated with 50% dextran 80 solution if the protein concentration was below 4 mg per ml.

Total delipidization was carried out according to the following procedure: ApoB-LP solution (2-3 ml) was added drop by drop to a 50 ml glass-stoppered centrifuge tube containing ethanol and peroxide-free diethyl ether (3:1 v/v) which was precooled to -30°C. Care was taken to limit the air space. The tube was stoppered and shaken gently and allowed to set at -30°C for two hours. Prior to the separation of the precip-

itated protein, the tube was shaken for a few minutes and centrifuged at 840 x g for 20 minutes. The organic solvent was decanted. The protein was further extracted five times with the same solvents but by rotating gently (15-20 rpm) for 30 minutes at 4°C. Extraction was continued in the same manner for 16 hours using a mixture of ethanol-diethyl ether (1:3, v/v). The protein was then washed 4-5 times with peroxide-free diethyl ether. The tubes containing proteins were covered with aluminum foil and placed in an evacuated desiccator overnight at 4°C; the samples were stored in a desiccator at room temperature.

In some cases, attempts were made to retain the solubility of the delipidized proteins by the following modification of the delipidization procedure: the salt-free ApoB-LP solution was concentrated to 15-25 mg protein per ml. Eight to five volumes of ethanol-diethyl ether (3:1 v/v), precooled to 4°C, were added to one volume of ApoB-LP solution, followed by gentle shaking. The mixture was stored for two hours at 4°C, and then centrifuged at 840 x g for 15 minutes. The organic solvent was decanted. The precipitate was extracted four times with the same solvent by gentle shaking at 4°C. Then, it was washed five times with peroxide-free diethyl ether at 4°C. The diethyl ether was removed, and the protein was stored in the same manner as described previously.

## Characterization of LDL Subfractions

The LDL subfractions obtained ultracentrifugally were characterized by the following techniques: (1) immunodiffusion and immunoelectrophoresis, (2) cellulose acetate electrophoresis, (3) starch gel electrophoresis, (4) chemical composition of ApoB-LP, (5) analytical ultracentrifugation, (6) amino acid composition of protein moiety, and (7) other

studies on delipidized ApoB-LP.

Immunodiffusion and immunoelectrophoresis. Ouchterlony's (72) method of the double diffusion was used as follows: a 1% agar solution in veronal buffer, pH 8.6, μ 0.1, was prepared by heating the mixture in a boiling water bath for 20 minutes followed by cooling to 56°C. The solution was then transferred at 56°C to a precleaned, leveled, glass plate greased and supported by a plastic frame. The solution was allowed to cool and gel at room temperature for four hours. Then it was stored in a closed chamber saturated with moisture. The agar plate, when equilibrated with the moisture after two hours, was ready to use. Wells of diameter 3 mm of different arrangements were made with a gel punch (LKB, Stockholm, Sweden). They were correspondingly filled with antibodies and antigen. The plate was allowed to develop for 24 hours in the closed chamber at room temperature. A different pattern was used for immunoelectrophoresis. A center trough (1.2 x 65 mm) was made with two holes (dia. 1 mm) on each side of it. The two holes were filled with antigens. Electrophoresis was performed at a constant voltage (7 volts per cm) in a veronal buffer, pH 8.6, μ 0.1, for 90 minutes. Upon finishing the electrophoresis, an antibody or a mixture of antibodies were added to the center trough. The reaction was allowed to develop for 24 hours at room temperature. For staining the patterns, the agar plate was first washed with saline to remove the excess proteins or lipoproteins, then washed with distilled water to remove salts followed by drying with filter paper and stained either with Amido Black 10B for protein or with Red Oil O for lipid at 37°C.

Cellulose acetate electrophoresis. Two different apparatuses

were used. One was the Colab Tank surrounded with the cooling system. Two hundred volts were applied for 2-3 hrs to eight 3 x 14 cm strips of cellulose acetate in Michaelis buffer of pH 8.6, μ 0.15. Either 0.2% Ponceau Red in 3% TCA or 0.001% nigrosin in 2% acetic acid was used for staining. Another apparatus, Beckman Microzone Cell Model R-101, was also used. Here, eight micro samples were applied on one piece of cellulose acetate (5.5 x 14 cm). Electrophoresis was performed at constant voltage (250 volts) in veronal buffer, pH 8.6, μ 0.075, for 45-55 minutes at room temperature. The cellulose acetate strips were stained with Ponceau Red.

Starch gel electrophoresis. The starch gel was prepared according to the procedure of Smithies (115). Hydrolyzed starch was molded (1 x 14 x 15 cm) in Tris buffer, pH 8.6, μ 0.075, and allowed to set overnight. Samples were applied on small pieces of filter paper (Whatman No. 1) and placed in a slit in the gel 3 cm from the cathode end. Borate buffer, pH 8.2-8.6, was used as buffer in the electrode vessels. The electrophoresis was conducted with a potential gradient 6 v/cm for 16 hours at room temperature. The gel was then stained with Amido Black 10B.

Chemical composition of ApoB-LP. ApoB-LP subfractions were subjected to lipid and protein analysis. Samples for both determinations were aliquoted simultaneously. Protein was determined according to the method by Lowry et al. (116). Where a sufficient quantity of sample was available, nitrogen content was also determined by the method of Kjeldahl (117). Protein content determined by latter method was estimated by subtracting lipid nitrogen from total nitrogen and multiplying by the factor 6.25 as shown in the following formula (48):

$$6.25(N\text{-}P \times \frac{14}{31}) \qquad [1]$$

where N = concentration of nitrogen in mg per ml

P = concentration of lipid phosphorus in mg per ml

Aliquots for lipid determinations were extracted successively with chloroform-methanol (2:1, v/v), acetone-ethanol (1:1, v/v), ethanol-diethyl ether (3:1, v/v), and diethyl ether. Aliquots for the determination of free and total cholesterol, phospholipids, triglycerides and free fatty acids were taken in duplicate, and each was evaporated to dryness prior to redissolving in appropriate solvents. Total and free cholesterol were determined by the modified method of Schoenheimer and Sperry (118); cholesterol esters were estimated as cholesterol linoleate. Triglycerides were determined by the method of Van Handel and Zilversmit (119), free fatty acids by the modified method of Dole (120), and lipid phosphorus by the method of Fiske and Subbarow (121). A factor of 25 was used to convert the lipid phosphorus to phospholipid.

Analytical ultracentrifugation. Sedimentation, flotation and diffusion coefficients on each ApoB-LP subfraction were measured in a Spinco Model E analytical ultracentrifuge equipped with a schlieren optical system and RTIC unit for indicating and regulating temperature. All experiments were carried out at 25°C. Whereas sedimentation and flotation experiments were performed generally at rotor speed of 52,640 rpm and only occasionally at 56,100 rpm, 59,780 rpm, 47,660 rpm, or 42,040 rpm, the diffusion experiments were carried out at 12,590 rpm. Photographs of the schlieren patterns were taken by the automatic mechanism of

the ultracentrifuge. Usually 10 pictures were taken for each experiment. A Nippon Kogaku KK Shadowgraph Model 6 which gave an accuracy of 0.0001 cm was used to magnify and measure the plates.

Samples of 3-4 ml were equilibrated with a NaCl solution of known density containing 0.01% EDTA, pH 6.8-7.0, by dialyzing the samples against it for 32-40 hours with three changes of external solution daily.

Specific gravity of each solvent was measured at 25°C by hydrometer (Fisher) which was calibrated against water at 15°C. The density of each solvent at 25°C was calculated from the value of specific gravity multiplied by 0.9991, the density of water at 15°C.

Viscosity of solvents was measured with an Ostwald viscometer and calculated by equation [2] (122)

$$\eta = A\rho t - B(\rho/t) \qquad [2]$$

where $\rho$ is the density of solvent and t is the time of flow in seconds. A and B are constants determined by two solutions of known viscosity values. Distilled water and 0.15 M NaCl were used as references. Viscosity values of NaCl solutions were taken from International Critical Tables. All experiments were carried out in a constant temperature water bath (Circu-Temp Chemical Rubber Co.) maintained at 25° ± 0.1°C.

Determination of sedimentation and flotation coefficients. The sedimentation coefficient, S, was calculated according to Svedberg and Katsurai (123) by using the following equation:

$$S = \frac{dx / dt}{\omega^2 x} \text{ or } \frac{d \ln x / dt}{\omega^2} \qquad [3]$$

where x is the distance in cm from boundary to the center of rotation at

time t (in seconds) and $\omega$ is the angular velocity of centrifugation in radians per second. The flotation coefficient, $S_f$, was calculated by the same equation, but it contains a negative slope, d ln x / dt.

A Fortran computer program (see Appendix B) was written to give the values of log x in parallel with ln x from the data measured from schlieren patterns. The slope d log x / dt (or d ln x / dt) was obtained by plotting log x (or ln x) values against time t.

Three to four different lipoprotein concentrations were used for each determination. The flotation and sedimentation coefficients at infinite dilution, $S_f^o$ and $S^o$, were obtained by extrapolating the $S_f$- and S-values at three different protein concentrations.

The observed S value was corrected to $S_{20,w}$, the sedimentation coefficient in water at 20°C, according to Svedberg and Pedersen (124), and expressed in seconds or in Svedberg units (1 S = $10^{-13}$ cgs. units).

$$S_{20,w} = S_{obs}\left(\frac{\eta_t^o}{\eta_{20}^o}\right)\left(\frac{\eta_t}{\eta_t^o}\right)\left(\frac{1 - \bar{V}_{20}\rho_{20}^o}{1 - \bar{V}_t\rho_t}\right) \qquad [4]$$

where $\eta_t$ = viscosity of solvent at t°C

$\eta_t^o$ = viscosity of water at t°C

$\eta_{20}^o$ = viscosity of water at 20°C

$\bar{V}_t$ = partial specific volume of solute in the investigated solvent at t°C

$\bar{V}_{20}$ = partial specific volume of solute in the same solvent at 20°C

$\rho_t$ = density of solvent at t°C

$\rho_{20}^o$ = density of water at 20°C

$\bar{V}_t$ was determined experimentally at 25°C (see hydrated density). $\bar{V}_{20}$ was

calculated from $\bar{V}_{25}$ by assuming the temperature variation of the partial specific volume of ApoB-LP to be similar to that of other common proteins (125). The sedimentation coefficient at 25°, $S_{25,w}$, was obtained by extrapolating the S and $S_f$ to 0.9971 g/ml, density of water at 25°C. This extrapolated $S_{25,w}$ could be checked by equation [5].

$$S_{25,w} = S_{obs}\left(\frac{\eta_t}{\eta^o_{25}}\right)\left(\frac{1 - \bar{V}_{25}\rho^o_{25}}{1 - \bar{V}_t\rho_t}\right) \qquad [5]$$

where $\eta^o_{25}$ = viscosity of water at 25°C

$\rho^o_{25}$ = density of water at 25°C

The standard flotation coefficient $S^o_{f,26}$ at 26°C in a NaCl solution of density 1.063 g/ml should be calculated from that at 25°C, $S^o_{f,25}$, theoretically according to equation [6]

$$S^o_{f,26} = S^o_{f,25}\left(\frac{\eta^o_{25}/\eta^o_{20}}{\eta^o_{26}/\eta^o_{20}}\right)\left(\frac{\eta_{25}}{\eta^o_{25}}\right)\left(\frac{\eta^o_{26}}{\eta_{26}}\right)\left(\frac{1 - \bar{V}_{26}\rho_{26}}{1 - \bar{V}_{25}\rho_{25}}\right) \qquad [6]$$

where $\eta^o_{25}/\eta^o_{20}$ and $\eta^o_{26}/\eta^o_{20}$ are the viscosities of water at 25°C and 26°C relative to that at 20°C. Assuming that the variations of the viscosity and density of the solvent as well as the change in partial sepcific volume of ApoB-LP from 25° to 26° are negligible, the difference between $S^o_{f,25}$ and $S^o_{f,26}$ would be very small.

Hydrated density determination. Four to five flotation and/or sedimentation determinations were performed on same sample at different solvent densities. The observed S's and $S_f$'s multiplied by the viscosity of each solvent were a linear function of solvent density. The density at which the solute had a zero sedimentation rate was defined as the hy-

drated density of the solute.

Calculation of partial specific volume. Since sedimentation coefficient is proportional to $(1 - \bar{V}\rho)$ as shown by equation [7]

$$S = \frac{M(1 - \bar{V}\rho)}{Nf} \qquad [7]$$

where M is the molecular weight, N is the Avogadro's number and f is the frictional factor, S, $\bar{V}$ and $\rho$ have the same meaning as previously described.

If $S = 0$,

$1 - \bar{V}\rho = 0$

Then, $\bar{V} = \frac{1}{\rho}$ [8]

Therefore, the reciprocal value of the hydrated density is the partial specific volume of the lipoprotein.

Diffusion coefficient determination. Diffusion experiments were performed according to the procedure described by Schachman (126) with a synthetic boundary cell. The apparent diffusion coefficient, $D_{app}$, was calculated from schlieren patterns according to equation [9]

$$D_{app} = \frac{1}{4\pi t} \left(\frac{A}{H_{max} F}\right)^2 (1 - \omega^2 St) \qquad [9]$$

where A is the area in $cm^2$ between the gradient curve and the baseline at time t measured from the instant when the synthetic boundary was formed, and $H_{max}$ is the maximum height of that curve. F is the magnification along the baseline, t is the time in seconds, and $\omega$ is the angular velocity of the centrifugation in radian per second. The factor $(1 - \omega^2 St)$

was introduced by Lamm (127) in order to correct for the influence of the variation of the centrifugal field in the different parts of the diffusing boundary. The area was measured through the ordinates of the gradient curve at a definite interval. For the purpose of time saving a Fortran computer program (see Appendix B) was developed for the calculation of the area A and the $D_{app}$. The $D_{app}$ was calculated usually for 4-8 pictures taken at different times. The values of $D_{app}$ showed a slight time dependence because the initial boundary was not infinitely sharp. "Zero time correction" was made from a plot of the $D_{app}$ versus the reciprocal of time. The intercept of this linear plot at $1/t = 0$, corresponding to infinite time, is the true diffusion coefficient for zero time. Diffusion coefficients at infinite dilution, $D^o_{app}$, were extrapolated from $D_{app}$ values obtained at two to three different lipoprotein concentrations. $D^o_{app}$ was then further corrected to the diffusion coefficient, $D^o_{20,w}$, according to the standard (126) equation [10]

$$D^o_{20,w} = D^o_{app}\left(\frac{293}{273+t}\right)\left(\frac{\eta_t}{\eta^o_t}\right)\left(\frac{\eta^o_t}{\eta^o_{20}}\right) \qquad [10]$$

where t is temperature in centigrade and $\eta$'s are as defined in equation [4].

Molecular weight calculations. Molecular weights were calculated from sedimentation velocity and diffusion coefficient according to the Svedberg equations:

$$M = \frac{RTS}{D(1 - \bar{V}\rho)} \qquad [11]$$

where R is the gas constant, $8.314 \times 10^7$ erg/mole/degree, T is the abso-

lute temperature, $^{o}K$, at which the S and D values were determined, $\bar{V}$ and $\rho$ are the partial specific volume of the solute and the density of the solvent at the temperature T. S is expressed in seconds.

Amino acid analysis. Three to four mg of delipidized lipoprotein (Apolipoprotein B) of each subfraction was hydrolyzed with 1 ml 5.7 N glass distilled HCl in an evacuated, sealed tube at $110^{o} \pm 1^{o}C$ for 24 hours. One of the samples was also hydrolyzed for 72 hours in order to determine the percentage of destruction during hydrolysis. The hydrolysates were dried in a rotary evaporator at $37^{o}C$, redissolved in distilled water, and evaporation was repeated. The residues were then dissolved in 3 ml of 0.2 N Na-citrate buffer, pH 2.2. Aliquots were analyzed in triplicate by ion exchange chromatography with a Beckman Model 120 C amino acid analyzer. The neutral and acidic amino acids were chromatographed on a 50 cm "long" column containing Beckman PA 28 resin and eluted with citrate buffer, pH 3.28, first for 85 minutes, and followed then with the same buffer, pH 4.25, for 105 minutes. The basic amino acids were placed on a 6 cm "short" column packed with Beckman resin PA 35 and eluted with citrate buffer, pH 5.25, for 57 minutes.

The analyzer was calibrated with the type 1 amino acid calibration mixture and, in studies of the performic acid-oxidized protein, with the oxidized calibration mixture and cysteic acid.

Half-cystine and methionine were determined as cysteic acid and methionine sulfoxide after oxidizing the apolipoprotein B with performic acid. The values for cysteic acid were corrected for the 94% recovery found by Moore (128).

Tryptophan was determined spectrophotometrically by the method

of Spies and Chambers (129) with the p-dimethylaminobenzaldehyde-$H_2SO_4$ reagent.

Glucosamine was determined on a separate hydrolysate with the amino acid analyzer. Glucosamine · HCl standard and the samples were hydrolyzed in 2 N HCl at $110^{\circ} \pm 1^{\circ}C$ for 16 hours. Hydrochloric acid was removed and the hydrolysates were redissolved in the citrate buffer as previously described. After filtration, the hydrolysates were chromatographed on the short column and eluted in the same manner as described already for basic amino acids.

The amino acid analysis of HCl-hydrolysates of ApoB-LP subfractions revealed on several occasions the presence of a small peak at the position assigned usually to cysteic acid (17 minutes), and indicated thus the possible presence of another compound at this position. The apolipoprotein B was extracted exhaustively with chloroform-methanol (2:1, v/v) and the extract was subjected to phosphorus determination by the micromethod of Gerlach and Deuticke (130); even the application of this highly sensitive method failed to detect the presence of phosphorus in the extract. Subsequently, the apolipoprotein B dissolved in the 0.018 N sodium hydroxide was precipitated by 10% trichloroacetic acid and sedimented by low speed centrifugation. The phosphorus determination of a non-digested aliquot of supernate showed the absence of inorganic phosphorus and that of a digested aliquot the presence of organic phosphorus. Although the occurrence of a small amount of non-extractable phospholipid could not be ruled out, it was thought necessary to test apolipoprotein B for the possible presence of phosphoserine. A reference sample of phosphoserine (Calbiochem, Los Angeles, Calif.) dissolved in citrate buffer,

pH 2.2, was chromatographed on the long column in the Beckman Model 120 C amino acid analyzer. The phosphoserine peak overlapped with that of cysteic acid (17 minutes). Qualitative determination of phosphoserine was performed by the following procedure. Apolipoprotein B samples were hydrolyzed in 5.7 N HCl in open tube for one hour at 100°. The HCl was removed by evaporating the hydrolysate to dryness over NaOH in vacuo. The residue was redissolved in distilled water and the solution was subjected to high voltage electrophoresis in the Vanguard Model 905 apparatus. Cysteic acid and phosphoserine were used as the reference compounds. The hydrolysate was applied to Whatman No. 1 paper and the electrophoresis was performed with a buffer system composed of pyridine-acetic acid-water (1:10:289, v/v/v), pH 3.6, for 50 minutes at 3000 volts. The papers were stained with 0.05% ninhydrin in ethanol and the color was developed by heating the papers in oven at 100°.

Other studies of delipidized ApoB-LP subfractions. Attempts were made to solubilize the apolipoprotein B without any additives. It was found that the apolipoprotein obtained by delipidization of ApoB-LP under mild condition (as described in delipidization procedure) would dissolve in Tris buffer, pH 8.3, μ 0.1, and in 0.018 N sodium hydroxide at room temperature. A further increase in the solubility of apolipoprotein B could be achieved by heating the sample for 30 minutes at 37°; the protein could be dissolved almost completely by heating it slowly in a sand bath for 30 minutes at 70-75°. The proteins obtained by vigorous delipidization (as described previously) would dissolve only slightly in Tris buffer, but would dissolve almost completely in dilute sodium hydroxide, pH 12, after heating at 70-75°C. Immunochemical properties of

these ApoB solutions were studied by immunodiffusion technique with rabbit sera containing antibodies to human ApoB-LP, human ApoA-LP and human albumin. Sedimentation and diffusion coefficients were determined at 2-3 different protein concentrations at pH 8.3 and pH 12 with and without heating. Also, one sample was determined at pH 7.0, obtained from pH 12 solution by adding HCl. Cellulose acetate electrophoresis was performed on the solubilized samples at pH values of 12, 8.3, 7.0 and 1, with and without heating.

# CHAPTER III

# RESULTS

## Isolation and Fractionation of LDL

Low-density lipoproteins were isolated from plasma of fasting healthy human donors by sequential preparative ultracentrifugation. Very low-density lipoproteins were first isolated by layering plasma under NaCl solution of density 1.006 g/ml and removing the material which floated at the top of the ultracentrifuge tubes. The solvent density (excluding the density of lipoproteins and proteins) of the bottom fractions was successively increased to 1.009, 1.019, 1.030, 1.040, 1.053 and 1.063 g/ml. It was found that the density contributed by proteins and lipoproteins was usually 0.010 to 0.013 g/ml depending on the concentration of the protein in the remaining plasma. The six lipoprotein fractions collected from the tops of the tubes were designated according to the solvent density at which they were isolated: (1) Fraction I (1.006-1.009 g/ml); (2) Fraction II (1.009-1.019 g/ml); (3) Fraction III (1.019-1.030 g/ml); (4) Fraction IV (1.030-1.040 g/ml); (5) Fraction V (1.040-1.053 g/ml); and (6) Fraction VI (1.053-1.063 g/ml). It was realized, however, that the true densities of the lipoproteins floating at the tops of the tubes were considerably less than the original densities of the salt solutions due to the formation of density gradients during centri-

fugation.

The purity of the LDL fractions was determined by standard procedures, and the findings will be presented in the section on characterization.

## Characterization of LDL

### Immunochemical Methods

Immunodiffusion. All six subfractions of low-density lipoproteins isolated ultracentrifugally gave identical precipitin lines against rabbit serum containing antibodies to human ApoB-LP using Ouchterlony's technique of double diffusion. Results are shown in Figure 3. At low antigen concentration only a single precipitin line was usually observed. But, at higher antigen concentration two and, occasionally, three precipitin lines were found in the same sample. This is illustrated in Figure 4.

The isolated LDL fractions were usually contaminated with albumin as detected by immunological tests. However, this contamination could be eliminated by recentrifugations. No γ-globulin contamination has ever been observed (Figures 5 and 6). However, the presence of apolipoprotein A-containing lipoprotein (ApoA-LP) was observed in practically all fractions at one time or another. In the upper density ranges such as 1.030-1.063 g/ml, ApoA-LP has been detected constantly (Figure 7). Washing and repeated centrifugation of the lipoprotein could not completely remove the ApoA-LP as demonstrated in Figure 8 where both the unwashed and the washed LDL fractions gave reactions with rabbit serum containing antibodies to human ApoA-LP. This reaction was nonidentical to the antigen-antibody re-

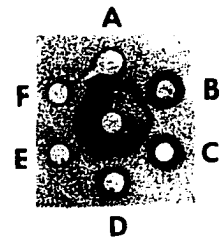


Figure 3. Immunological identity of six LDL subfractions.

Center well: rabbit serum containing anti-human ApoB-LP. A to F: LDL Fractions I to VI, characterized by densities 1.006 to 1.063 g/ml.

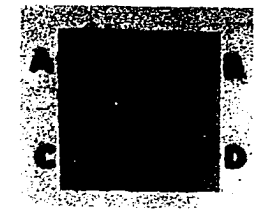


Figure 4. Multiple precipitin lines observed in antigen-antibody reaction.

Center well: rabbit serum containing anti-human ApoB-LP. A and C: LDL (1.009-1.063 g/ml). B and D: LDL (1.009-1.019 g/ml). Note three precipitin lines in D but only two in B.



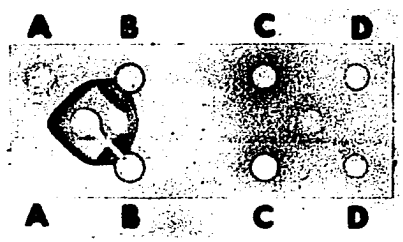


Figure 5. Contamination by albumin observed in LDL before washing.

Center wells: left, rabbit serum containing anti-human albumin; right, anti-human γ-globulin. A: human albumin; B and D: LDL fractions before washing; C: human serum.

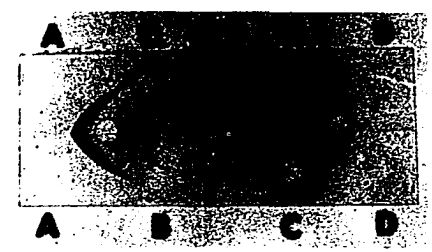


Figure 6. Contamination by albumin eliminated in LDL after washing.

Same arrangement of antigens and antibodies as in Figure 5.

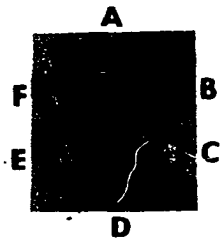


Figure 7. Presence of ApoA-LP in the LDL fractions.

Center well: rabbit serum containing anti-human ApoA-LP. A: Human ApoA-LP; B to F: LDL Fractions I + II, III, IV, V and VI, respectively. Note that the precipitin lines at C, D, E and F are identical to A (ApoA-LP).

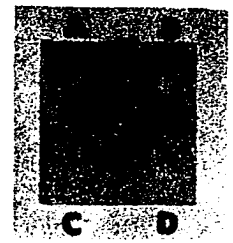


Figure 8. Washing and recentrifugation of LDL Fractions IV and V.

Center well: rabbit serum containing anti-human ApoA-LP. A and B: LDL Fraction IV after washing; C and D: LDL Fraction V after washing.



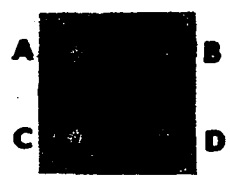


Figure 9. Interaction between human LDL and rabbit sera containing antibodies to human ApoA-LP and to human ApoB-LP.

Center well: LDL. A and C: rabbit serum containing antibodies to human ApoA-LP; B and D: rabbit serum containing antibodies to human ApoB-LP. Note the identity reaction between A and C, B and D, and the nonidentity reaction between A and B, and C and D.

action with anti-human ApoB-LP indicated by the crossing of the two precipitin lines (Figure 9). This crossing indicated also that the reaction with anti-ApoA-LP was not due to contamination of that antiserum with anti-ApoB-LP. In addition, it was demonstrated that anti-ApoB-LP did not give a precipitin reaction with ApoA-LP. Therefore, all precipitin lines obtained with anti-ApoB-LP must be due to the presence of ApoB-LP.

Removal of ApoA-LP from the LDL subfractions was accomplished by precipitation with the γ-globulin fraction of rabbit serum containing antibodies to human ApoA-LP. Each step of the procedure was followed closely by immunodiffusion. As the concentration of ApoA-LP in an LDL sample was reduced, the corresponding immunological precipitin line moved closer to the antigen-containing well. When the precipitin line disappeared, the degree of purity was considered satisfactory. The results of this stepwise purification procedure are shown in Figures 10 and 11.

After precipitating ApoA-LP by anti-human ApoA-LP, the added foreign rabbit proteins were removed from the LDL solutions by ultracentrifugation during which they sedimented to the bottom of the tubes. The top portions of the tubes contained the lipoproteins free of rabbit proteins. Removal of rabbit proteins was checked by immunodiffusion utilizing goat serum containing antibodies to rabbit γ-globulin and rabbit total globulin as shown in Figure 12. It was noticed that human albumin had been added to the commercial preparations of rabbit serum containing antibodies to human ApoA-LP. Fortunately, it was found that the added human albumin was removed simultaneously with rabbit proteins by ultracentrifugation (Figure 13). The purified LDL fractions are now called ApoB-LP in order to distinguish them from the immunochemically-heterogeneous

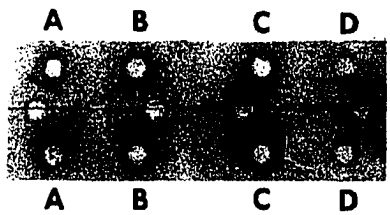


Figure 10. Stepwise purification of LDL

Center wells: rabbit serum containing antibodies to human ApoA-LP. A: LDL before purification; B, C: LDL under purification; D: LDL purified. Note that the precipitin lines are closer to B and C than to A, and disappear at D indicating the removal of ApoA-LP.

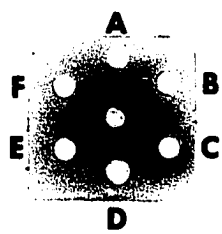


Figure 11. Comparison of LDL before and after precipitation of ApoA-LP by anti-human ApoA-LP.

Center well: rabbit serum containing antibodies to human ApoA-LP. A and D: human ApoA-LP; C and E: LDL before purification; B and F: LDL after purification.

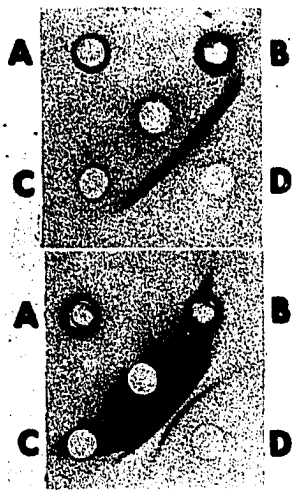


Figure 12. Testing for the presence of rabbit proteins in purified LDL fractions.

Center wells: top - goat serum containing antibodies to rabbit γ-globulin; bottom - anti-rabbit total globulin. A, B: samples from top portion of centrifuge tubes of purified LDL Fraction III; C: sample from top portion of LDL Fraction IV; D: sample from bottom portion of centrifuge tubes of LDL Fraction IV.

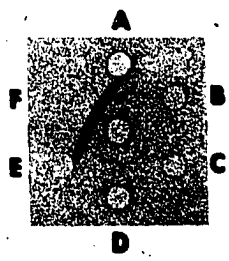


Figure 13. Testing for the presence of human albumin in purified LDL fractions.

Center well: rabbit serum containing antibodies to human albumin. A to E: the purified LDL Fractions I + II, III, IV, V and VI, respectively. F: human albumin.

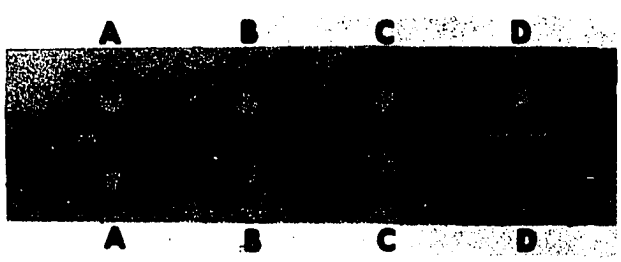


Figure 14. Comparison of the immunochemical properties of LDL fractions before and after purification.

Center trough: rabbit serum containing antibodies to ApoB-LP. A, B, C, D (bottom row): samples from LDL Fractions II to V before purification. A, B, C, D (top row): samples from corresponding Fractions after purification.

LDL. The purified ApoB-LP subfractions were still reactive to anti-human ApoB-LP as demonstrated in Figure 14.

Immunoelectrophoresis. All subfractions with the exception of Fraction I (1.006-1.009 g/ml) were submitted to immunoelectrophoresis. There was practically no difference among the patterns obtained with each ApoB-LP subfraction and rabbit serum containing antibodies to human ApoB-LP or human VLDL. A single precipitin line with or without tailing from the origin appeared at a position behind albumin and ApoA-LP but overlapping partially with the reaction given by ApoA-LP. When the center trough contained anti-human whole serum mixed with anti-human ApoA-LP and anti-human albumin, the purified LDL subfractions showed none of the precipitin lines characteristic of ApoA-LP or albumin. Some typical patterns are shown in Figures 15, 16, 17, 18 and 19. However, even before purification, the presence of ApoA-LP which was demonstrated clearly by immunodiffusion could not be detected by this method. Therefore, immunoelectrophoresis has been used throughout this study only as a method of characterization, but not as a criterion of purity.

## Cellulose Acetate Electrophoresis

By cellulose acetate electrophoresis each ApoB-LP subfraction showed a single band, close to the origin if the sample was applied between anode and cathode, and migrated toward anode at the position of β-globulin if applied near the cathode. Prior to the purification of subfractions, albumin was also detectable whereas ApoA-LP was not.

## Starch Gel Electrophoresis

Each ApoB-LP subfraction exhibited a single band on starch gel

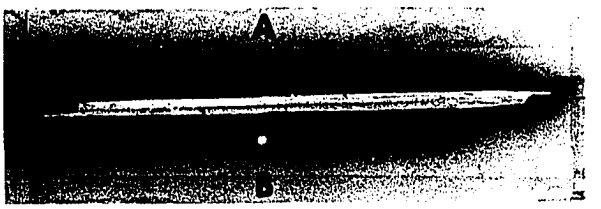


Figure 15. Immunoelectrophoresis of human whole serum and ApoB-LP Fraction II.

Center trough: mixture of rabbit sera containing antibodies to human serum, human ApoB-LP, and human VLDL. A: human whole serum; B: purified ApoB-LP Fraction II.

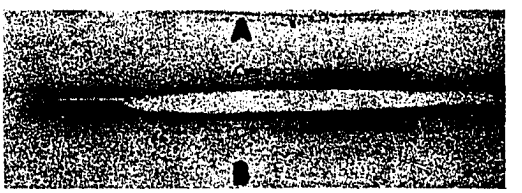


Figure 16. Immunoelectrophoresis of ApoB-LP fractions.

Center trough: rabbit serum containing antibodies to human ApoB-LP. A: Fraction III; B: Fraction V.

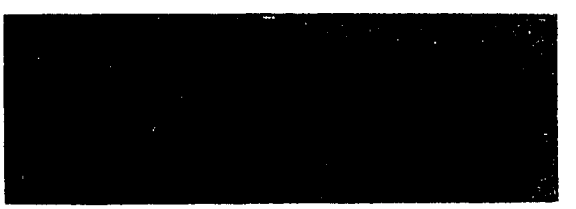

Figure 17. Immunoelectrophoresis of ApoB-LP fractions.

Center trough: rabbit serum containing antibodies to human VLDL. A: Fraction III; B: Fraction V.

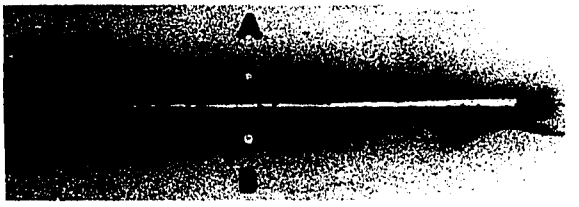


Figure 18. Immunoelectrophoresis of ApoB-LP and human albumin.

Center trough: mixture of rabbit sera containing antibody to human serum, human ApoA-LP, and human albumin. A: ApoB-LP (1.009-1.053 g/ml); B: human albumin.

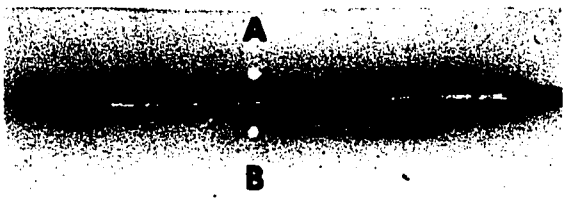


Figure 19. Immunoelectrophoresis of ApoB-LP and ApoA-LP.

Center trough: mixture of rabbit sera containing antibodies to human serum, human ApoA-LP, and human albumin. A: ApoA-LP; B: ApoB-LP (1.019-1.030 g/ml). Note absence of ApoA-LP in ApoB-LP Fraction.

electropherograms. The bands migrated toward the anode. There was no significant difference among the mobilities of various subfractions. A typical pattern of starch gel electrophoresis of ApoB-LP subfractions is shown in Figure 20.

## Chemical Composition of ApoB-LP Subfractions

Determinations of free cholesterol, cholesterol ester, triglyceride, phospholipid, and protein were performed on each ApoB-LP subfraction. The results are presented in Table 5. Six such analyses were done on each of Fractions IV and V; for these standard deviations are included. The results of analysis of Fraction II were not satisfactory due to the extremely small amount of material available, and therefore have not been included in the table. Since all figures represent an average value of several determinations the totals for each subfraction are not necessarily equal to one hundred percent. Triglyceride content was the most variable, as would be expected from its close dependence on sex, age, dietary habits and fasting interval of the donors.

Cholesterol ester increases slightly with increasing density while free cholesterol remains fairly constant in all subfractions. Triglyceride decreases drastically with increasing density. Both phospholipid and protein content are constant from Fraction III through Fraction VI. They are lower in Fraction I, but the phospholipid/protein ratio is the same for all fractions including Fraction I. The neutral lipid/(phospholipid + protein) ratio for Fraction I is 1.9; for Fractions III through VI it is close to a constant value of 1.18.

Free fatty acid values were not included in the table. However, the fatty acids of Fractions IV and V were determined; the weight percent-


Figure 20. Starch gel electrophoresis of purified ApoB-LP Fractions.

A: Fraction V; B: Fraction III; C: Fraction IV; D: Fraction II, and E: whole serum. Stained for protein. Origin at lower edge. ApoB-LP band migrated slower than β-globulin in the sample of whole serum.

TABLE 5

PERCENT CHEMICAL COMPOSITION OF HUMAN PLASMA ApoB-LP SUBFRACTIONS

| Fraction | Density Range g/ml | CE | FC | TG | PL | PR | PL/PR | N.L./PL-PR |
|---|---|---|---|---|---|---|---|---|
| I | 1.006-1.009 | 24.9 | 7.2 | 30.4 | 15.7 | 17.2 | 0.97 | 1.90 |
| II | 1.009-1.019 | - | - | - | - | - | - | - |
| III | 1.019-1.030 | 34.97 | 7.55 | 8.75 | 23.13 | 24.00 | 0.96 | 1.09 |
| IV | 1.030-1.040 | 41.30±1.48 | 6.70±.42 | 6.35±1.71 | 21.60±1.86 | 22.60±1.56 | 0.96 | 1.23 |
| V | 1.040-1.053 | 40.64±3.49 | 7.41±.73 | 5.60±2.32 | 21.60±2.09 | 24.35±2.40 | 0.89 | 1.16 |
| VI | 1.053-1.063 | 44.30 | 6.05 | 3.78 | 21.60 | 22.9 | 0.98 | 1.23 |

CE = Cholesterol ester expressed in terms of cholesterol-linoleate = 1.68 (Total cholesterol-Free cholesterol).

FC = Free cholesterol.

TG = Triglyceride.

PL = Phospholipid.

PR = Protein

N.L. = Neutral lipid = CE + FC + TG.

ages were 1.8 and 1.4, respectively.

## Physical-Chemical Characterization of ApoB-LP Subfractions

Determination of sedimentation and flotation coefficients. Qualitatively, all LDL fractions isolated and purified by preparative ultracentrifugation gave a single symmetric peak in the analytical ultracentrifuge. An asymmetric peak, indicating contamination by ApoA-LP, appeared only when the total lipoprotein concentration of a sample was very low. For this reason, the analytical ultracentrifugation was used only for ApoB-LP subfractions which showed no detectable ApoA-LP by immunodiffusion tests. All fractions characterized by analytical ultracentrifugation satisfied this criterion except Fraction VI, which contained a relatively high concentration of ApoA-LP. Precipitation of ApoA-LP with the γ-globulin fraction of rabbit anti-ApoA-LP serum removed the major part of impurities, but traces of ApoA-LP did remain in this subfraction. Sedimentation and flotation coefficients of every ApoB-LP subfraction were determined at various solvent densities. Figure 21 shows a graph of the slope d ln x/dt for ApoB-LP Fraction I in a solvent density of 1.063 g/ml, centrifuged at rotor speed 42,040 rpm. The $S_f$ value was calculated from the slope d ln x/dt according to equation [3]. Figures 22, 23, 24, 25, 26 and 27 are representative graphs for determination of flotation coefficients of ApoB-LP subfractions. Included are determinations for Fractions I + II, II, III, IV, V and VI in various solvent densities at a rotor speed of 52,640 rpm. The majority of points fall on straight lines indicating the reliability of the runs. Some typical ultracentrifugal flotation patterns are shown in Figures 28 and 29.

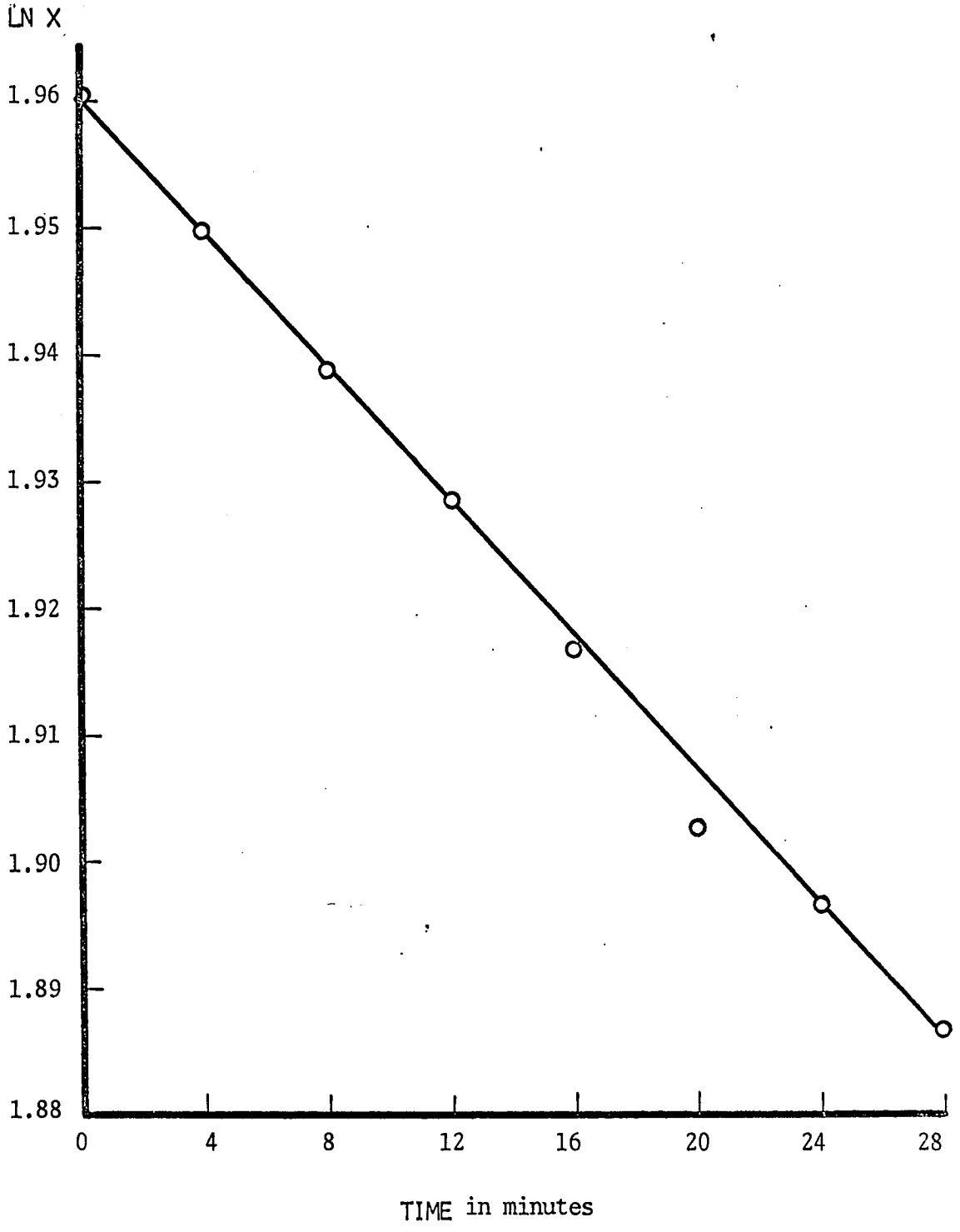


Figure 21. Determination of $S_f$ of ApoB-LP Fraction I, 1.006-1.009 g/ml in solvent density 1.063 g/ml. Measurements were taken after rotor speed reached 42,040 rpm.

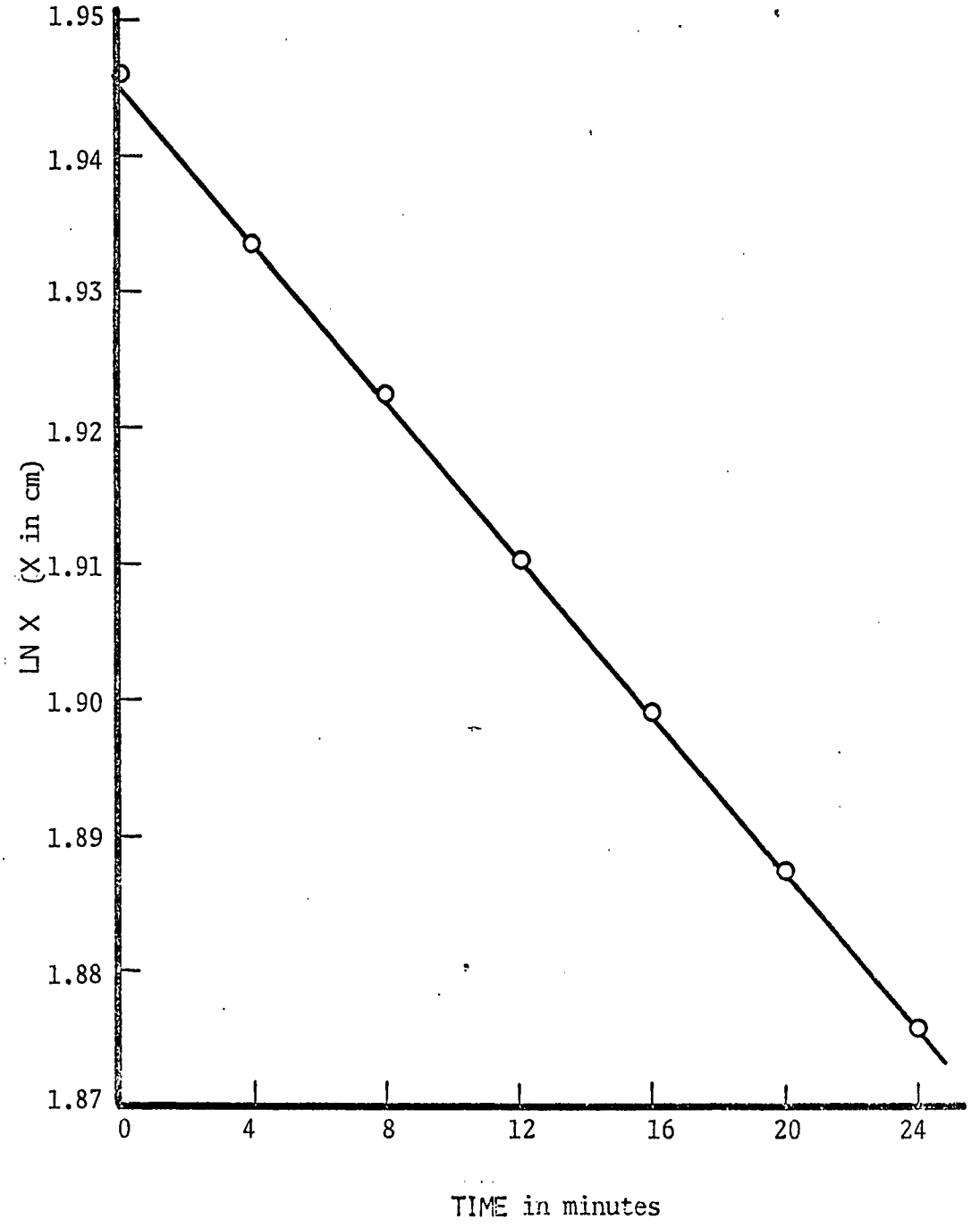


Figure 22. Determination of $S_f$ of ApoB-LP Fraction (I and II), 1.006-1.019 g/ml in solvent density 1.0770 g/ml. Measurements were taken after the rotor speed reached 52,640 rpm.

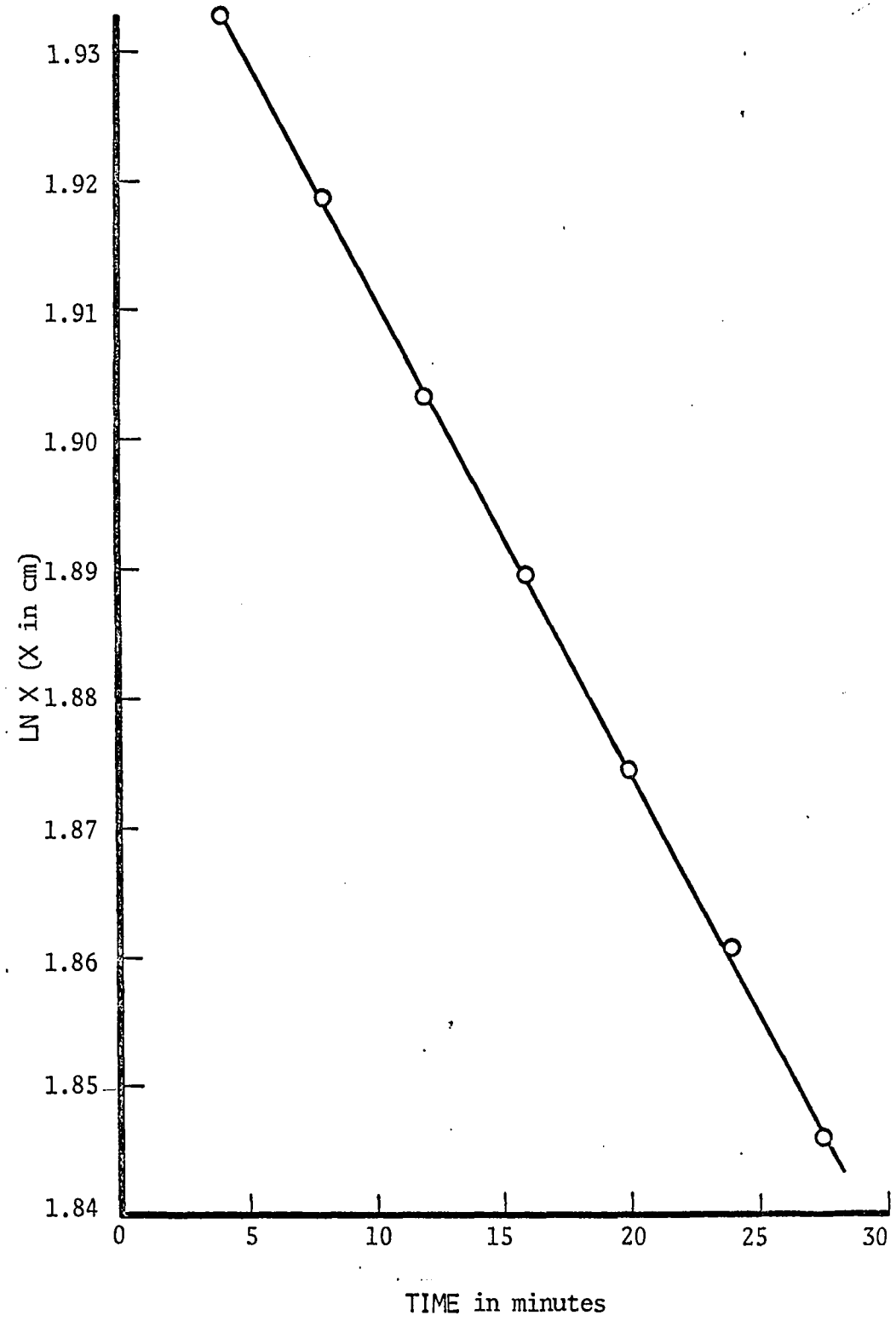


Figure 23. Determination of $S_f$ of ApoB-LP Fraction II, 1.009-1.019 g/ml in solvent density 1.0772 g/ml. Measurements were taken after the rotor speed reached 52,640 rpm.

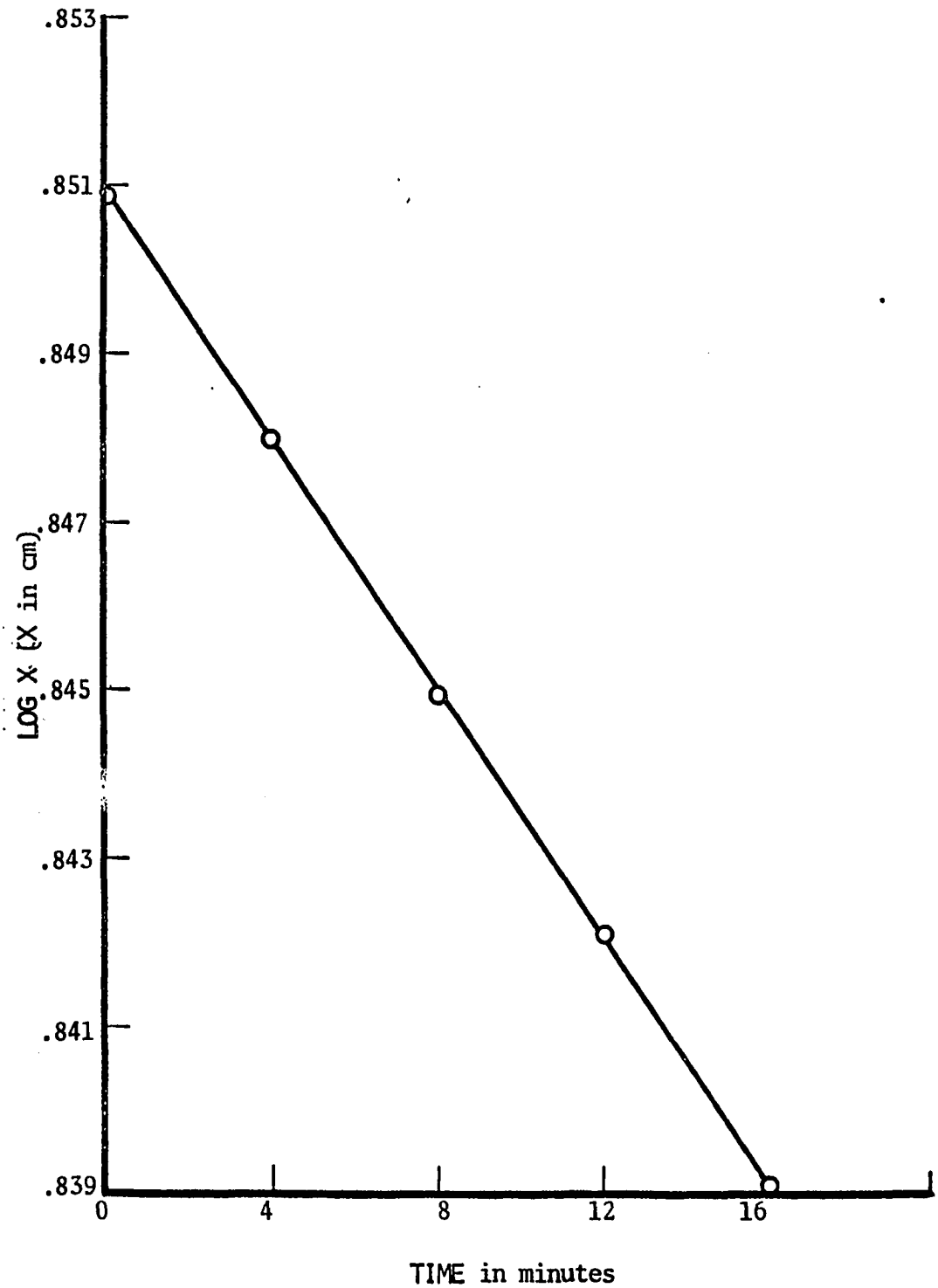


Figure 24. Determination of $S_f$ of ApoB-LP Fraction III, 1.019-1.030 g/ml in solvent density 1.063 g/ml. Measurements were taken after the rotor speed reached 52,640 rpm.

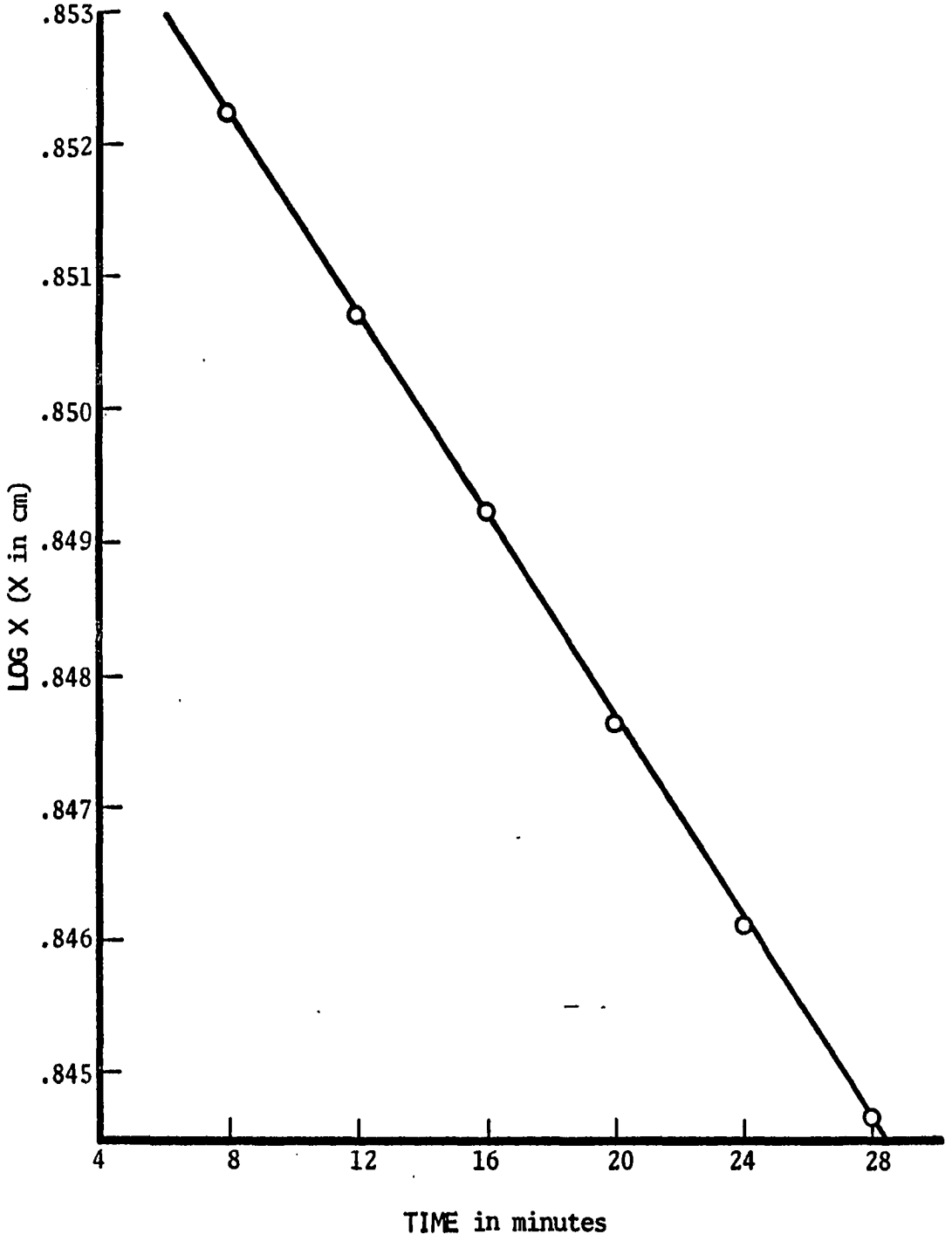


Figure 25. Determination of $S_f$ of ApoB-LP Fraction IV, 1.030-1.040 g/ml in solvent density 1.0480 g/ml. Measurements were taken after rotor speed reached 52,640 rpm.

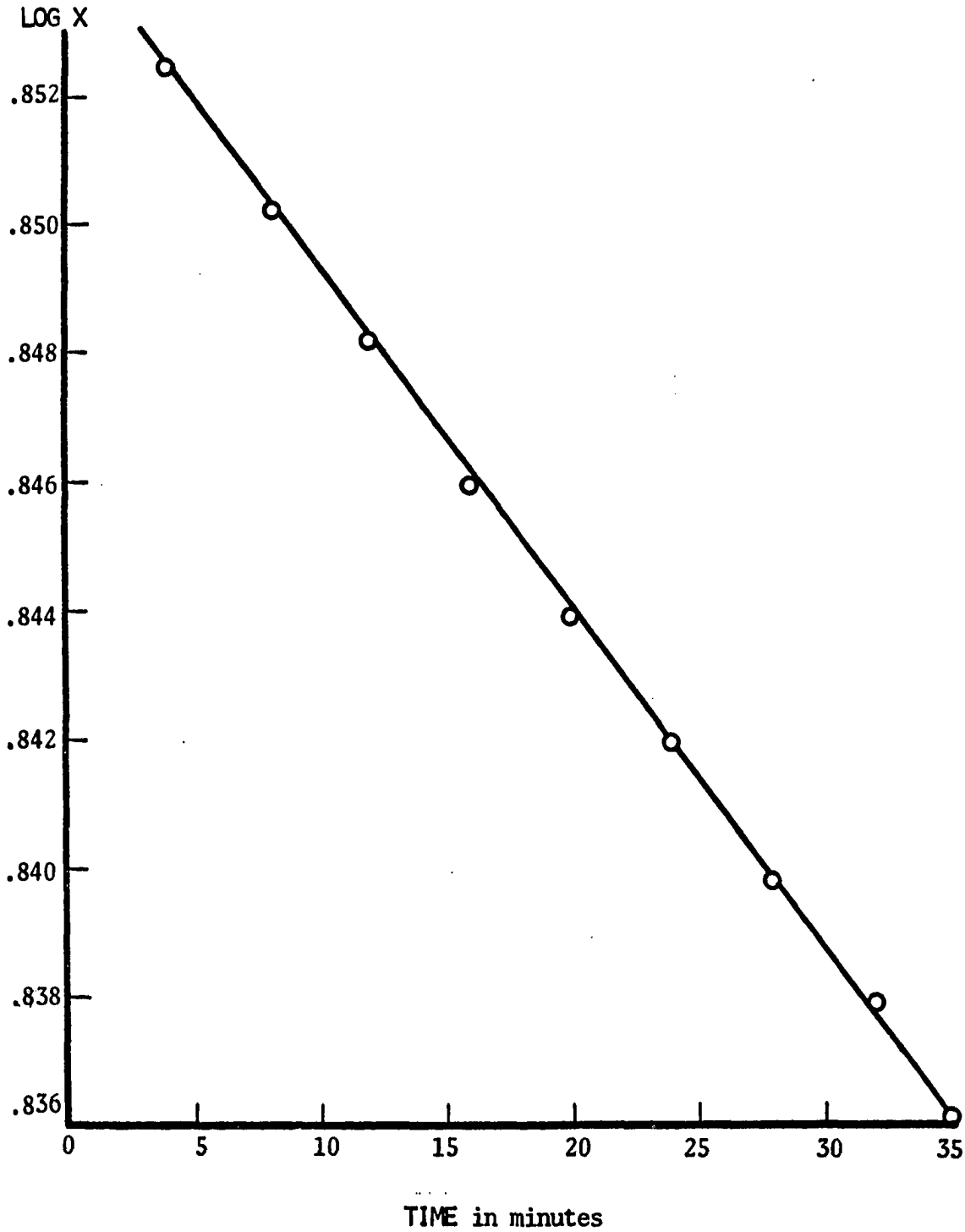


Figure 26. Determination of $S_f$ of ApoB-LP Fraction V, 1.040-1.053 g/ml in solvent density 1.063 g/ml. Measurements were taken after the rotor speed reached 52,640 rpm.

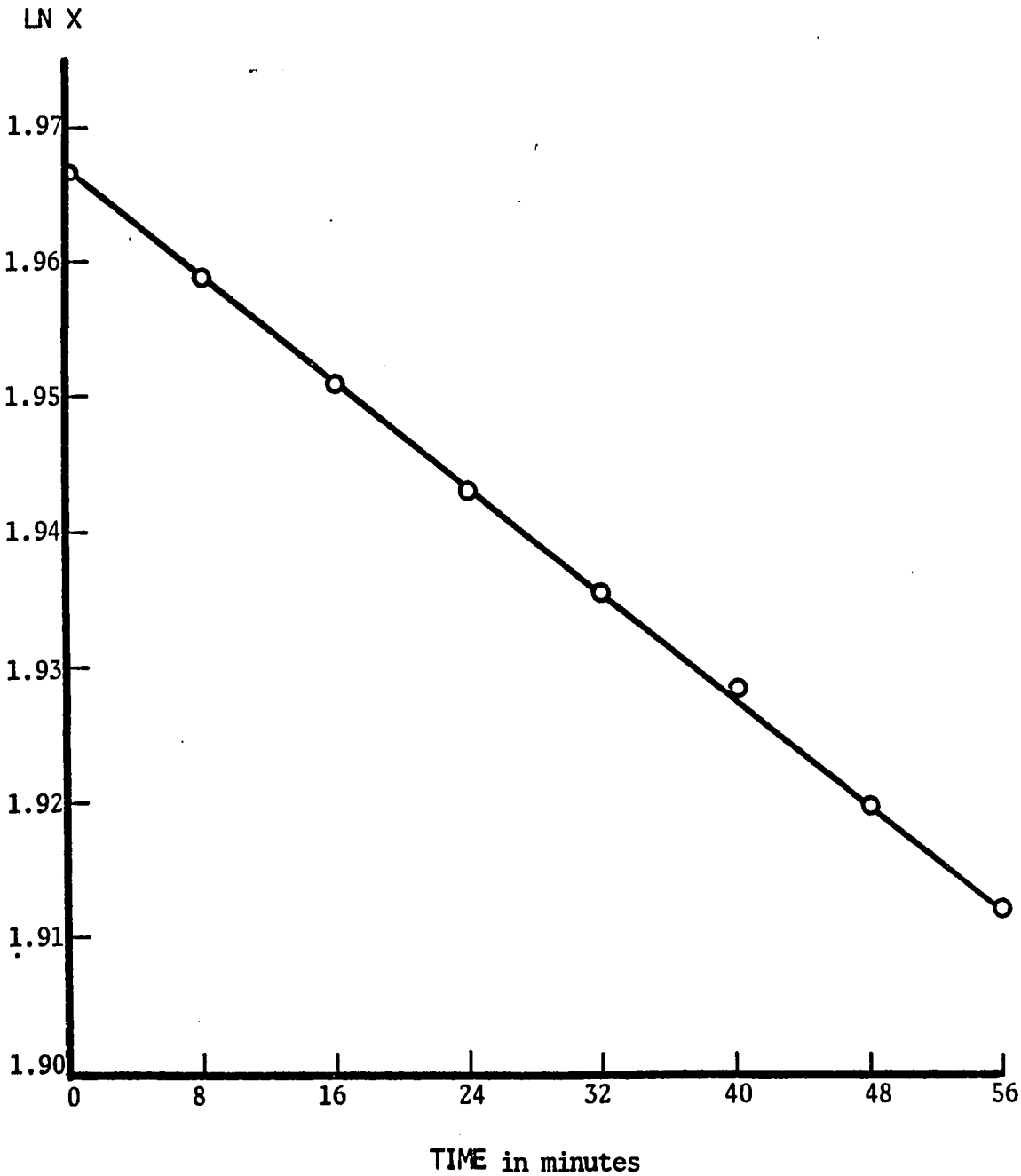


Figure 27. Determination of $S_f$ of ApoB-LP Fraction VI, 1.053-1.063 g/ml in solvent density 1.063 g/ml. Measurements were taken after rotor speed reached 52,640 rpm.

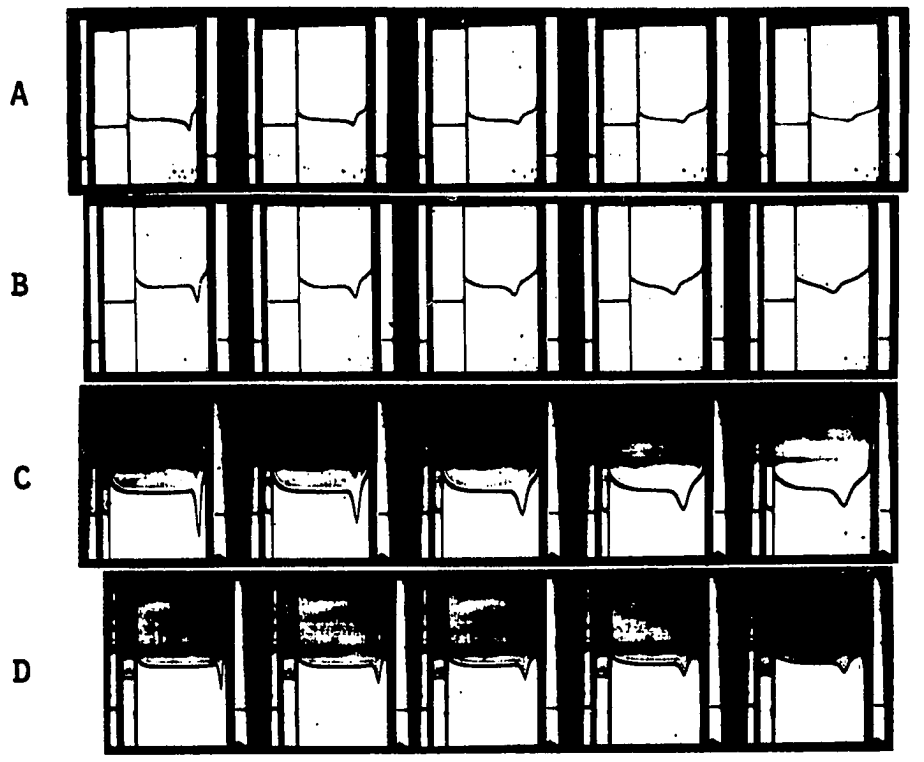


Figure 28. Ultracentrifugal flotation patterns of ApoB-LP Fractions I (A), I + II (B), II (C), and III (D).

Experimental conditions: rotor speeds 42,040 rpm (A) and 52,640 rpm (B, C, D); solvent density 1.063 g/ml; temperature 25°C. Pictures were taken from left to right at 4-minute intervals after rotor attained desired speed.

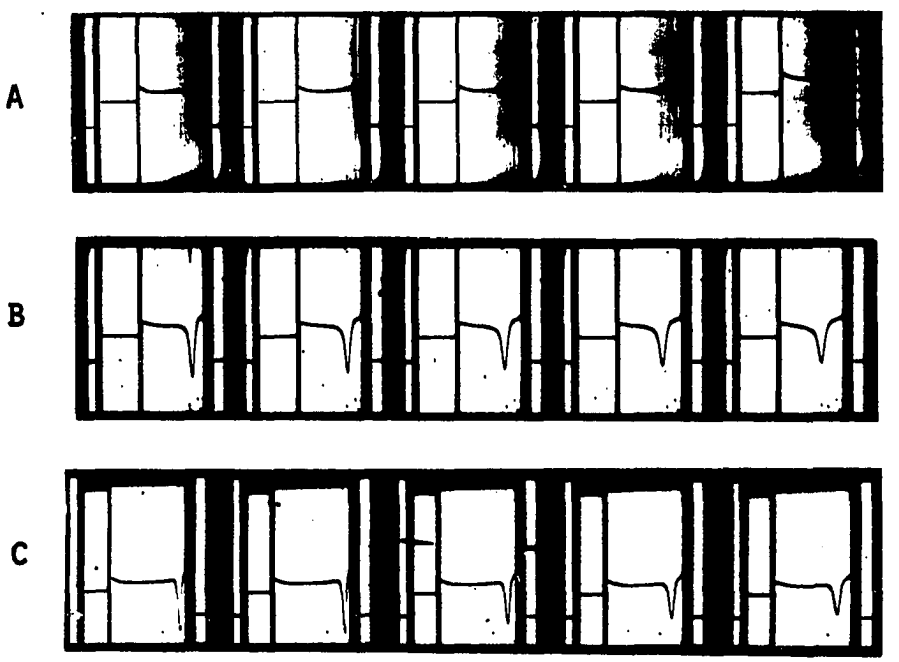


Figure 29. Ultracentrifugal flotation patterns of ApoB-LP Fractions IV (A), V (B), and VI (C).

Experimental conditions: rotor speed 52,640 rpm; solvent density 1.063 g/ml (A, B) and 1.077 g/ml (C); temperature 25°C. Pictures were taken from left to right at 4-minute intervals after rotor reached full speed.

Sedimentation experiments were performed by the same principle. Figures 30 and 31 are representative graphs for the determination of sedimentation coefficients of ApoB-LP Fractions V and VI at solvent density 1.0028 g/ml and 1.0052 g/ml, respectively. Again, all points fall on straight lines. Schlieren patterns of sedimentation experiments are shown in Figure 32.

Both flotation and sedimentation coefficients are concentration dependent. Figure 33 shows the determination of flotation coefficients at infinite dilution for ApoB-LP Fraction II, 1.009-1.019 g/ml, and ApoB-LP Fraction III, 1.019-1.030 g/ml. The flotation coefficients, $S_f^o$, for Fraction II and Fraction III were 16.7S and 12.4S, respectively. The slopes of $S_f$ versus concentration for both fractions are not identical, but are both positive. This indicates that there are no aggregation or association phenomena observed at higher ApoB-LP concentrations. Figure 34 illustrates the determination of the sedimentation coefficient at infinite dilution of ApoB-LP Fraction V, 1.040-1.053 g/ml. Four out of five points lie on a straight line obtained by plotting the S-values versus lipoprotein concentration. The S-values also increase with decreasing concentration. Again, this suggests that no aggregation or association occurs at higher ApoB-LP concentrations. Figure 35 shows the determination of $S^o$ of ApoB-LP Fraction VI, 1.053-1.063 g/ml. This subfraction has also a positive slope.

Values of $S_{20,w}$ were calculated according to equation [4] from the values of the observed sedimentation coefficients ($S_{obs.}$). Both $S_{20,w}$ and $S_f$ values for each subfraction are presented together with hydrated density ($\bar{d}$) and other physical-chemical parameters in Tables

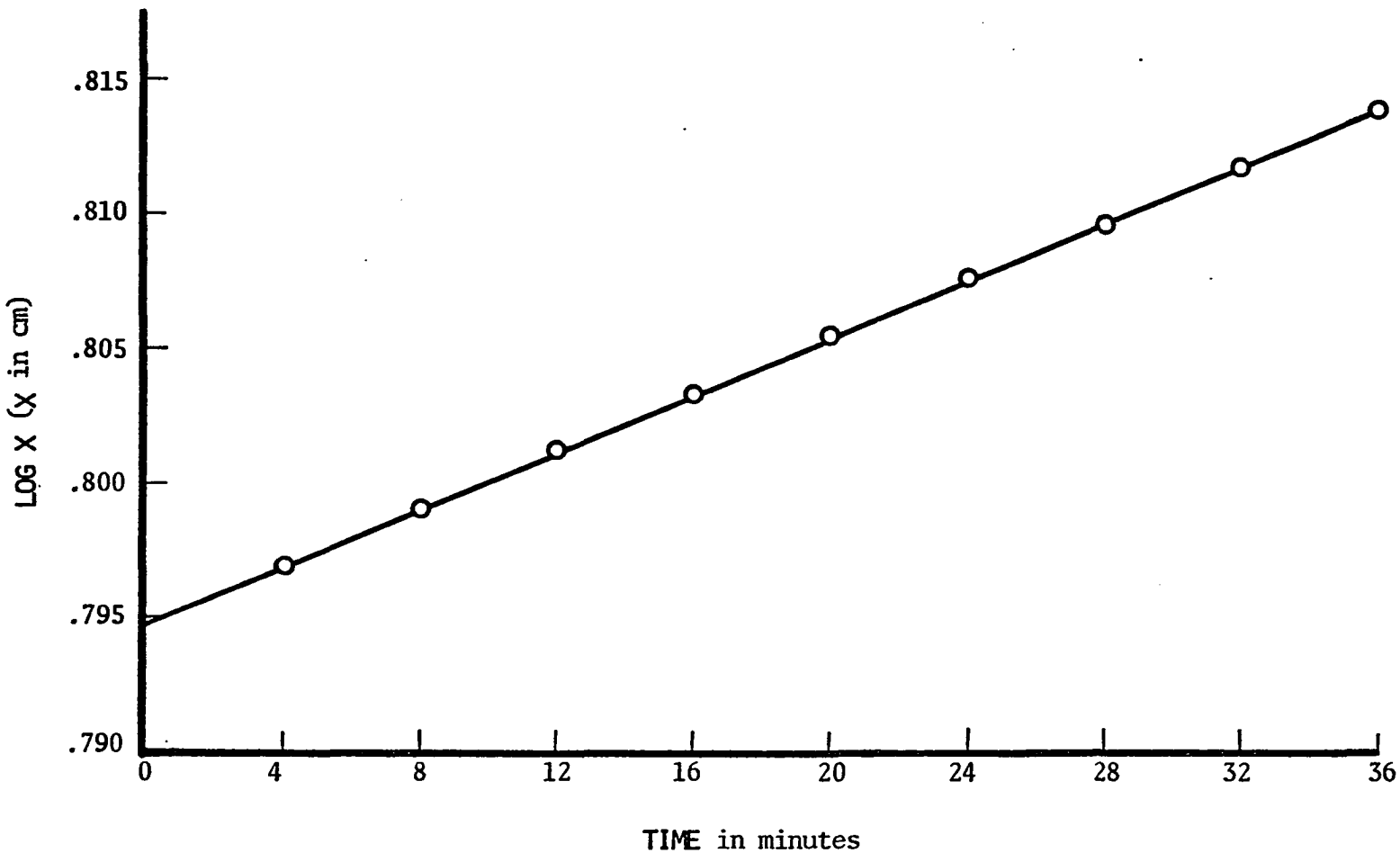


Figure 30. Determination of S of ApoB-LP Fraction V, 1.040-1.053 g/ml in solvent density 1.0028 g/ml. Measurements were taken after the rotor speed reached 52,640 rpm.

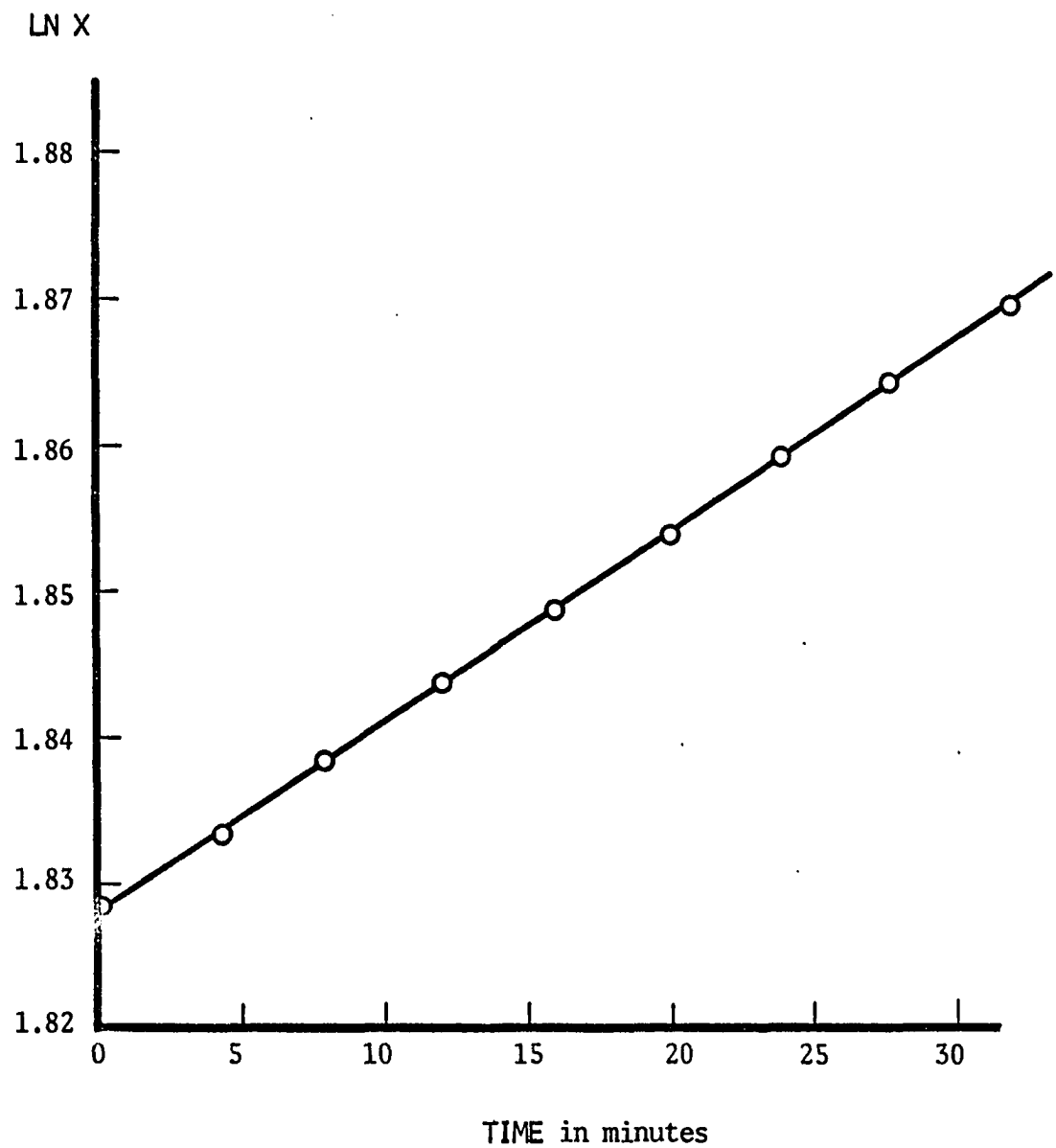


Figure 31. Determination of S of ApoB-LP Fraction VI, 1.053-1.063 g/ml in solvent density 1.0052 g/ml. Measurements were taken after the rotor speed reached 52,640 rpm.

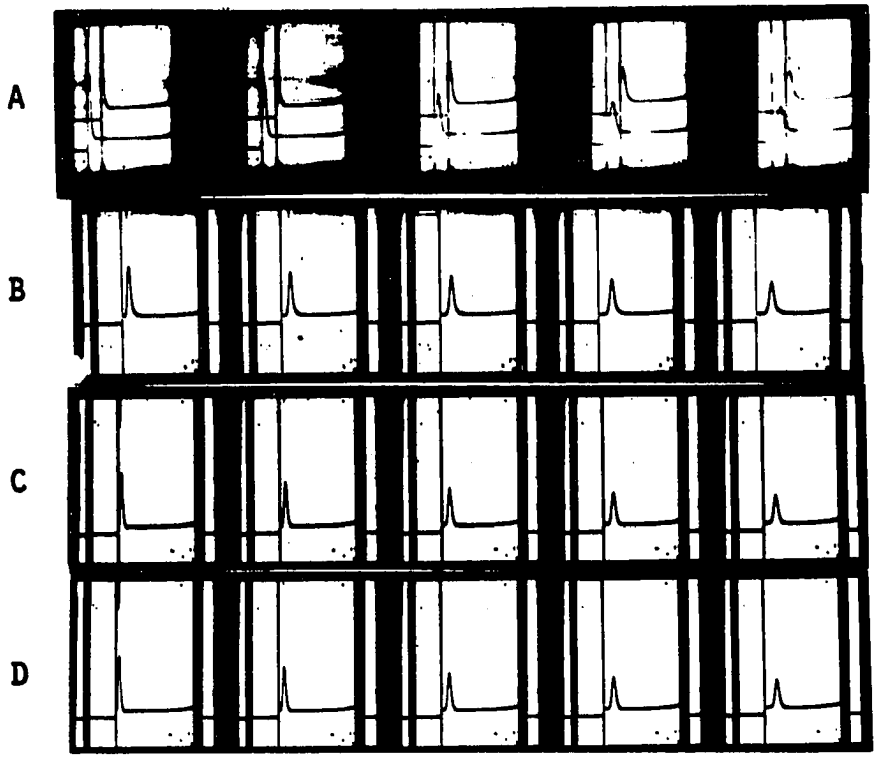


Figure 32. Ultracentrifugal sedimentation patterns of ApoB-LP Fractions II (A, top), III (A, bottom), IV (B), V (C), and VI (D).

Experimental conditions: rotor speed 52,640 rpm; solvent density 1.003 g/ml (A, B, C), 1.005 g/ml (D); temperature 25°C. Pictures were taken from left to right at 0, 4, 8, 16 and 24 minutes (A) and 4-minute intervals (B, C, D) after rotor attained full speed.

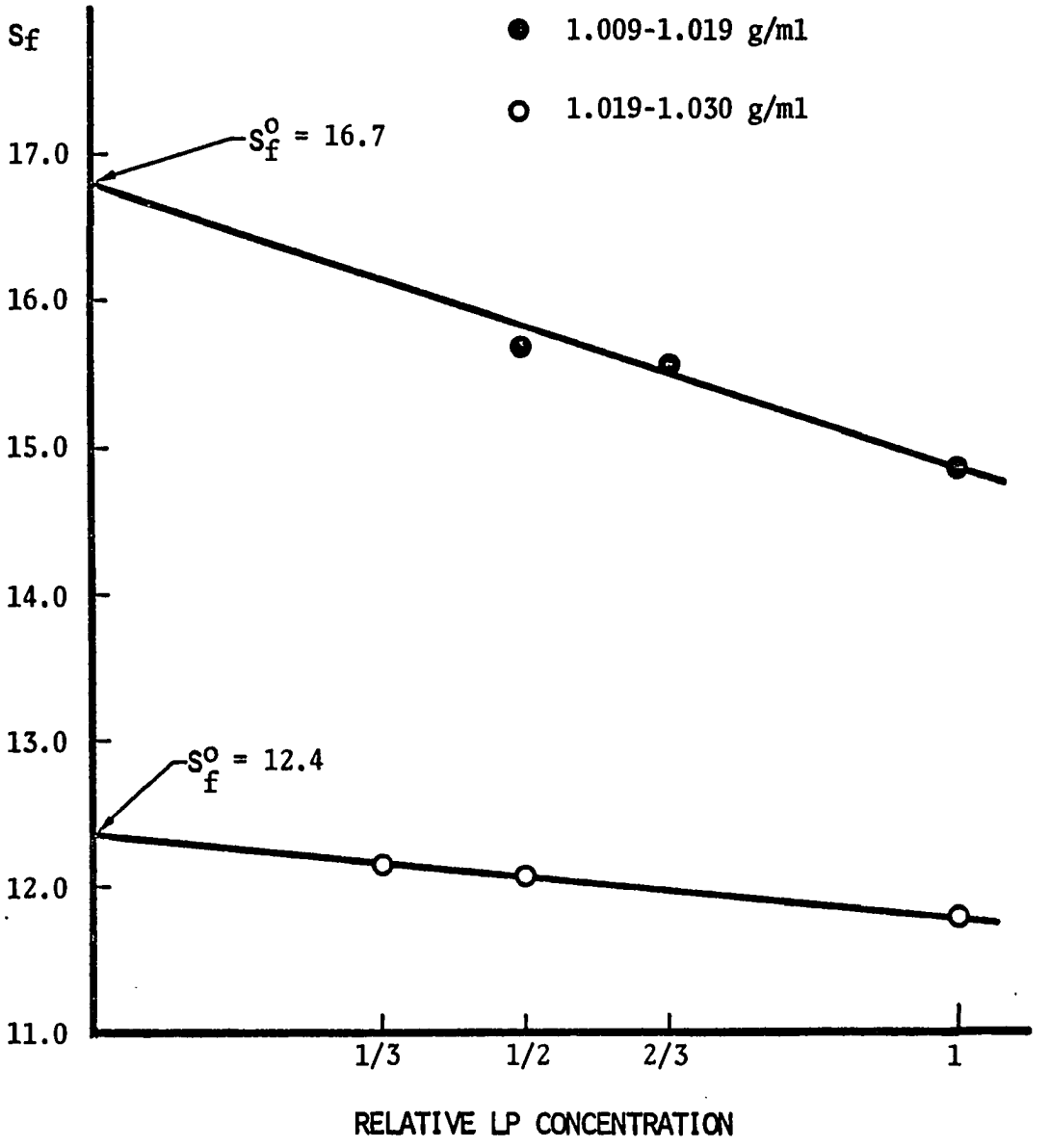


Figure 33. Determination of $S_f^o$ of ApoB-LP Fractions II, 1.009-1.019 g/ml and III, 1.019-1.030 g/ml.

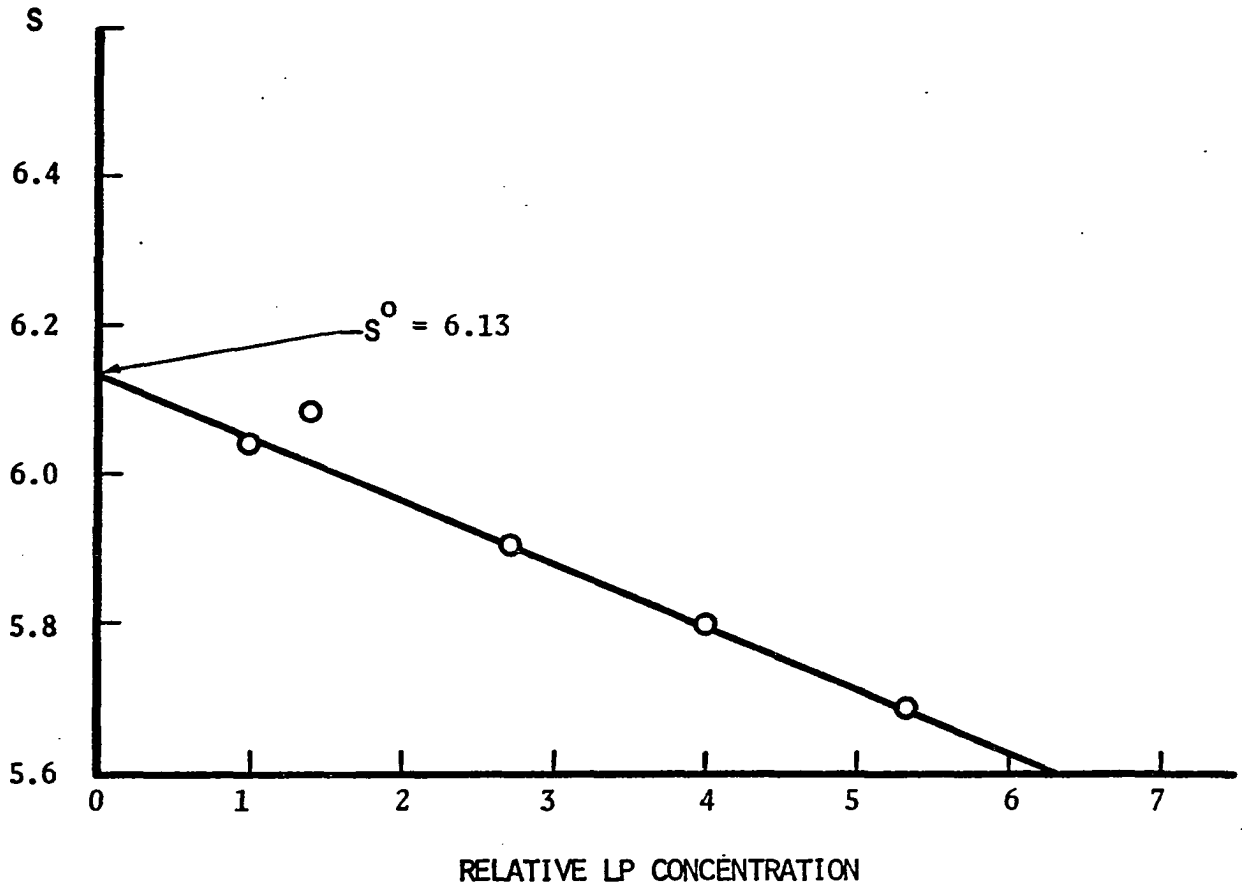


Figure 34. Determination of $S^o$ of Apo-LP Fraction V, 1.040-1.053 g/ml.

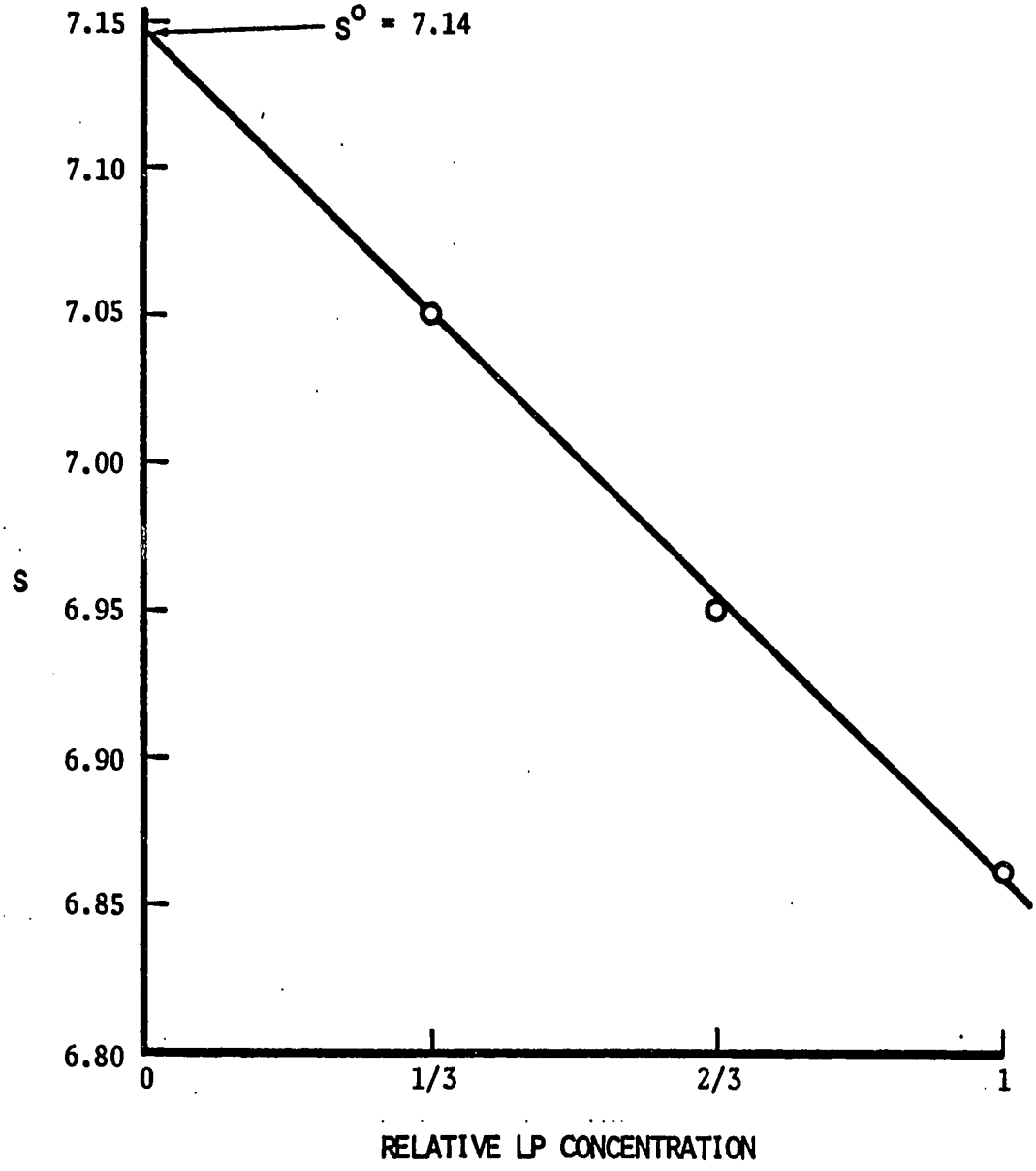


Figure 35. Determination of $S^o$ of ApoB-LP Fraction VI, 1.053-1.063 g/ml.

6-12.

Hydrated density determinations. Usually, two or three preparations of each ApoB-LP subfraction obtained on different occasions were used independently for the determination of hydrated densities. Each sample was subjected to three to six flotation and/or sedimentation determinations at different solvent densities. The solvent density at which zero sedimentation occurs represents the hydrated density of the subfraction. Figure 36 shows the determination of hydrated densities of two different preparations of ApoB-LP Fraction I, 1.006-1.009 g/ml. One was isolated from the plasma of a male donor, the other was from a female subject. It is observed that the hydrated density values are different for the two preparations. Although both preparations were isolated at the same solvent density range of 1.006-1.009 g/ml, the preparation from the male donor had a value of 0.9993 g/ml, and that from female donor had a value of 1.0016 g/ml. Both hydrated density values are considerably lower than the density used for the isolation of subfraction. This subfraction would actually represent a subfraction of VLDL, if a hydrated density of 1.006 g/ml represents a boundary line between VLDL and LDL as suggested originally by Lindgren et al. (19).

The different values of S and $S_f$ obtained at the same solvent density for these two preparations can be explained mainly by the differences in distribution of the lipoproteins between the sexes at this density range. Differences in concentration of the lipoprotein samples may also play a small role, since the S and $S_f$ values shown on the graph are not values obtained at infinite dilution. However, the concentration of lipoproteins would not affect the hydrated density value. Altered con-

TABLE 6

PHYSICAL CHEMICAL CHARACTERIZATION OF HUMAN PLASMA
ApoB-LP FRACTION I, 1.006-1.009 g/ml

| Preparation | Sex of Donors | $\bar{d}$ g/ml | $S_{25,w}$ x$10^{13}$ cgs | $S_f^o$ | Diameter Å |
|---|---|---|---|---|---|
| I | F | 1.0016 | 1.60 | 18.20[a] | - |
| II | M | 0.9993 | 0.90 | 21.17 | 250 |

[a] $S_f$ value.

TABLE 7

PHYSICAL CHEMICAL CHARACTERIZATION OF HUMAN PLASMA
ApoB-LP FRACTIONS (I + II), 1.006-1.019 g/ml

| Sex of Donors | $\bar{d}$ g/ml | $S_{25,w}$ x$10^{13}$ cgs | $S^o_{20,w}$ x$10^{13}$ cgs | $S^o_f$ | Diameter Å |
|---|---|---|---|---|---|
| M, F | 1.0030 | 1.80 | 2.96 | 19.05 | 244 |

TABLE 8

PHYSICAL CHEMICAL CHARACTERIZATION OF HUMAN PLASMA
ApoB-LP FRACTION II, 1.009-1.019 g/ml

| Preparation | Sex of Donors | $\bar{d}$ g/ml | $S_{25,w}$ x$10^{13}$ cgs | $S^{o}_{20,w}$ x$10^{13}$ cgs | Relative LP Concentration | $S_f$ | $D_{obs}$ x$10^{7}$ cm$^2$/sec | $S^{o}_f$ | $D^{o}_{25,w}$ x$10^{7}$ cm$^2$/sec | $D^{o}_{20,w}$ x$10^{7}$ cm$^2$/sec | Diameter $^{o}$A | M. W. x$10^{-6}$ g/mole |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I | M, F | 1.013 | 4.90 | 5.14[a] | 1<br>2/3<br>1/3 | 12.82<br>13.35<br>15.40 | 1.90<br>-<br>- | 15.75 | 1.93[a] | 1.68[a] | 244 | 4.70 |
| II | M, F | 1.012 | 4.90 | 5.14[a] | 1<br>2/3<br>1/2 | 14.87<br>15.60<br>15.70 | - | 16.68 | - | - | 248 | 4.55 |
| III | M, F | - | - | - | 1<br>1/2<br>1/4 | -<br>-<br>- | 1.95<br>1.95<br>1.95 | - | 1.98 | 1.73 | - | - |
| IV | M | 1.0055 | 2.75 | 4.24 | 1<br>1/2 | 14.71<br>15.90 | 2.17<br>- | 17.09 | 2.18[a] | 1.90[a] | 238 | 4.58 |

[a]Value obtained from a single LP concentration.

TABLE 9

PHYSICAL CHEMICAL CHARACTERIZATION OF HUMAN PLASMA
ApoB-LP FRACTION III, 1.019-1.030 g/ml

| Preparation | Sex of Donors | $\bar{d}$ g/ml | $S_{25,w}$ x$10^{13}$ cgs | $S^{o}_{20,w}$ x$10^{13}$ cgs | Relative LP Concentration | $S_f$ | $D_{obs}$ x$10^{7}$ $cm^2$/sec | $S^{o}_{f}$ | $D^{o}_{25,w}$ x$10^{7}$ $cm^2$/sec | $D^{o}_{20,w}$ x$10^{7}$ $cm^2$/sec | Diameter $^{o}$A | M. W. x$10^{-6}$ g/mole |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I | M, F | 1.019 | 5.75 | 5.30[a] | 1<br>2/3<br>1/3 | 9.28<br>10.12<br>10.32 | 2.2<br>-<br>- | 11.0 | 2.24[a] | 1.95[a] | 217 | 3.26 |
| II | M, F | 1.018 | 6.50 | 5.97[a] | 1<br>1/2<br>1/3 | 11.78<br>12.08<br>12.18 | 2.0<br>-<br>- | 12.39 | 2.05[a] | 1.79[a] | 227 | 3.91 |
| III | M, F | - | - | 5.05 | 1<br>1/2 | -<br>- | 1.93<br>1.93 | - | 1.93 | 1.73 | - | - |

[a]Value obtained from a single LP concentration.

TABLE 10

PHYSICAL CHEMICAL CHARACTERIZATION OF HUMAN PLASMA
ApoB-LP FRACTION IV, 1.030-1.040 g/ml

| Preparation | Sex of Donors | $\bar{d}$ g/ml | $S_{25,w}$ $x10^{13}$ cgs | $S^o_{20,w}$ $x10^{13}$ cgs | $S^o_f$ | $D^o_{25,w}$ $x10^{7}$ $cm^2/sec$ | $D^o_{20,w}$ $x10^{7}$ $cm^2/sec$ | Diameter $\AA^o$ | M.W. $x10^{-6}$ g/mole |
|---|---|---|---|---|---|---|---|---|---|
| I | M, F | 1.0266 | 7.30 | 7.49 | 8.31 | 2.10 | 1.83 | 209 | 3.23 |
| II | M, F | 1.0266 | 7.35 | 7.24 | 8.92 | - | - | 215 | 3.21 |

TABLE 11

PHYSICAL CHEMICAL CHARACTERIZATION OF HUMAN PLASMA
ApoB-LP FRACTION V, 1.040-1.053 g/ml

| Preparation | Sex of Donors | $\bar{d}$ g/ml | $S_{25,w}$ x$10^{13}$ cgs | $S^o_{20,w}$ x$10^{13}$ cgs | $S^o_f$ | $D^o_{25,w}$ x$10^7$ cm$^2$/sec | $D^o_{20,w}$ x$10^7$ cm$^2$/sec | Diameter Å | M.W. x$10^{-6}$ g/mole |
|---|---|---|---|---|---|---|---|---|---|
| I | M, F | 1.0335 | 8.25 | 7.44 | 5.90 | 2.11 | 1.84 | 194 | 2.77 |
| | | 1.0310[m] | 11.1[m] | 10.67[m] | | | | 244[m] | 4.10[m] |
| II | M, F | 1.0329 | 8.30 | 8.69 | 6.90 | 2.10 | 1.83 | 208 | 3.24 |

[m]Minor component due to incomplete removal of rabbit proteins added as anti-human ApoA-LP (d > 1.25 g/ml) for purification of the fraction.

TABLE 12

PHYSICAL CHEMICAL CHARACTERIZATION OF HUMAN PLASMA
ApoB-LP FRACTION VI, 1.053-1.063 g/ml

| Preparation | Sex of Donors | $\bar{d}$ g/ml | $S_{25,w}$ $x10^{13}$ cgs | $S^o_{20,w}$ $x10^{13}$ cgs | $S^o_f$ | $D^o_{25,w}$ $x10^7 cm^2/sec$ | $D^o_{20,w}$ $x10^7 cm^2/sec$ | Diameter $^oA$ | M.W. $x10^{-6}$ g/mole |
|---|---|---|---|---|---|---|---|---|---|
| I | M, F | 1.0357 | 8.75 | 8.60 | 5.45 | 2.12 | 1.85 | 194 | 2.88 |
| II | F | 1.0420 | 9.70 | - | 4.40 | 2.14 | 1.87 | 198 | 2.93 |

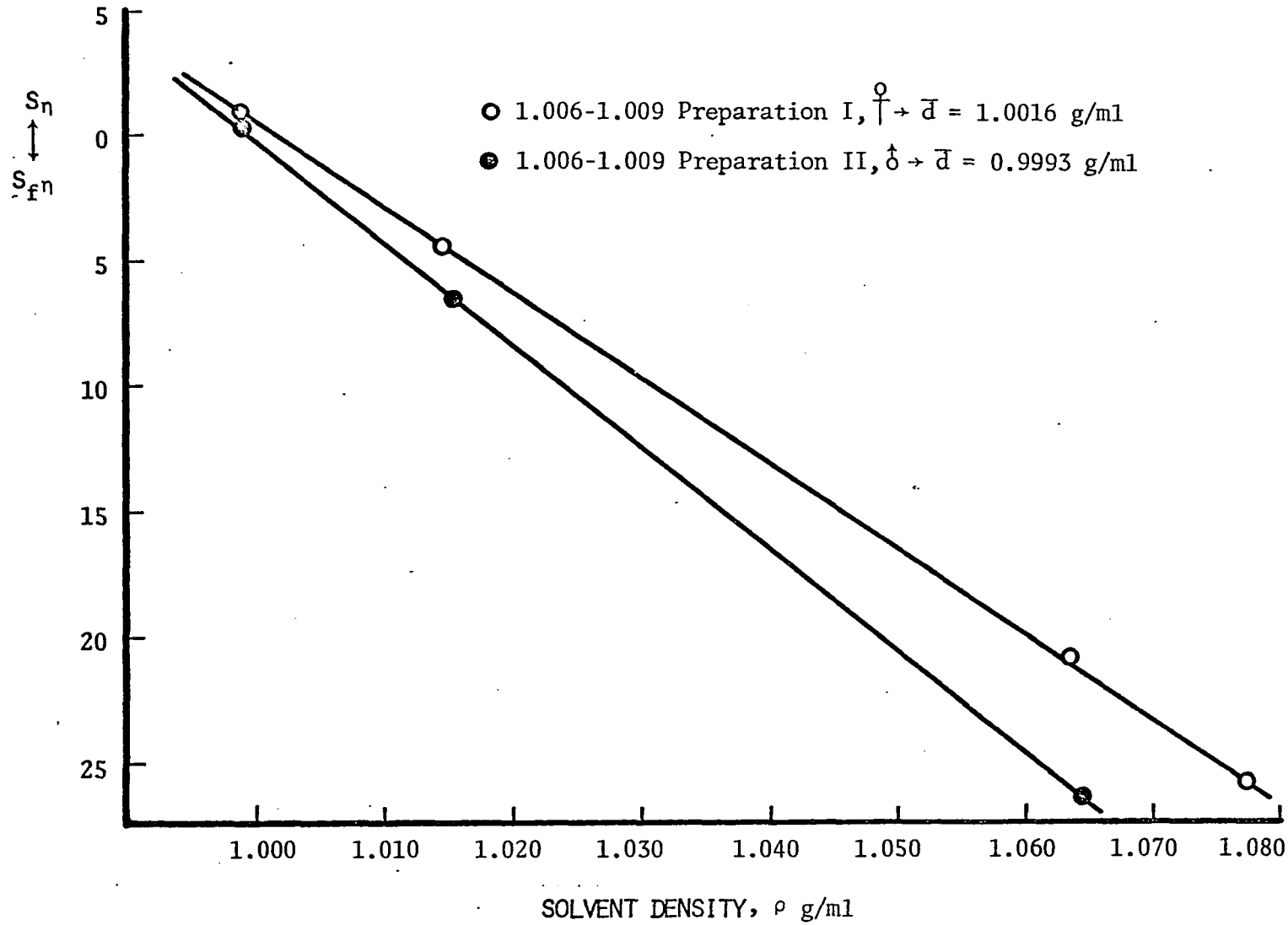


Figure 36. Determination of hydrated densities of ApoB-LP Fraction I of two preparations.

centrations of lipoproteins would merely give slightly different slopes of the straight lines of S and $S_f$ versus $\rho$ with an axis located at the same point of zero sedimentation. That the hydrated density of the male LP sample is lower than that of the female is to be expected, since the triglyceride content of male LP is known to be higher than that of female, and it is the relative triglyceride content which controls the density.

Figures 37 and 38 demonstrate the determination of hydrated densities of ApoB-LP Fractions II and III, each of which was obtained at two separate occasions (preparations I and II). Both preparations were isolated from a sample consisting of plasma from both sexes. Fraction II isolated at density range 1.009-1.019 g/ml gave hydrated densities of 1.013 and 1.012 g/ml, respectively. Fraction III, isolated at 1.019-1.030 g/ml gave values of 1.018-1.019 g/ml. Again, slight variations between two preparations isolated at the same density indicate differences in the triglyceride content and the lipoprotein distribution between the two groups of donors.

A third preparation of Fraction II is shown in Figure 39. The lipoprotein was isolated from male plasma. The hydrated density value is considerably lower than those of the other two preparations. This observation is consistent with the results of Fraction I: due to a higher triglyceride content the hydrated density of male lipoprotein is lower than that of female lipoprotein for the same isolation density range.

Figure 40 illustrates the determination of hydrated densities of ApoB-LP Fractions IV, 1.030-1.040 g/ml and V, 1.040-1.053 g/ml obtained from two different plasma samples. Both samples consisted of a mixture

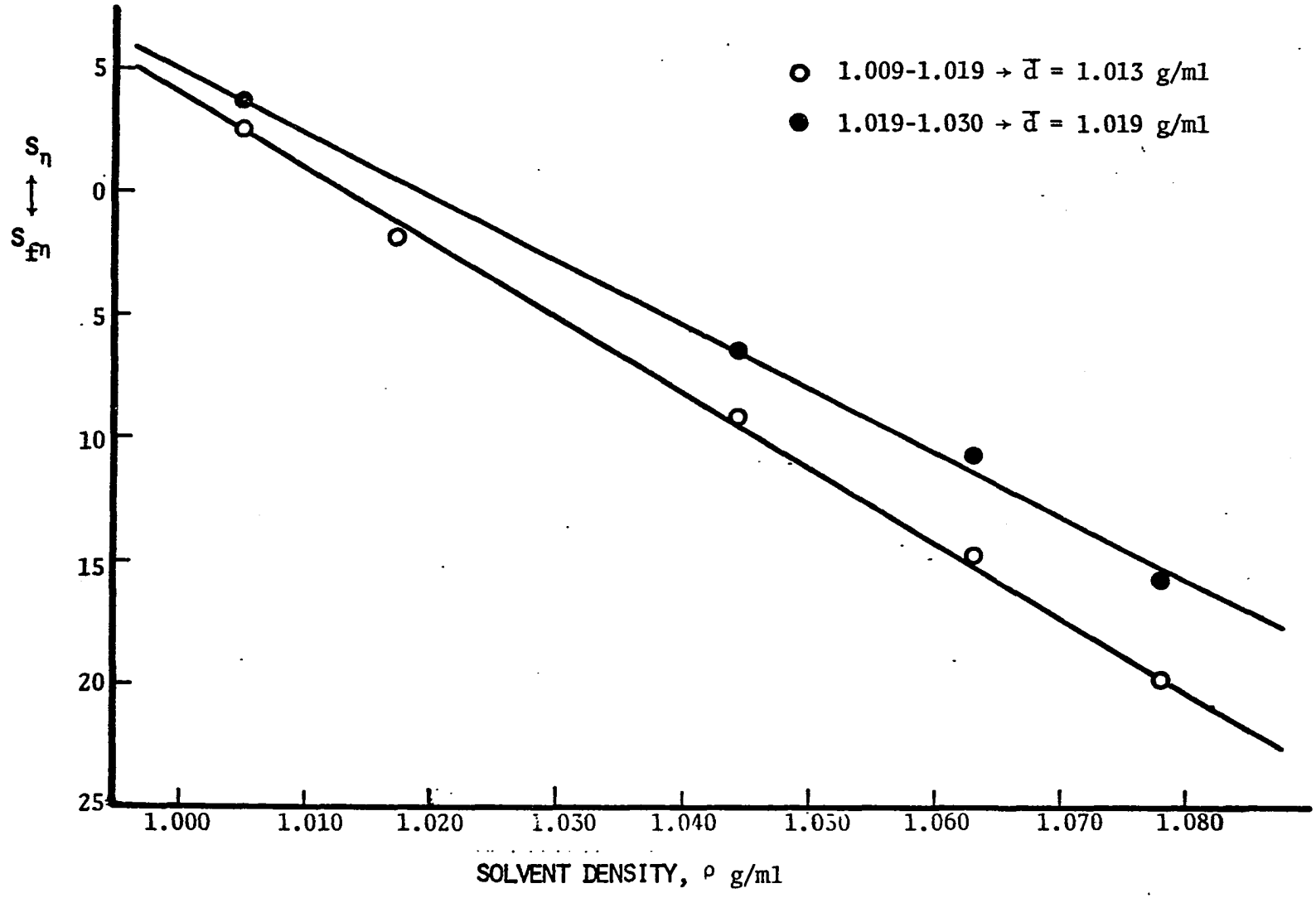


Figure 37. Determination of hydrated densities of ApoB-LP Fractions II and III (Preparation I).

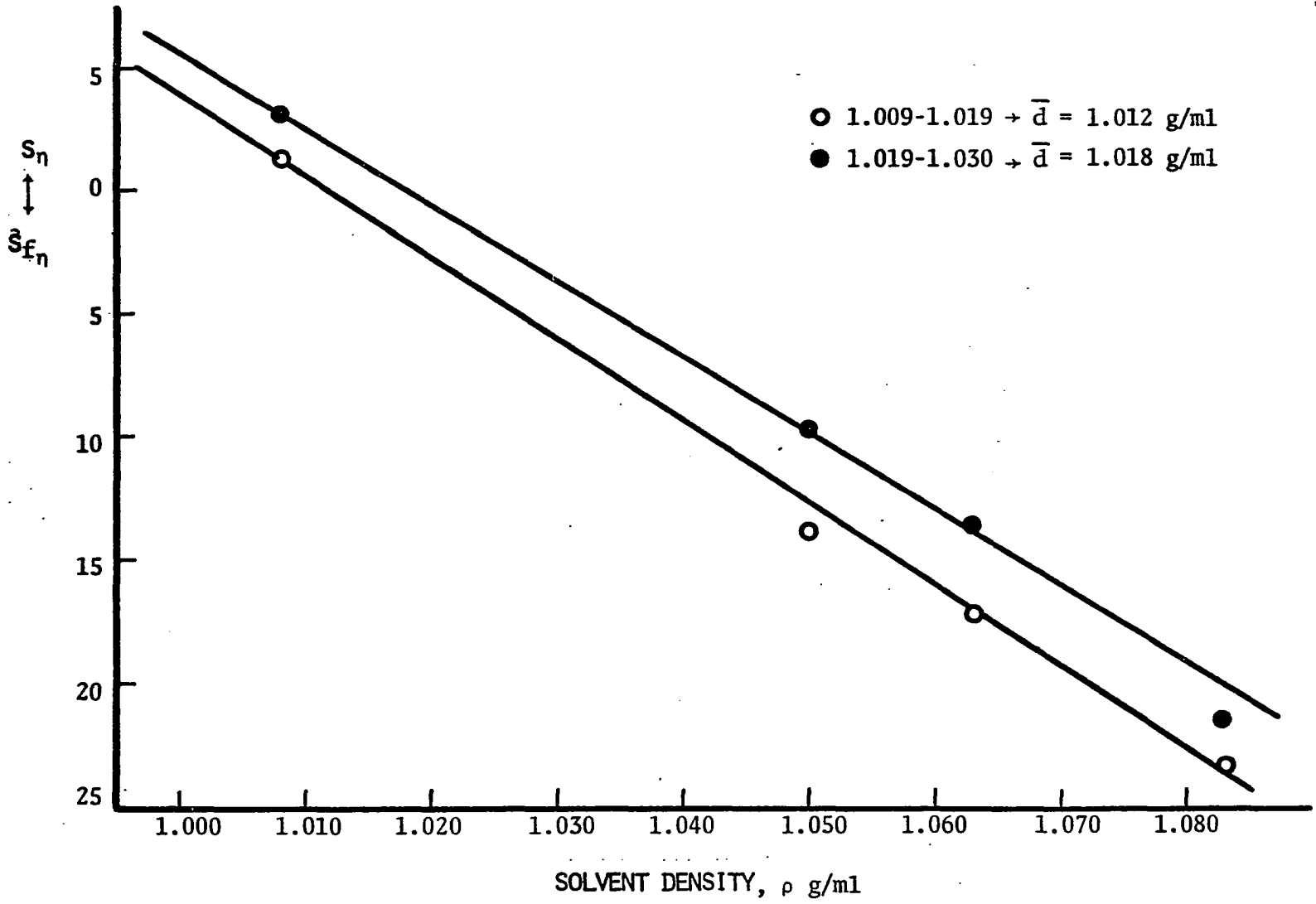


Figure 38. Determination of hydrated densities of ApoB-LP Fractions II and III (Preparation II).

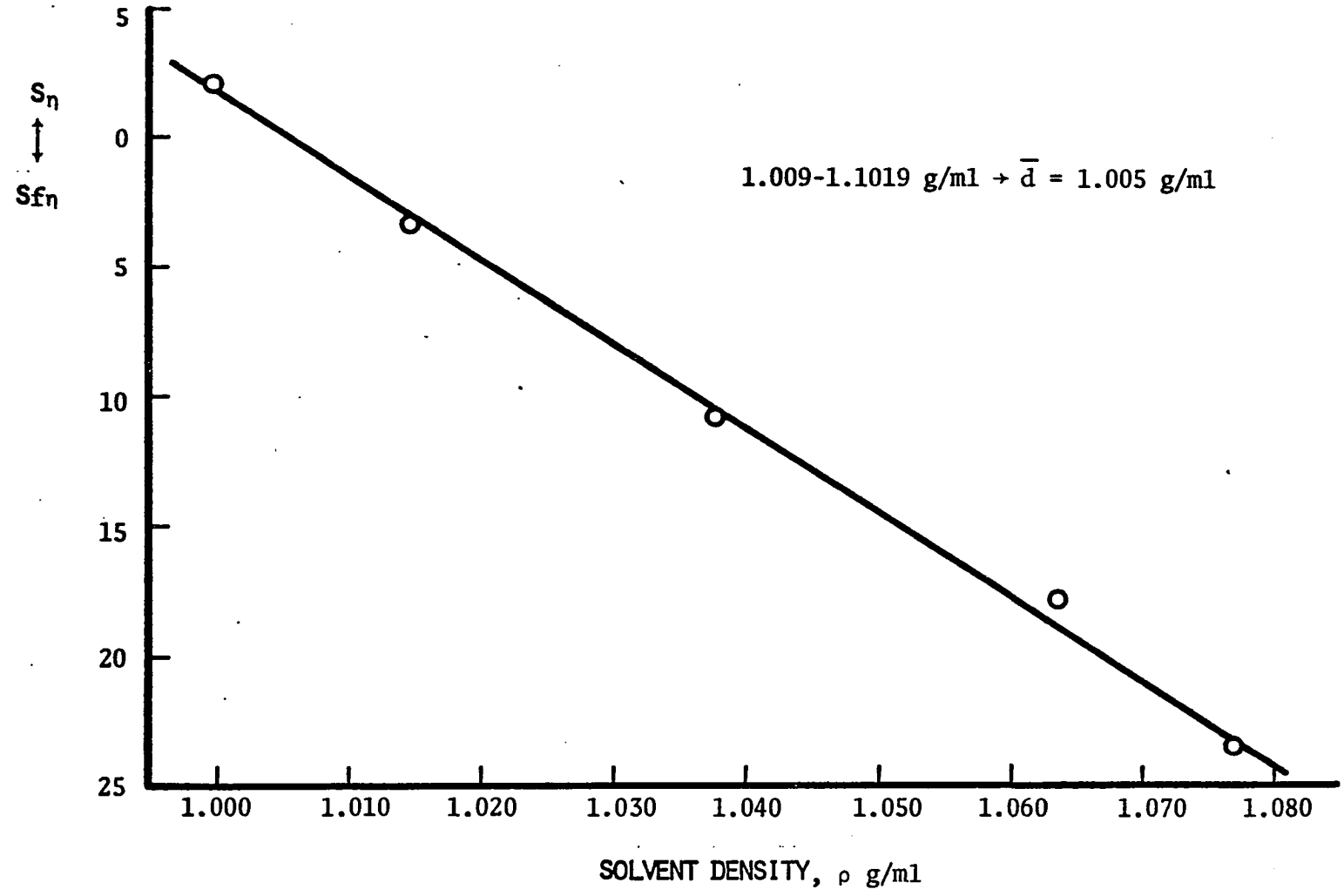


Figure 39. Determination of hydrated density of ApoB-LP Fraction II, 1.009-1.019 g/ml, (Preparation III, $\hat{o}$).

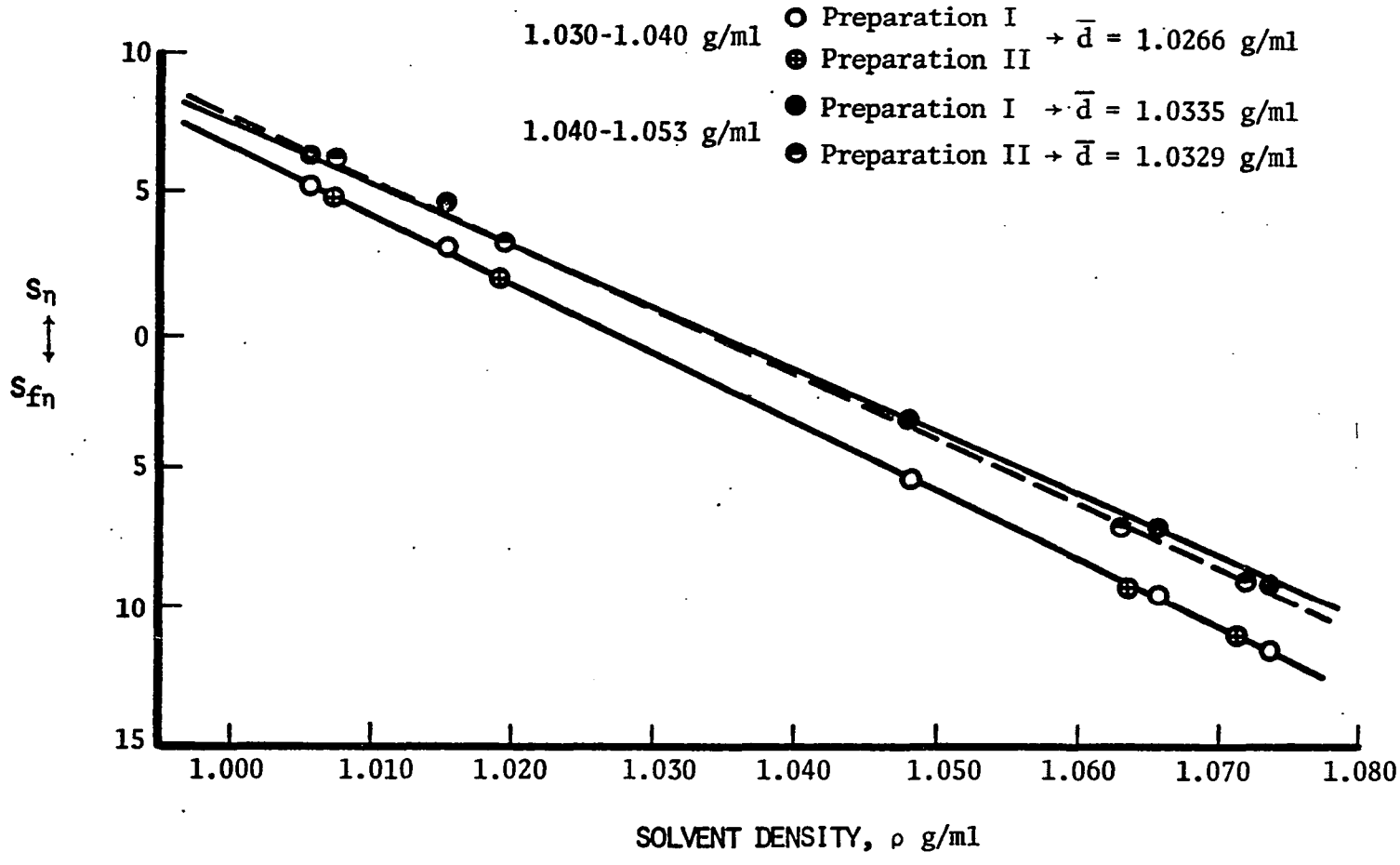


Figure 40. Determination of hydrated densities of ApoB-LP Fractions IV, 1.030-1.040 g/ml and V, 1.040-1.053 g/ml.

of plasma from both sexes. Sedimentation and flotation coefficients of both preparations of Fraction IV had identical values indicating that the lipoproteins of this density range possessed the same distribution for both preparations. The hydrated density value was found to be 1.0266 g/ml. However, Fraction V showed slight differences between the two preparations (1.0329 g/ml and 1.0335 g/ml, respectively), even though both were isolated from the same plasma pools as the Fraction IV preparations.

Figure 41 shows the determination of hydrated densities of ApoB-LP Fraction VI (1.053-1.063 g/ml) from two preparations. One preparation was from both sexes, and the other was from female donors only. The graph of the female preparation lies to the right of that of a mixed preparation. The hydrated density of the female sample was 1.0420 g/ml, and that of the mixed sample was 1.0357 g/ml. The observation that hydrated density values of lipoproteins isolated at any given isolation density range are dependent on sex is consistent throughout this study. For the same density range, the hydrated density of a female lipoprotein is greater than that of a male lipoprotein. The hydrated density of a mixed lipoprotein represents then the average value between that of female and male, depending on the proportion of the sample contributed by each sex.

Since the partial specific volume of a lipoprotein is the reciprocal of the hydrated density, the values for partial specific volume obtained in this study are also dependent on sex. Female lipoproteins have a lower value than male lipoproteins isolated under identical conditions. "Mixed" lipoproteins possess an intermediate value.

Determination of diffusion coefficients. Diffusion experiments

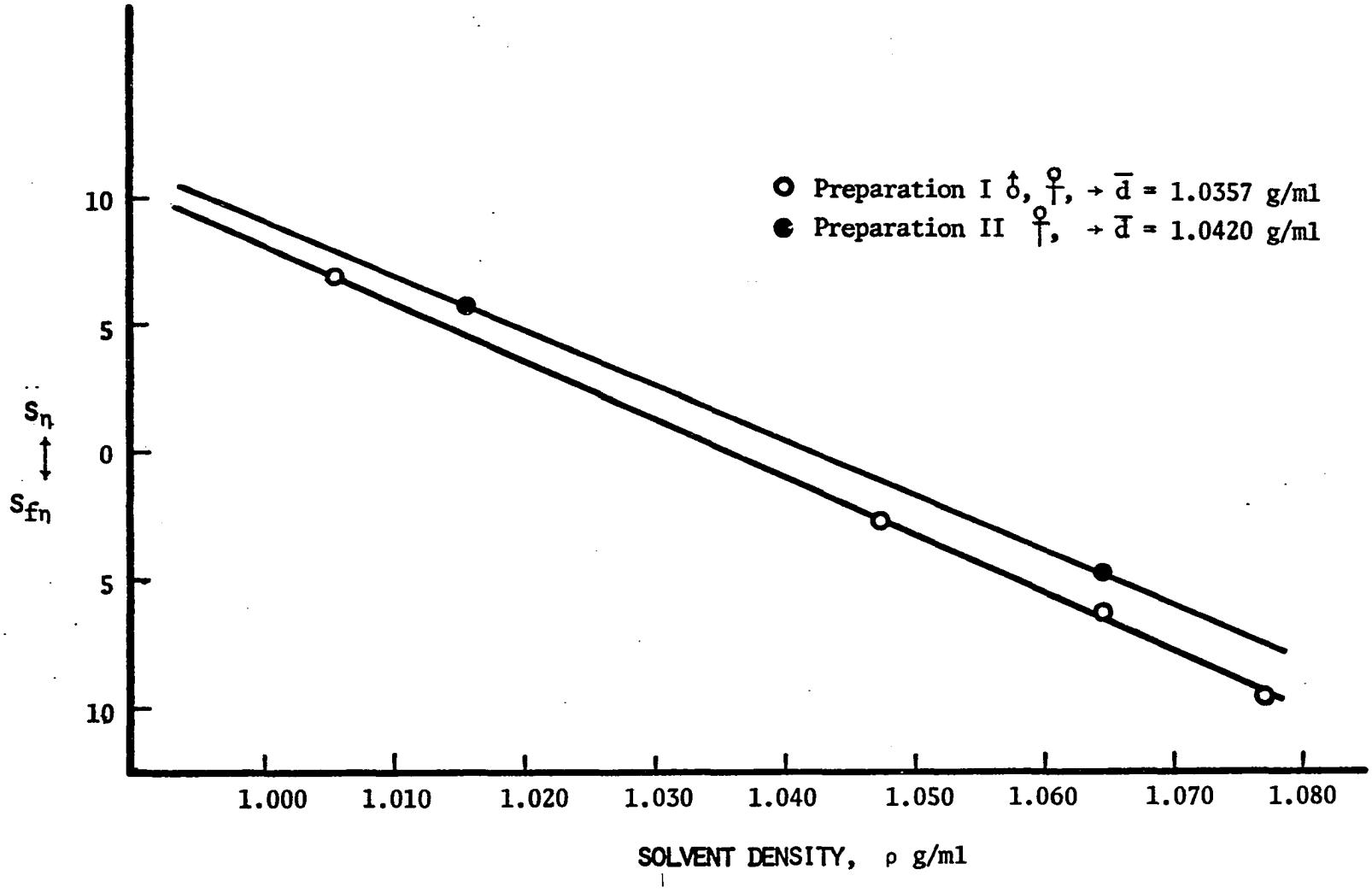


Figure 41. Determination of hydrated densities of ApoB-LP Fraction VI, 1.053-1.063 g/ml of two preparations.

were performed with every ApoB-LP subfraction except Fraction I, 1.006-1.009 g/ml and Fraction (I + II), 1.006-1.019 g/ml. Because of the extremely low hydrated density values of these two subfractions, it was difficult to obtain the sedimenting boundary in a salt solution required for such determination.

The apparent diffusion coefficient, $D_{app}$, which is measured from the instant when the synthetic boundary is formed, changes slightly with concentration and with time. Figure 42 shows that $D_{app}$ is a linear function of reciprocal time for different concentrations of ApoB-LP Fraction IV, 1.030-1.040 g/ml. Different concentrations of the same sample have a tendency to converge at the zero time. Figure 43 demonstrates the concentration dependence of the apparent diffusion coefficient, $D_{app}$. The apparent diffusion coefficient extrapolated to infinite dilution, $D^{o}_{app}$, was $2.06 \times 10^{-7}$ $cm^2/sec$. Figure 44 shows that $D_{app}$ is also slightly concentration dependent for ApoB-LP Fraction V, 1.040-1.053 g/ml. The $D^{o}_{app}$ of this subfraction was $2.07 \times 10^{-7}$ $cm^2/sec$. Figure 45 shows the determination of the apparent diffusion coefficient, $D_{app}$, at zero time for ApoB-LP Fraction VI, 1.053-1.063 g/ml at two different concentrations. These two straight lines converge to the same point at zero time. Therefore, $D_{app}$ becomes concentration independent. This phenomenon has been observed also in Fractions II and III.

Some ultracentrifugal diffusion patterns are shown in Figures 46 and 47. A pattern representative for each ApoB-LP subfraction from II through VI is presented.

All apparent diffusion coefficients at infinite dilution are corrected to $D^{o}_{20,w}$. The results for all subfractions are listed in Tables

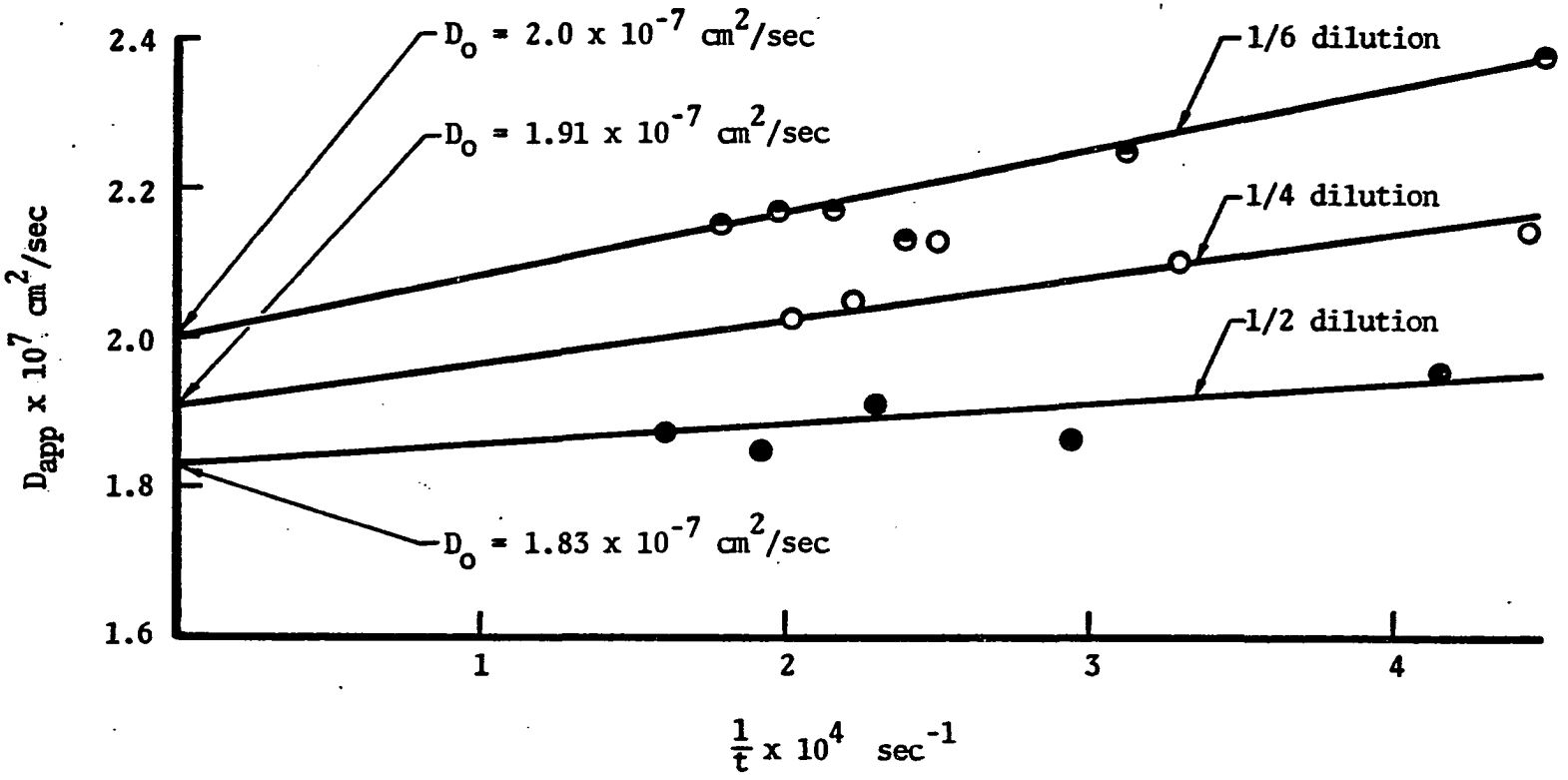


Figure 42. Determination of apparent diffusion coefficient at zero time of ApoB-LP Fraction IV, 1.030-1.040 g/ml of different concentrations.

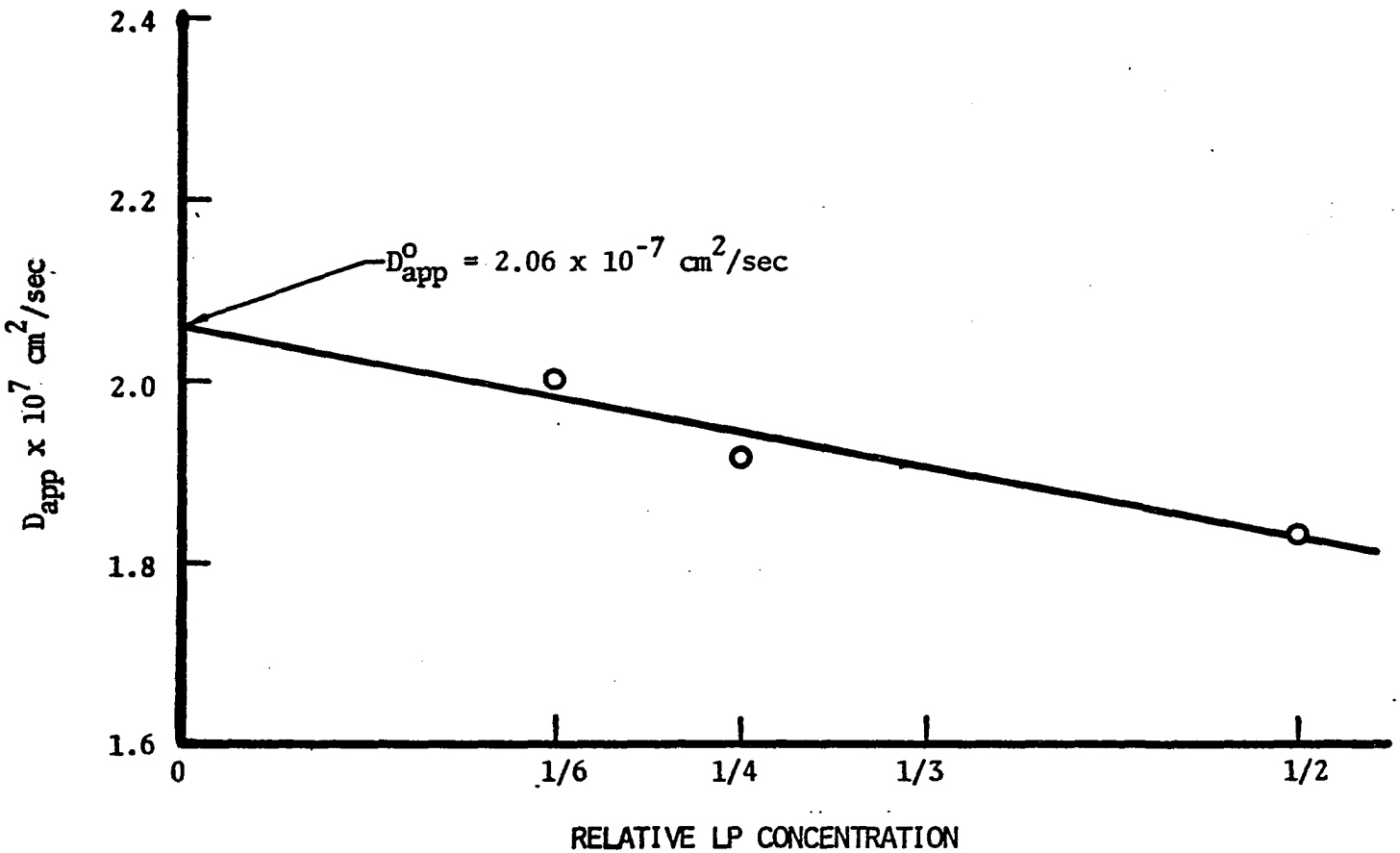


Figure 43. Determination of $D^o_{app}$ of ApoB-LP Fraction IV, 1.030-1.040 g/ml.

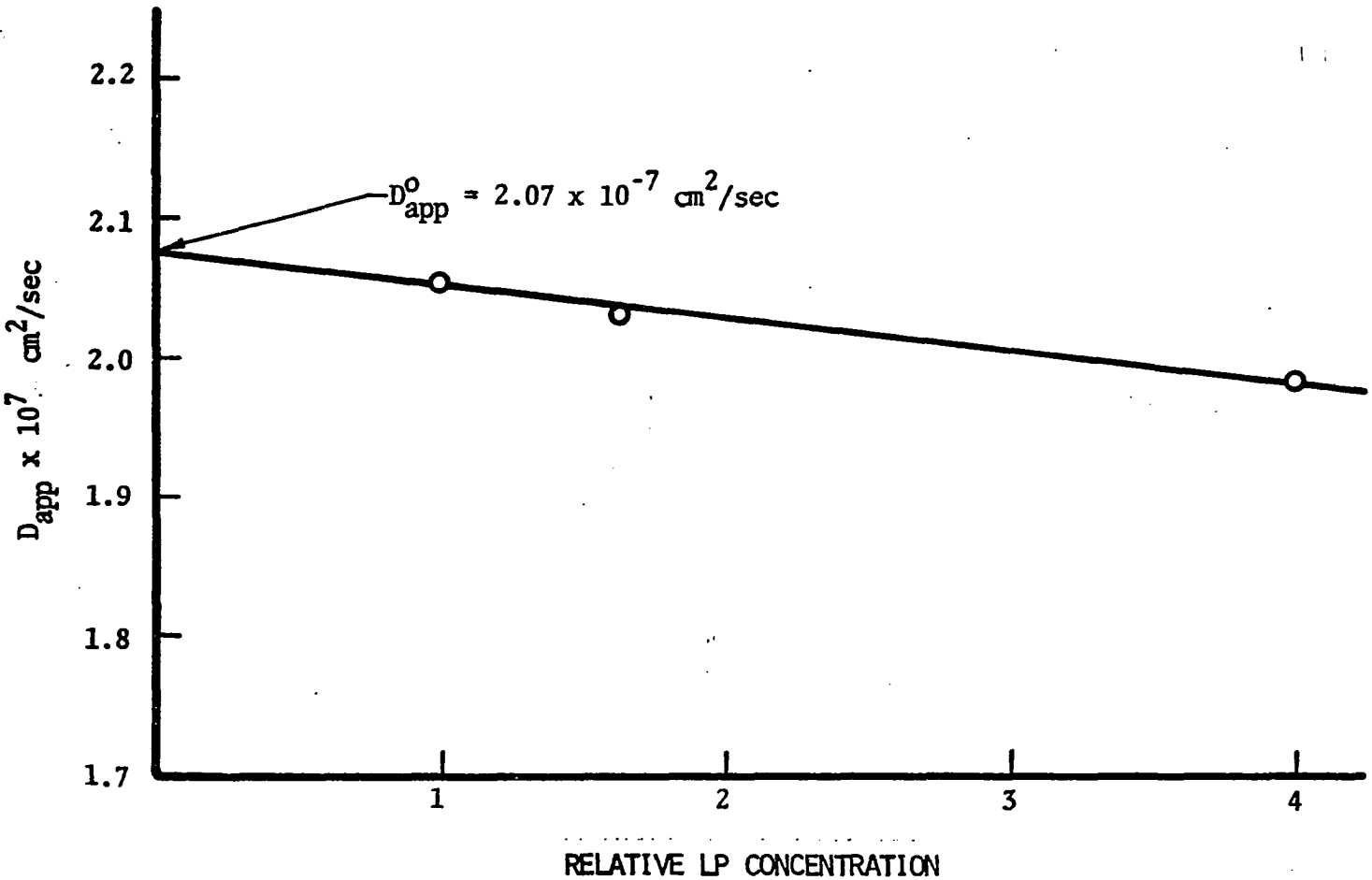


Figure 44. Determination of $D^o_{app}$ of ApoB-LP Fraction V, 1.040-1.053 g/ml.

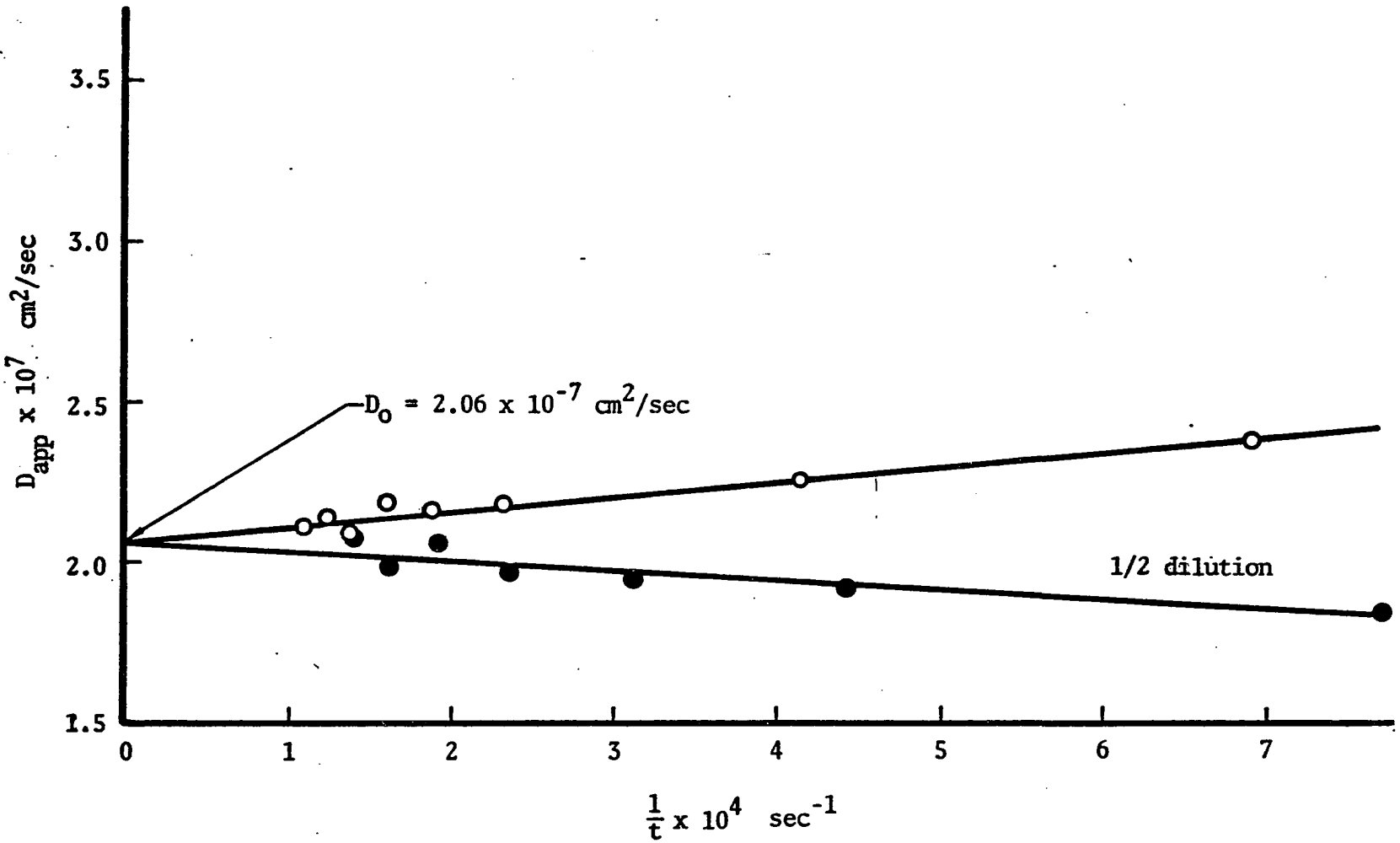


Figure 45. Determination of $D_{app}$ at zero time of ApoB-LP Fraction VI, 1.053-1.063 g/ml of two concentrations.

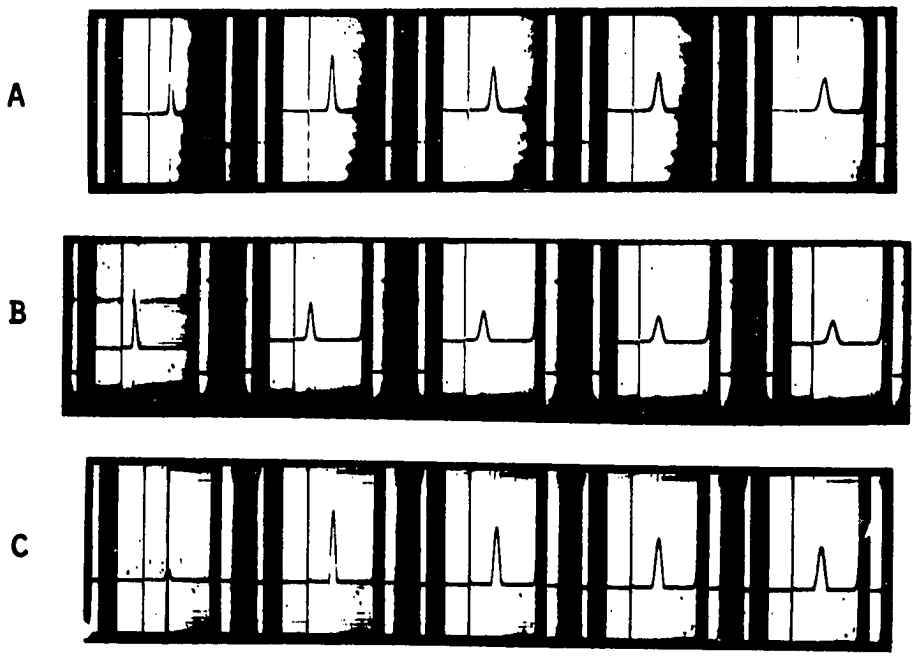


Figure 46. Ultracentrifugal diffusion patterns of ApoB-LP Fraction II (A), Fraction III (B), and Fraction IV (C).

Experimental conditions: synthetic boundary cell; temperature 25°C; solvent density 1.004 (A), 1.002 (B), and 1.003 g/ml (C). Pictures were taken at 16-minute intervals after the desired speed (12,590 rpm) was attained.

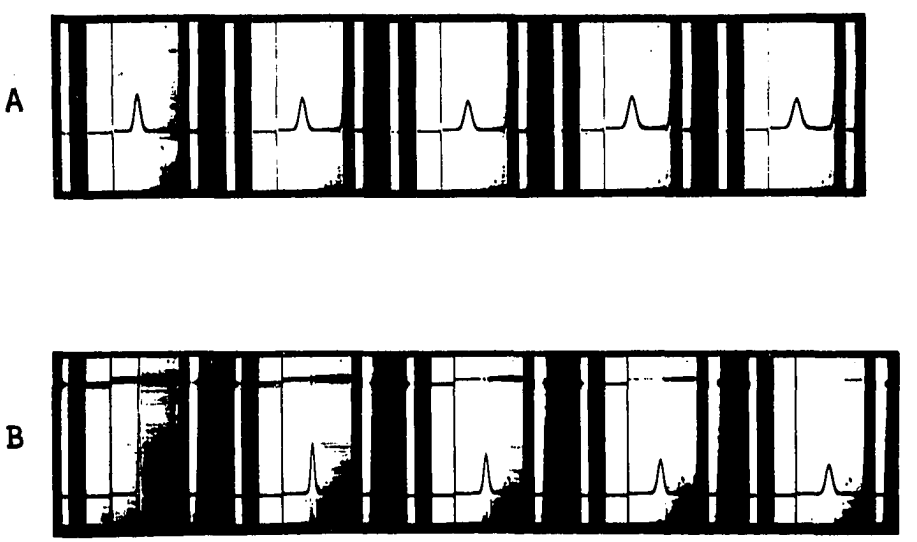


Figure 47. Ultracentrifugal diffusion patterns of ApoB-LP Fraction V (A) and Fraction VI (B).

Experimental conditions: synthetic boundary cell; temperature 25°C; solvent density 1.003 g/ml (A), 1.015 g/ml (B). Pictures were taken at 48, 64, 80, 96 and 112 minutes (A), and 0, 16, 32, 48 and 64 minutes (B) after the desired speed (12,590 rpm) was attained.

6-12. The diffusion coefficients of each ApoB-LP subfraction differ very slightly (1.7-1.9 x $10^7$ $cm^2$/sec).

Determination of average diameter. Average diameters of ApoB-LP subfractions were estimated according to the equation of Oncley and Gurd (131), assuming spherical particles:

$$S_f^o = \frac{1.063 - \bar{d}}{188} \text{Dia.}^2 \qquad [12]$$

Where Dia. is the lipoprotein diameter in Å. The values are presented in Tables 6-12.

Determination of molecular weight. Molecular weights were calculated from the sedimentation and diffusion coefficients according to Svedberg's equation. Values of $S_{25}^o$, $D_{25}^o$ and $\bar{V}_{25}$ obtained experimentally at 25° were used. Since there was no necessity to use assumed values, the possibility of errors was greatly reduced. Where $S_{25}^o$ was not available, the value $\frac{S_{fn}^o}{1 - \bar{V}\rho}$ was used as an equivalent for $\frac{S_n^o}{1 - \bar{V}\rho}$. The validity of this assumption can be proved as follows: Figures 36-41 show that plots of $S_n$ and $S_{fn}$ versus solvent density, $\rho$, represented linear functions for every subfraction studied. Hence within each subfraction

$$\frac{S_n}{\bar{d} - \rho} = \frac{-S_{fn}}{\rho - \bar{d}} \qquad [13]$$

The value of $\rho$ on the left side of equation [13] is the solvent density at which S was measured. The value of $\rho$ on the right is 1.063 g/ml. If each side of equation [13] is multiplied by $\bar{d}$

$$\frac{S_n \cdot \bar{d}}{\bar{d} - \rho} = \frac{S_{fn} \cdot \bar{d}}{\rho - \bar{d}} \qquad [14]$$

Rearranging equation [14] we obtain

$$\frac{S_\eta}{1 - \frac{\rho}{\bar{d}}} = \frac{S_{f\eta}}{1 - \frac{\rho}{\bar{d}}} \qquad [15]$$

By definition, $\bar{V} = \frac{1}{\bar{d}}$, therefore

$$\frac{S_\eta}{1 - \bar{V}\rho} = \frac{S_{f\eta}}{1 - \bar{V}\rho} \qquad [16]$$

This equation would also be true for S and $S_f$ at infinite dilution

$$\frac{S^o_\eta}{1 - \bar{V}\rho} = \frac{S^o_{f\eta}}{1 - \bar{V}\rho} \qquad [17]$$

When $\frac{S^o_{f\eta}}{1 - \bar{V}\rho}$ is used for $\frac{S^o_\eta}{1 - \bar{V}\rho}$, the diffusion coefficient is also corrected for viscosity of the solvent at 25°. The Svedberg equation is then modified to

$$M = \frac{RTS^o_{f\eta}}{D^o_\eta (1 - 1.063\ \bar{V})} \qquad [18]$$

If the diffusion coefficient was not available, the molecular weight was estimated according to Oncley's equation (16)

$$M = 0.317\ \bar{d}\ (\text{Dia.})^3 \qquad [19]$$

where Dia. is the average diameter of the ApoB-LP subfractions calculated from $S^o_f$ and $\bar{d}$ values as shown in equation [12].

The molecular weights calculated for each fraction are listed in Tables 6-12. A compiled tabulation of the physical data for all fractions is given in Table 13. The values of each ApoB-LP subfraction are

TABLE 13

PHYSICAL CHEMICAL CHARACTERIZATION OF HUMAN PLASMA
ApoB-LP SUBFRACTIONS

| Lipoprotein Fraction g/ml | $\bar{d}$ g/ml | $S_f^o$ | $S_{20,w}^o$ | $D_{20,w}^o$ x$10^7$ $cm^2$/sec | Diameter Å | $\frac{S_n^o}{1-\bar{V}\rho}$ or $\frac{S_{fn}^o}{1-\bar{V}\rho}$ | M.W. x$10^{-6}$ g/mole |
|---|---|---|---|---|---|---|---|
| I 1.006-1.009 | 1.0005 | 19.7 | - | - | 250 | 383 | 4.9[a] |
| I, II 1.006-1.019 | 1.0030 | 19.1 | 2.96 | - | 244 | 381 | 4.6[a] |
| II 1.009-1.019 | 1.0125 | 16.2 | 5.14[b] | 1.7 | 246 | 375 | 4.6 |
| III 1.019-1.030 | 1.0185 | 12.7 | 5.63[b] | 1.8 | 222 | 294-323 | 3.3-3.9 |
| IV 1.030-1.040 | 1.0266 | 8.6 | 7.36 | 1.8 | 212 | 279 | 3.2 |
| V 1.040-1.053[c] | 1.0329 | 6.9 | 8.30 | 1.8 | 208 | 275 | 3.2 |
| VI 1.053-1.063 | 1.0357 | 5.5 | 8.60 | 1.9 | 194 | 243 | 2.9 |

[a] M.W. calculated according to Oncley's equation $M = 0.317\ \bar{d}\ (Dia.)^3$ (16).

[b] Value obtained from a single LP concentration.

[c] Only data of preparation II are included.

averages calculated from data of several preparations from both male and female donors. Values of $\frac{S^o_\eta}{1 - \bar{V}\rho}$ or $\frac{S^o_{f\eta}}{1 - \bar{V}\rho}$ are also included.

It is obvious that the hydrated density of ApoB-LP subfractions increases with increasing isolating density. Flotation coefficient, $S^o_f$, decreases rapidly, while sedimentation coefficient, $S^o_{20,w}$, increases at a slower rate with increasing density. Diffusion coefficient, $D^o_{20,w}$, is almost a constant, and the particle size decreases slightly. The ApoB-LP subfractions fall into three distinct classes on the basis of data for $S^o_\eta$ and $S^o_{f\eta}$ with correction for buoyancy factor $1 - \bar{V}\rho$ and of molecular weights. The first group includes Fractions I, I + II, and II, of isolation density 1.006-1.019 g/ml. The values of $\frac{S^o_\eta}{1 - \bar{V}\rho}$ or $\frac{S^o_{f\eta}}{1 - \bar{V}\rho}$ fall within 375-383 for this group (with $S^o$ and $S^o_f$ expressed in Svedberg units). The molecular weights of these subfractions lie between 4.6-4.9 x $10^6$. The second group is represented by Fraction III of isolation density 1.019-1.030 g/ml. It has a lower $\frac{S^o_\eta}{1 - \bar{V}\rho}$ factor, ranging from 294-323. Correspondingly, its molecular weight lies between 3.3-3.9 x $10^6$. The third group is comprised of Fractions IV, V, and VI of isolation density 1.030-1.063 g/ml. This group has the lowest range of $\frac{S^o_\eta}{1 - \bar{V}\rho}$ values, 243-279. Molecular weights range from 2.9-3.2 x $10^6$. The three groups show a steady decrease of $\frac{S^o_\eta}{1 - \bar{V}\rho}$ or $\frac{S^o_{f\eta}}{1 - \bar{V}\rho}$ factors and of molecular weights with increasing density range of isolation.

It is believed that the molecular weight obtained for Fraction VI was slightly lower than the true value for ApoB-LP of this density range due to the presence of a small amount of ApoA-LP which could be removed completely. Pure ApoB-LP of that subfraction should have given a value closer to 3.2 x $10^6$ g/mole.

## Amino Acid Composition

Amino acid analyses were performed on the following subfractions: Fraction IV, 1.030-1.040 g/ml, Fraction V, 1.040-1.053 g/ml, Fraction VI, 1.053-1.063 g/ml and a subfraction of ApoB-LP isolated in the density range 1.009-1.050 g/ml. This latter subfraction will be designated as Fraction A; it includes Fractions II, III, IV, and most of V. Triplicate analyses were determined on a 24-hour hydrolysate of each subfraction and standard deviations were calculated. In addition, a 72-hour hydrolysate of Fraction A was analyzed in duplicate. It was found that the contents of serine, threonine, and tyrosine decreased with increasing hydrolysis time. The initial content of these amino acids was obtained by a linear extrapolation to zero time; serine showed an increase of 3.74%, threonine 5.10% and tyrosine 1.80% over those obtained after a 24-hour hydrolysis. Lysine, valine and isoleucine gave maximum values after a 72-hour hydrolysis. These values represented increases of 5.0%, 6.2%, and 6.36%, respectively, over those obtained with a 24-hour hydrolysate. Phosphoserine and cysteic acid could not be detected by the qualitative test described in the Methods. The qualitative test for cysteine was also negative. Therefore the small peak shown on the amino acid analyzer at the position of cysteic acid must have been due to a slight oxidation of cystine during hydrolysis. Half-cystine and methionine were determined as cysteic acid and methionine sulfoxide in the performic acid-oxidized protein. Tryptophan was determined spectrophotometrically in duplicate. The amino acid compositions of the four fractions studied are given in Table 14. Correction factors for amino acids changing with hydrolysis time were calculated from data obtained with Fraction A. In all frac-

TABLE 14

AMINO ACID COMPOSITION OF THE PROTEIN MOIETIES OF HUMAN PLASMA
ApoB-LP FRACTIONS A, IV, V AND VI

| Fraction | A | IV | V | VI | Average |
|---|---|---|---|---|---|
| Density Range g/ml | 1.009-1.050 | 1.030-1.040 | 1.040-1.053 | 1.053-1.063 | |
| Amino Acid | $M/10^5$ g $\pm$ $\sigma$ | $M/10^5$ g $\pm$ $\sigma$ | $M/10^5$ g $\pm$ $\sigma$ | $M/10^5$ g $\pm$ $\sigma$ | $M/10^5$ g |
| Lysine | [a]49.38 $\pm$ 1.68 | 54.99 $\pm$ 6.20 | 57.02 $\pm$ 0.69 | 55.72 $\pm$ 3.11 | 55.91 |
| Histidine | 17.51 $\pm$ .89 | 21.69 $\pm$ 1.02 | 18.16 $\pm$ 0.29 | [a] 7.69 $\pm$ 0.67 | 19.12 |
| Arginine | 21.91 $\pm$ 1.80 | 20.18 $\pm$ 3.23 | 23.82 $\pm$ 3.46 | 18.73 $\pm$ 0.49 | 21.16 |
| Aspartic Acid | 86.33 $\pm$ 3.36 | 90.30 $\pm$ 1.80 | 88.07 $\pm$ 7.92 | 88.86 $\pm$ 0 | 88.39 |
| Threonine | 54.57 $\pm$ 2.13 | 51.96 $\pm$ 0.31 | 51.55 $\pm$ 0.93 | 56.20 $\pm$ 0.50 | 53.57 |
| Serine | 67.01 $\pm$ 1.88 | 69.12 $\pm$ 1.87 | 62.10 $\pm$ 1.11 | 60.04 $\pm$ 3.0 | 64.57 |
| Glutamic Acid | 102.03 $\pm$ 2.66 | 95.35 $\pm$ 2.56 | 99.78 $\pm$ 0.99 | 102.31 $\pm$ 1.43 | 99.87 |
| Proline | 29.22 $\pm$ 1.54 | 33.30 $\pm$ 2.06 | 29.29 $\pm$ 1.26 | 27.86 $\pm$ 0.73 | 29.92 |
| Glycine | 36.46 $\pm$ 0.91 | 45.40 $\pm$ 0.77 | 43.15 $\pm$ 1.72 | 50.43 $\pm$ 0.30 | 43.86 |
| Alanine | 52.16 $\pm$ 1.25 | 55.49 $\pm$ 0 | 60.73 $\pm$ 1.03 | 61.00 $\pm$ 2.14 | 57.35 |
| Half Cystine | 4.47 | 7.06 | 4.69 | 6.24 | 5.61 |
| Valine | 37.67 $\pm$ 1.32 | 35.31 $\pm$ 0.53 | 36.52 $\pm$ 4.12 | 39.87 $\pm$ 1.04 | 37.34 |
| Methionine | 12.07 | 10.59 | 11.72 | 12.49 | 11.72 |
| Isoleucine | 39.84 $\pm$ 1.31 | 35.31 $\pm$ 0.53 | 37.49 $\pm$ 0.52 | 40.04 $\pm$ 0.28 | 38.17 |
| Leucine | 90.92 $\pm$ 1.91 | 85.76 $\pm$ 2.31 | 82.99 $\pm$ 0.99 | 86.46 $\pm$ 0.95 | 86.53 |
| Tyrosine | 24.87 $\pm$ 0.32 | 24.72 $\pm$ 0.79 | 23.82 $\pm$ 0.31 | 24.02 $\pm$ 1.30 | 24.36 |
| Phenylalanine | 36.58 $\pm$ 0.72 | 35.31 $\pm$ 1.34 | 33.78 $\pm$ 0.30 | 35.06 $\pm$ 0.29 | 35.18 |
| Tryptophan | 5.07 | 3.03 | 6.44 | 5.18 | 4.93 |

[a]Not included in the calculation of average.

tions, glutamic acid, leucine, and aspartic acid are present in highest amounts, and cystine, tryptophan, and methionine are present in lowest amounts. Standard deviations for most of the values are less than 3%. However, greater variability (8-14%) in the determinations of lysine, arginine and histidine has been observed in most of the fractions. Ammonia was not determined.

The results of amino acid analyses of all subfractions were critically compared. Except for lysine in Fraction A, histidine in Fraction VI and glycine in Fractions A and VI, the amino acid compositions of all subfractions appear to be identical. The variations in the content of lysine, histidine and glycine most probably reflect the experimental difficulties rather than intrinsic differences in the amino acid composition of the samples. An average value has been calculated for each amino acid residue, the averages representing the amino acid composition of apolipoprotein B of low-density lipoproteins.

The results of glucosamine determinations are presented in Table 15. Values are expressed as percentages of apolipoprotein B dry weight. Fraction III, 1.019-1.030 g/ml and Fraction IV, 1.030-1.040 g/ml appeared to have a higher content than the other fractions. The significance of these differences is not known at the present time.

## Other Studies on Delipidized ApoB-LP

Preliminary studies were carried out on solubilized ApoB. A protein residue obtained by mild delipidization of ApoB-LP Fraction IV, 1.030-1.040 g/ml, was found to be partially soluble in Tris buffer, pH 8.3, $\mu$ 0.1, at room temperature. Ultracentrifugal study showed that the $S_{20,w}$ value for this compound was 2.57. Viscosity of the Tris buffer

TABLE 15

GLUCOSAMINE CONTENT OF HUMAN PLASMA ApoB-LP
FRACTIONS A, III, IV, V AND VI

| Fraction | A | III | IV | V | VI |
|---|---|---|---|---|---|
| Density Range g/ml | 1.009-1.050 | 1.019-1.030 | 1.030-1.040 | 1.040-1.053 | 1.053-1.063 |
| Weight percent of ApoB | 1.45 | 2.12 | 3.16 | 1.52 | 1.53 |

measured to be 1.019 relative to that of water at 25° by using an Ostwald viscometer. The protein solution was then heated slowly in a sand bath for one hour at 80°. The apparent solubility increased substantially. The sedimentation value, $S_{20,w}$, of the heated protein was found to be 2.39. Another sample of the same protein was solubilized in a 0.018 M NaOH and 0.15 M NaCl solution, pH 12, $\mu$ 0.17, by heating slowly for 20 minutes at 70°. The solubility of the protein appeared to be even greater than in Tris buffer. The sedimentation coefficient, $S^{o}_{20,w}$, was found to be 2.43. The value for sedimentation coefficient increased slightly at infinite dilution. Apolipoprotein B obtained by vigorous delipidization of ApoB-LP Fraction IV was then investigated. It was solubilized successfully in the NaOH-NaCl solution at pH 12 but with heating for 30 minutes at 37°. Sedimentation and diffusion coefficients of this sample were determined at infinite dilution. The pH of the protein solution was then adjusted to 7 by adding HCl. The final solvent became 0.17 M NaCl of density 1.0063 g/ml. Sedimentation and diffusion coefficients of this solution were also determined and the results are shown in Table 16.

Figures 48 and 49 show the determination of sedimentation coefficients of ApoB at pH 12 and pH 7, respectively. The two slopes of $\frac{d \ln X}{dt}$ are almost parallel to each other. Figure 50 illustrates the ultracentrifugal sedimentation patterns of three sedimentation experiements on ApoB: at pH 8.3 with heating for one hour at 80°, at pH 12 with heating for 30 minutes at 37°, and at pH 7 after solubilization at pH 12. Each sedimentation pattern indicates a single, symmetric peak. Figure 51 shows the ultracentrifugal diffusion pattern of ApoB in dilute alkaline

TABLE 16

PHYSICAL-CHEMICAL CHARACTERIZATION OF ApoB UNDER VARIOUS CONDITIONS OF SOLUBILIZATION

| Sample | ApoB-LP Fraction | Conditions of Solubilization | | | | | $S^{o}_{20,w}$ | $D^{o}_{20,w}$ x$10^{7}$ $cm^{2}$/sec | M. W. S/D x10-4 g/mole |
|---|---|---|---|---|---|---|---|---|---|
| | | pH | Solvent | $\mu$ | $\rho$ | Temperature and length of time | | | |
| A | IV | 8.3 | Tris buffer | 0.1 | 1.0011 | Room temperature | 2.57[a] | - | - |
| A | IV | 8.3 | Tris buffer | 0.1 | 1.0011 | 80°C, 1 hr. | 2.39[a] | - | - |
| A ($\frac{1}{\infty}$ dil.) | IV | 12 | NaOH + NaCl | 0.17 | 1.0043 | 70°C, 20 min. | 2.43 | - | - |
| B | IV | 12 | NaOH + NaCl | 0.17 | 1.0043 | 37°C, 30 min. | 2.47[a] | 4.00[a] | - |
| B ($\frac{1}{2}$ dil.) | IV | 12 | NaOH + NaCl | 0.17 | 1.0043 | 37°C, 30 min. | 2.56[a] | 3.72[a] | - |
| B ($\frac{1}{\infty}$ dil.) | IV | 12 | NaOH + NaCl | 0.17 | 1.0043 | 37°C, 30 min. | 2.65 | 3.44 | 7.0 |
| B | IV | 7 | NaCl | 0.17 | 1.0063 | 37°C, 30 min. | 2.44[a] | 4.93[a] | - |

[a]Value obtained from a single protein concentration.

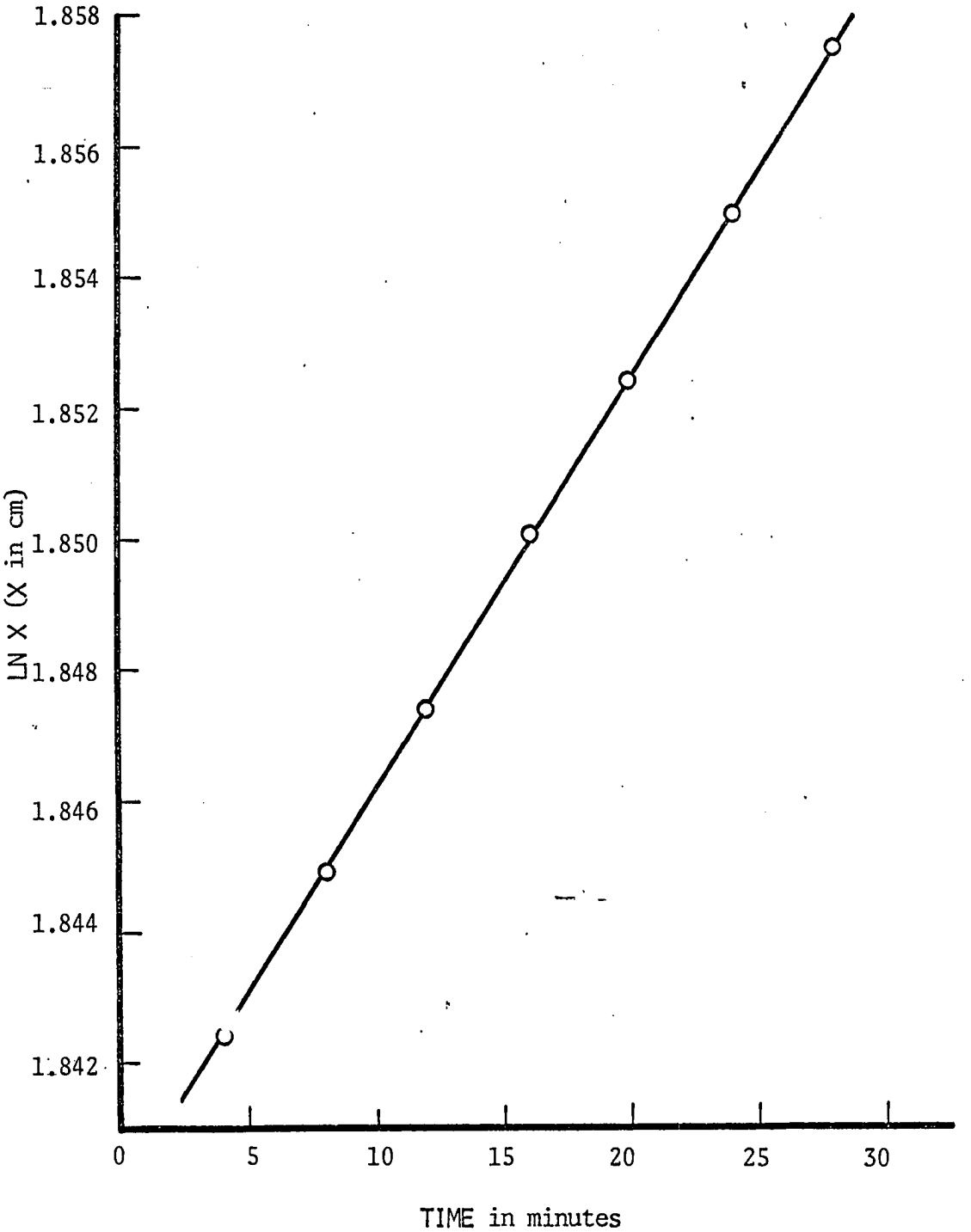


Figure 48. Determination of S of Apolipoprotein B obtained from ApoB-LP Fraction 1.030-1.040 g/ml. Protein was dissolved in 0.018 M NaOH and 0.15 M NaCl ($\rho$ = 1.0043 g/ml) at 37°C for 30 minutes. Measurements were taken after the rotor speed reached 59,780 rpm.

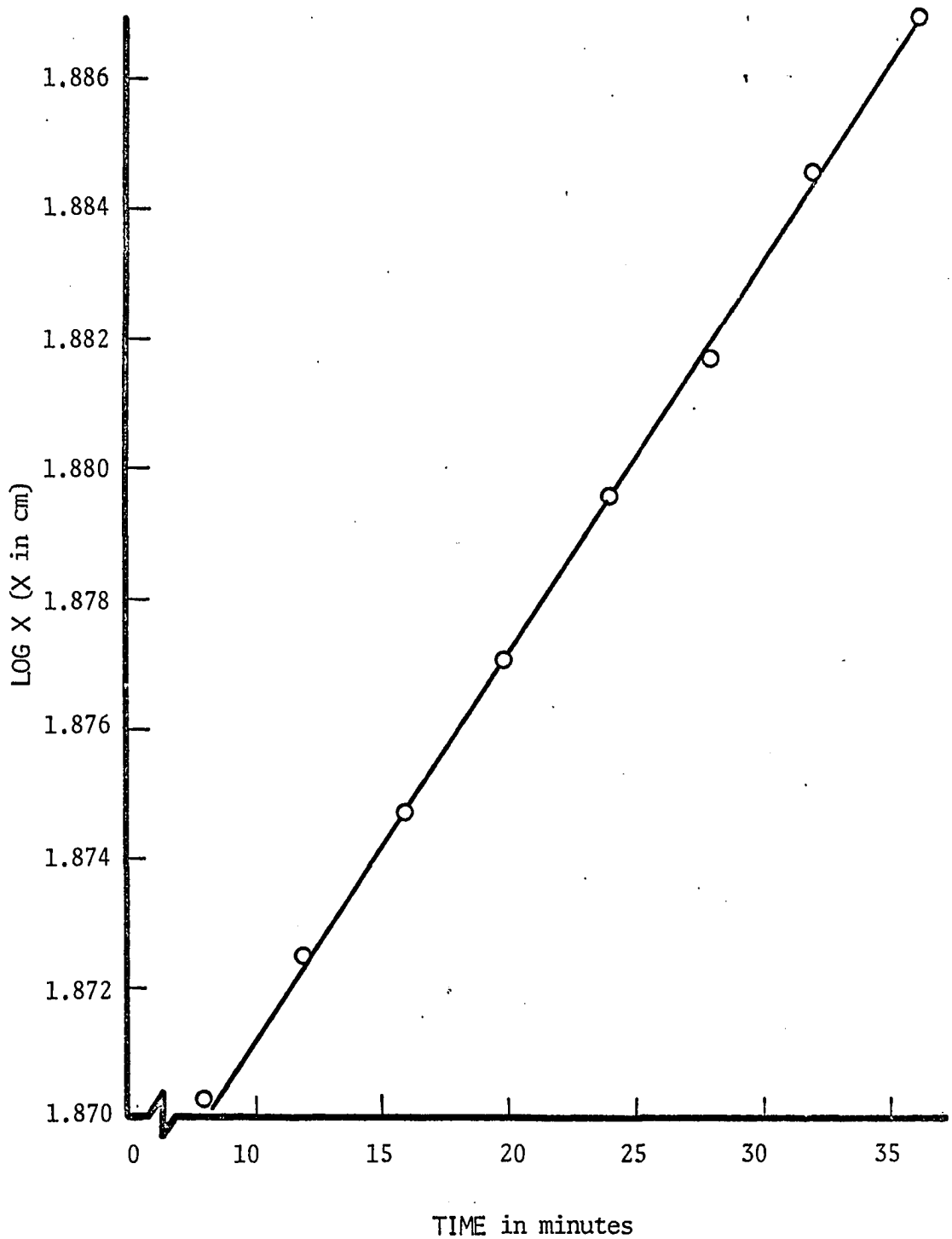


Figure 49. Determination of S of Apolipoprotein B obtained from ApoB-LP Fraction 1.030-1.040 g/ml. Protein was solubilized in 0.018 M NaOH and 0.15 M NaCl at 37°C for 30 minutes and then pH reduced to 7 with HCl ($\rho$ = 1.0063 g/ml). Measurements were taken after the rotor speed reached 59,780 rpm.

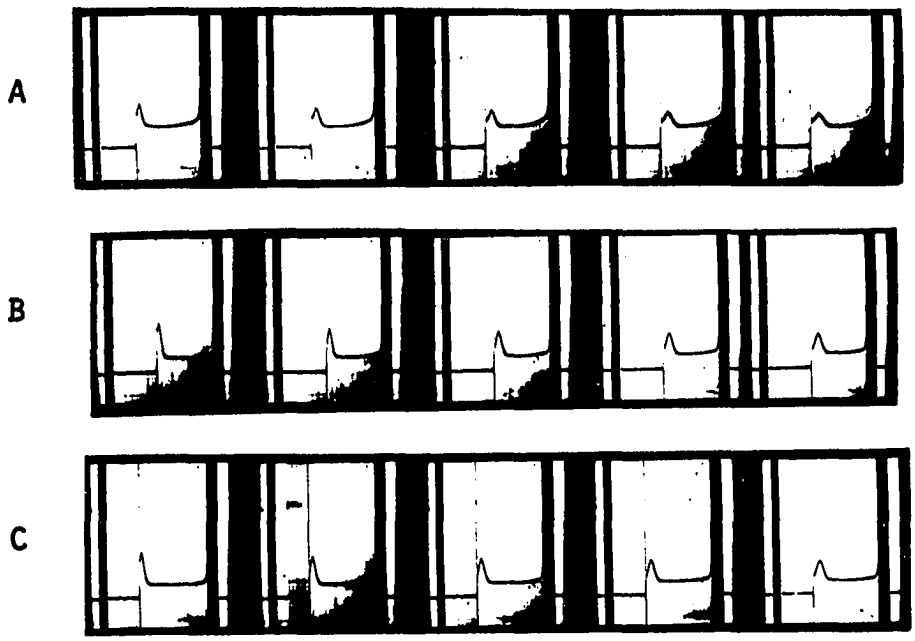


Figure 50. Ultracentrifugal sedimentation patterns of ApoB.

A: ApoB from ApoB-LP Fraction IV, 1.030-1.040 g/ml, in 0.1 M Tris buffer, pH 8.3, with heating for one hour at 80°. B: ApoB from the same lipoprotein, in 0.018 M NaOH and 0.15 M NaCl, pH 12 with heating for 30 min. at 37°. C: Same protein as in pattern B with pH reduced to 7. Experimental conditions: rotor speed 59,780 rpm; temperature 25°. Pictures were taken from left to right at 4-minute intervals after full speed was attained.

Figure 51. Ultracentrifugal diffusion pattern of ApoB.

Protein, solvent and treatment are the same as in pattern B of Figure 50. Pictures were taken from left to right at 4-minute intervals after desired speed was attained (12,590 rpm).

solution at pH 12 with heating at 37°C.

Table 17 shows the partial specific volume, $\bar{V}$, of ApoB calculated from the weight percentages of the amino acids. The values for partial specific volumes of each amino acid residue were taken from Cohn and Edsall (132). The calculated $\bar{V}$ of ApoB was 0.733 ml/g. Since the $\bar{V}$ values for both asparagine and glutamine are very close to those of aspartic acid and glutamic acid, it was decided not to make an assumption on the distribution of ammonia between glutamic and aspartic residues. The molecular weight of ApoB, estimated from S, D and $\bar{V}$ was $7.0 \times 10^4$. This value was correlated with the amino acid composition and the nearest integral number of residues of each amino acid in a mole of protein was estimated. Results are shown in Table 18. Molecular weight summarized from the weight of amino acids is $7.07 \times 10^4$.

Figure 52 shows the immunochemical properties of ApoB solubilized in Tris buffer, pH 8.3, $\mu$ 0.1, at room temperature and after heating for one hour at 80°. ApoB under both conditions gave two precipitin lines against rabbit serum containing antibodies to human ApoB-LP. One of the two precipitin lines was identical to that of ApoB-LP. However, the ApoB solubilized in dilute alkaline solution with heating failed to show any reaction against the same antiserum.

Figure 53 shows the electrophoretic mobility of ApoB on cellulose acetate paper. It is noted that ApoB solubilized at pH 12 showed a single band with $\alpha_2$-mobility, regardless of the final pH. ApoB solubilized in Tris buffer at room temperature gave 2 bands; however, after heating to 70° the same sample gave a single band with electrophoretic mobility slower than that of the samples resolubilized at pH 12. The

TABLE 17

PARTIAL SPECIFIC VOLUME OF ApoB

| Amino Acid | $M/10^5$ g | Wt. % | $\bar{V}$ ml/g | $\bar{V}$ · Wt. % |
|---|---|---|---|---|
| Lysine | 55.91 | 8.12 | 0.82 | 6.66 |
| Histidine | 19.12 | 2.95 | 0.67 | 1.98 |
| Arginine | 21.16 | 3.66 | 0.70 | 2.56 |
| Aspartic Acid | 88.39 | 11.68 | 0.60 | 7.01 |
| Threonine | 53.57 | 6.34 | 0.70 | 4.44 |
| Serine | 64.57 | 6.74 | 0.63 | 4.25 |
| Glutamic Acid | 99.87 | 14.59 | 0.66 | 9.63 |
| Proline | 29.92 | 3.42 | 0.76 | 2.60 |
| Glycine | 43.86 | 3.27 | 0.64 | 2.09 |
| Alanine | 57.35 | 5.07 | 0.74 | 3.75 |
| Half Cystine | 5.61 | 0.67 | 0.63 | 0.42 |
| Valine | 37.34 | 4.34 | 0.86 | 3.73 |
| Methionine | 11.72 | 1.74 | 0.75 | 1.31 |
| Isoleucine | 38.17 | 4.97 | 0.90 | 4.47 |
| Leucine | 86.53 | 11.27 | 0.90 | 10.14 |
| Tyrosine | 24.36 | 4.38 | 0.71 | 3.11 |
| Phenylalanine | 35.18 | 5.77 | 0.77 | 4.44 |
| Tryptophan | 4.93 | 1.00 | 0.74 | 0.72 |

$\bar{V}$ of ApoB = $\Sigma\bar{V}$ · Wt. % = 0.733 ml/g.

TABLE 18

AMINO ACID COMPOSITION OF ApoB

| Amino Acid | $M^*/7.0 \times 10^4$ |
|---|---|
| Lysine | 39 |
| Histidine | 13 |
| Arginine | 15 |
| Aspartic Acid | 62 |
| Threonine | 37 |
| Serine | 45 |
| Glutamic Acid | 70 |
| Proline | 21 |
| Glycine | 31 |
| Alanine | 40 |
| Cystine | 2 |
| Valine | 26 |
| Methionine | 8 |
| Isoleucine | 27 |
| Leucine | 61 |
| Tyrosine | 17 |
| Phenylalanine | 25 |
| Tryptophan | 3 |

*Nearest integral no. of amino acid residues based on the molecular weight obtained by physical-chemical method.

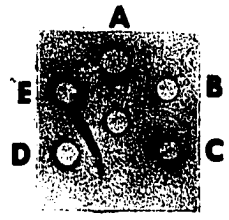


Figure 52. Immunochemical properties of ApoB in Tris buffer.

Center well: rabbit serum containing antibodies to human ApoB-LP. A, B: ApoB solubilized in 0.1 M Tris buffer, pH 8.3. C, E: ApoB solubilized in 0.1 M Tris buffer, pH 8.3, by heating for one hour at 80°. D: ApoB-LP. Note 2 precipitin lines at A, B, C, and E. One of the precipitin lines is identical to that of D.


Figure 53. Cellulose acetate electrophoretic pattern of ApoB under various solubilization conditions.

A: ApoB from ApoB-LP, 1.019-1.053 g/ml, at pH 1, solubilized at pH 12 with heating for 30 min. at 75°, pH reduced to 1 with HCl; B: same protein as A but at pH 7; C: ApoB from ApoB-LP Fraction IV, 1.030-1.040 g/ml, at pH 12, solubilized by heating 20 min. at 70°; D: same protein as A at pH 12; E: ApoB from ApoB-LP Fractions I + II, 1.006-1.019 g/ml, solubilized in 0.1 M Tris buffer, pH 8.3, with heating for 10 min. at 70°; F: same protein as C but solubilized in 0.1 M Tris buffer at room temperature; G: ApoB-LP, 1.019-1.040 g/ml; H: human whole serum. Mobilities: A, B, D migrated as $\alpha_2$-globulin, C as $\alpha_1$-globulin, E stayed at origin, F at origin and at $\beta$-globulin and ApoB-LP migrated as $\beta$-globulin.

protein solutions A, B, D, and F were also subjected to high voltage electrophoresis. No amino acids were observed in samples A, B, and D. However, glutamic acid, aspartic acid, and some neutral amino acids were observed in protein F solubilized in Tris buffer. This same protein (C) solubilized in NaOH and NaCl at pH 12 migrated further (to an $\alpha_1$-position) than other similarly treated proteins. This indicates that this particular protein was degraded before it was solubilized.

# CHAPTER IV

# DISCUSSION

## Heterogeneity of Ultracentrifugal Isolates of LDL

It has long been assumed that ultracentrifugal preparations of LDL are homogeneous with respect to protein moiety. Apolipoprotein B has been considered to be the only protein present in the low-density lipoprotein range. It was not until 1963 that Freeman et al. (23) found a high-density lipoprotein ($HDL_1$) present in the highest density range of LDL. No further characterization of this $HDL_1$ has been reported. Nearly all the workers studying ApoB-LP still use the solvent density range 1.006-1.063 g/ml as the criterion for isolating pure ApoB-LP. Most methods currently in use for determining the homogeneity of the protein moiety of LDL are not sensitive enough to detect the small amounts of ApoA-LP present. It has been demonstrated in this study that electrophoresis on cellulose acetate, on starch gel, and even immunoelectrophoresis all fail to detect the trace amount of ApoA-LP. Since analytical ultracentrifuge shows a single symmetric peak at reasonable lipoprotein concentration for any LDL fraction, this has been considered as a reasonable indication for the homogeneity of LDL. Only in highly dilute solution would the broadening or assymetry of a peak become apparent in a schlieren pattern. However, since LDL itself is a polydisperse system, a broadened

peak would not necessarily lead to the conclusion that ApoA-LP was present.

The antigenicity of ApoB-LP is much greater than that of ApoA-LP. When human LDL are used for immunizing rabbits ApoA-LP antibodies appear much slower than ApoB-LP antibodies. Therefore, the homogeneity in the antibody production within a certain time limit does not necessarily indicate the homogeneity of antigen. Even when a whole human serum is used for immunization the concentration of ApoA-LP antibodies produced is generally too weak to detect the small amount of ApoA-LP in the LDL range. For this reason, rabbit serum containing antibodies to human ApoA-LP rather than that containing antibodies to human whole serum has been used throughout the present study. It is probably for the same reason that conflicting findings on antigenicity of the LDL are reported in the literature. Gitlin et al. (68), Aladjem et al. (12, 69), and Lawrence et al. (70) have found antigenic heterogeneity, while Korngold et al. (65), Levine (67), Scanu et al. (71), and Blumberg et al. (73) have reported antigenic homogeneity of LDL.

It was found that the ApoA-LP detectable in samples by the micro immunodiffusion technique, as described in Methods, was not observed when Petri dishes were used for immunodiffusion experiments.

Since washing by recentrifugation did not remove ApoA-LP from the LDL fractions completely, it is suggested that the ApoA-LP found in the LDL range was not a contaminant, but rather a lipoprotein with a hydrated density characteristic of the LDL range (1.006-1.063 g/ml). However, the amount of ApoA-LP within the LDL range is sufficiently small to render quantitative study very difficult.

It has been noticed frequently that the removal of traces of ApoA-LP resulted in a decreased stability of ApoB-LP solutions as shown by an increased rate of turbidity formation. The significance of the ApoA-LP presence in LDL is not known. It appears that, similarly to albumin, it may serve as a stabilizing agent for ApoB-LP.

## Chemical Composition and Physical-Chemical Characterization

Results of the chemical analysis of ApoB-LP subfractions were compared with those reported in the literature. The composition of ApoB-LP Fraction I (Table 5) is surprisingly close to that of a subfraction isolated at the density 1.006-1.019 g/ml (Table 3) by Korn (76). For the higher density ranges of LDL, the results of the present study show good agreement with most of the data in the literature. Exceptions are the results of Lindgren *et al.* (81) which varied considerably from the values in other literature reports, and the rather low protein and phospholipid values reported by Smith (83). It is interesting to note (Table 5) that within the density range 1.019-1.063 g/ml, the phospholipid and protein values do not increase with increasing density, but remain rather constant. The phospholipid/protein ratio is a constant in all subfractions from d 1.006 g/ml to 1.063 g/ml, in spite of the fact that the total phospholipid and protein values are lower in the first subfraction. The results reported in the literature confirm this finding. The neutral lipid content of the last four fractions (1.019-1.063 g/ml) represents also a rather constant value [neutral lipid = 100 - (phospholipid+protein)]. Since the free cholesterol content is practically independent of density, it is the cholesterol ester/triglyceride ratio that controls actually the

density of low-density lipoproteins. The neutral lipid/phospholipid-protein ratio of the ApoB-LP subfractions have two distinct values: 1.90 for the first fraction and approximately 1.18 for the last four fractions. The content of triglyceride is higher than that of cholesterol ester in the first fraction and the opposite is true for the last four fractions. It is regrettable that the values for Fraction II are lacking. However, by comparison with a study compiled by Korn on the LDL subfraction of d 1.006-1.019 g/ml (76), one would expect that the neutral lipid/phospholipid-protein ratio of Fraction II, 1.009-1.019 g/ml, would be closer to that of Fraction I, 1.006-1.009 g/ml than to that of higher density fractions.

The correlation between the increased hydrated densities of ApoB-LP subfractions and the decreased $S_f^o$ values found in the present study agrees very well with the report of Lindgren et al. (19). No studies have been reported in the literature regarding the comparison of the hydrated density, and flotation and sedimentation coefficients between female and male lipoproteins isolated at the same solvent densities. It has been demonstrated that the hydrated densities of all male ApoB-LP subfractions are consistently lower than those of female lipoproteins. Despite the variations in hydrated densities, sedimentation and flotation coefficients, and diameter of the particles between male and female lipoproteins isolated at the same density range, molecular weights are the same for similarly isolated preparations regardless of sex (Tables 8 and 12). This suggests that the molecular weights could be identical for any lipoproteins of different hydrated densities, even those isolated at different solvent densities. Indeed, the molecular weights of ApoB-LP

Fractions IV, V and VI are the same, as are those of ApoB-LP Fractions I, I + II and II. Fraction III appears to have an intermediate value.

One of the preparations of ApoB-LP Fraction III had a hydrated density of 1.019 g/ml and a molecular weight of $3.3 \times 10^6$, while another preparation of the same subfraction with a hydrated density of 1.018 g/ml possessed a molecular weight of $3.9 \times 10^6$. Thus, these two preparations differed slightly in their hydrated densities, but appreciably in their molecular weights. Since subfractions of ApoB-LP can be separated into two groups characterized by molecular weights of $4.6 \times 10^6$ and $3.2 \times 10^6$, it would appear that the dividing line between these two groups must be within the hydrated densities 1.018-1.019 g/ml.

The values for "hydrated densities" of lipoprotein subfractions isolated within a certain solvent density range represent actually <u>average</u> hydrated densities of all lipoprotein molecules occurring in that particular density range. The true hydrated densities of individual lipoprotein molecules must lie on both sides of an average value. Therefore, lipoproteins of ApoB-LP Fraction III with average hydrated densities of 1.018 and 1.019 g/ml contain certain number of molecules with true hydrated density values lower and higher than those of their average hydrated density. It is postulated, on the basis of results obtained in this study, that the intermediate subfractions of ApoB-LP represent mixtures of lipoproteins of only two molecular weights, $4.6 \times 10^6$ and $3.2 \times 10^6$, rather than of molecules characterized by many gradually changing molecular weights. The variation in the percent distribution of these two groups of lipoprotein molecules results in preparations characterized by molecular weights falling within the two extreme values.

There is an excellent agreement (Table 13) between values for the molecular weight of various LDL subfractions reported in this study and in the literature regardless of some important differences in the isolation and purification procedures.

Despite differences in the flotation and sedimentation coefficients the values for terms $\frac{S^o_\eta}{1 - \bar{V}\rho}$ or $\frac{S^o_{f\eta}}{1 - \bar{V}\rho}$ ($S^o_\eta$ and $S^o_{f\eta}$ corrected for buoyancy factors) for ApoB-LP Fractions IV, V and VI (Table 13) are the same (the lower value for ApoB-LP Fraction VI is due to the presence of small amount of ApoA-LP). This result indicates clearly that the variation in the sedimentation and flotation coefficients at infinite dilution depends only on the buoyancy factor which is, in turn, controlled exclusively by the hydrated density of lipoprotein subfractions. It folfows then that lipoprotein molecules of ApoB-LP subfractions isolated at different density ranges must have the same shape. Since the diffusion coefficients of all three subfractions have almost identical values, the molecular weights calculated according to the Svedberg's equation at constant temperature, must be also identical.

The results of the chemical composition of ApoB-LP subfractions seem to favor the conclusion that the Fraction III should have most probably the same molecular weight as Fractions IV, V and VI, since its neutral lipid/phospholipid-protein ratio is in the same range. If all four subfractions consisted of the same basic phospholipid-protein structural unit, their molecular weights would be expected to have similar or identical values.

A comparison of chemical composition between certain subfractions of LDL from this study and that of Korn (76) and an apolipoprotein

B-containing VLDL ($S_f$ 20-50) isolated and characterized by Gustafson et al. (20) discloses that the phospholipid/protein ratio is very similar, if not identical, for all apolipoprotein B-containing lipoproteins of different densities or classes (Table 19). On the other hand, the values for the neutral lipid/phospholipid-protein ratio differ for three major density groups of apolipoprotein B-containing lipoproteins; the density subfractions $S_f$ 20-50, 1.006-1.019 g/ml and 1.019-1.063 g/ml are characterized by ratios 3.0, 2.0 and 1.18, respectively. Similarly, the molecular weight of these subfractions decreases with increasing density. However, the calculated molecular weight of the phospholipid-protein structural units of all these subfractions is $1.5 \times 10^6$. This indicates that the absolute amount of phospholipid-protein unit is constant in all subfractions of apolipoprotein B-containing lipoproteins thus far studied. Therefore, it is postulated that each segment of the polydisperse system of ApoB-LP consists of the same basic phospholipid-protein structural unit. The increase in size and molecular weight of ApoB-LP subfractions is due solely to the increased amount of triglycerides or triglycerides and cholesterol esters. A further support for this hypothesis will be forthcoming from the discussion of studies on the protein moiety.

It appears from this study as well as from literature reports that the absolute amount of free cholesterol remains also quite constant in every ApoB-LP subfraction. Since free cholesterol can be removed more readily than phospholipid by partial delipidization (51, 109), it is not quite clear whether it is directly bound to the protein or whether it represents a "bridge" between the inner core of triglyceride and cholesterol ester and the outside shell or membrane composed of protein-

TABLE 19

COMPARISON OF APOLIPOPROTEIN B-CONTAINING FRACTIONS FROM VLD- AND LD-LIPOPROTEINS

| Lipoprotein Fraction Density Range g/ml | Apolipoprotein | Phospholipid / Protein Ratio | Neutral Lipids / PL + PR Ratio | Molecular Weight x10$^{-6}$ | Molar Weight PL + PR x10$^{-6}$ | Reference |
|---|---|---|---|---|---|---|
| VLD $S_f$ 20-50 | B | 1.10 | 3.0 | 6.0 | 1.5 | (20) |
| I, 1.006-1.009 | B | 0.97 | 1.9 | 4.9 | 1.69 | a |
| I + II, 1.006-1.019 | B | 1.20 | 2.0 | 4.6[a] | 1.5 | (76) |
| IV, 1.030-1.040 | B | 0.96 | 1.18[b] | 3.2 | 1.5 | a |

[a]Data from present study.

[b]Average value of Fraction III - VI.

phospholipid units.

The results of amino acid analysis of ApoB-LP subfractions are generally in excellent agreement with those reported by Margolis and Langdon (95). The only significant difference (20%) is found in the contents of alanine, valine and isoleucine. This similarity in the amino acid composition of apolipoprotein B-containing lipoproteins covering flotation ranges such as $S_f$ 10-400 (95) or $S_f$ 5-16 (present study) could be interpreted as another evidence for the possible identity of protein moieties and, indirectly, the phospholipid-protein units of ApoB-LP subfractions.

Results of amino acid analysis confirm also the already available evidence (100, 113, 134) for a difference between the amino acid composition of ApoA and ApoB. The striking difference between these two proteins was noticed primarily in their content of isoleucine, aspartic acid and phenylalanine.

## Physical-Chemical Studies on Apolipoprotein B

This study describes also the first attempt to redissolve a totally delipidized ApoB-LP in absence of additives and to determine the sedimentation and diffusion coefficients, and the molecular weight of apolipoprotein B. Granda and Scanu (101) have demonstrated the feasibility of solubilizing the apolipoprotein B by a method of total delipidization of ApoB-LP in the presence of sodium dodecyl sulfate. However, these authors obtained a sedimentation value which appeared to be too high for a protein deprived of lipids. Since sodium dodecyl sulfate forms a complex with the apolipoprotein B, such a "model" offers little or no advantage for physical-chemical characterization of the protein moiety.

In order to avoid the use of complex-forming, solubilizing agents, a study of apolipoprotein B solubility in dilute alkaline solutions has shown that the protein moiety is fairly soluble in both Tris buffer, pH 8.3, and in a 0.018 M NaOH solution, pH 12, at 70-80°. The similarity in the sedimentation coefficients of apolipoprotein B solutions indicated little or no effect of pH-values on the structure of protein moiety. Short heating intervals, up to 20-30 minutes, resulted in no detectable degradation fragments such as small peptides or amino acids as tested by high voltage electrophoresis or two-dimensional thin-layer chromatography of apolipoprotein B solutions.

The preliminary study shows that the molecular weight of apolipoprotein B dissolved in 0.018 M NaOH solution, pH 12, at 37° is $7.0 \times 10^4$. The smallest possible molecular weight calculated from the amino acid composition of apolipoprotein B is $3.5 \times 10^4$, assuming the presence of only one cystine residue. The theoretical value of the molecular weight based on the presence of two moles of cystine would correspond exactly to the experimental value.

The appearance of a single peak at various concentrations of apolipoprotein B in the ultracentrifugal patterns, seems to indicate absence of impurities and aggregates. If a molecular weight of $7.0 \times 10^4$ represents the smallest protein unit and the average value for the phospholipid/protein ratio is close to unity, the molar weight of the protein moiety of all apolipoprotein B-containing lipoproteins must be approximately $7.5 \times 10^5$ (calculated from the molar weight of phospholipid-protein unit, $1.5 \times 10^6$, Table 19). The apolipoprotein B consists then of ten or eleven identical subunits.

# CHAPTER V

## SUMMARY

Human plasma low density lipoproteins (LDL) of normal subjects were separated by sequential preparative ultracentrifugation into six arbitrary subfractions (d 1.006-1.009, 1.009-1.019, 1.019-1.030, 1.030-1.040, 1.040-1.053 and 1.053-1.063 g/ml). Small amounts of apolipoprotein A-containing lipoproteins associated with this density range were removed by immuno-precipitation. The homogeneous, apolipoprotein B-containing subfractions were characterized by immunochemical methods, by cellulose acetate and starch gel electrophoresis, by chemical and amino acid composition, by determination of hydrated density, sedimentation, flotation and diffusion coefficients, and by estimation of particle diameter and molecular weight.

All subfractions had practically the same diffusion coefficients ($D^{o}_{20,w}$ = 1.7-1.9 x $10^{-7}$ $cm^2$/sec.). On the basis of gross chemical composition, molecular weights, and sedimentation coefficients corrected for solvent viscosity and buoyancy factors the LDL can be separated into two distinct groups occurring within the density ranges 1.006-1.019 g/ml (Class I) and 1.030-1.063 g/ml (Class II). The lipoproteins of density range 1.019-1.030 g/ml represent a mixture of both major groups. Subfractions of both groups had a constant percentage of neutral

lipids (triglyceride + cholesterol) and phospholipid and protein. The phospholipid/protein ratio of all subfractions was the same. Subfractions of Class I and Class II were characterized by molecular weights of $4.6 \times 10^6$ and $3.2 \times 10^6$, respectively. All subfractions had an identical total amount of phospholipid and protein ($1.5 \times 10^6$). An apolipoprotein B-containing subfraction ($S_f$ 20-50) of VLDL was characterized by the same phospholipid/protein-ratio and the same absolute amount of phospholipid and protein, even though the gross chemical composition and physical-chemical properties (molecular weight $6 \times 10^6$) were different from those of LDL subfractions. It has been postulated, therefore, that each segment of the polydisperse system of apolipoprotein B-containing lipoproteins consists of the same basic phospholipid-protein structural unit. The absolute amount of this unit is practically constant throughout the spectrum of apolipoprotein B-containing lipoproteins and is characterized by the capacity to maintain the structural integrity of triglyceride-cholesterol micelles of different sizes and molecular weights. In support of this hypothesis, it was found that the protein moiety of each subfraction had an identical amino acid composition.

It has been demonstrated that apolipoprotein B can be solubilized in the absence of detergents. The sedimentation coefficient, $S^o_{20,w}$, was found to be 2.6 at various pH values and was not altered by heating the protein solution at $70^o$. The value for the molecular weight of apolipoprotein B calculated by Svedberg's equation was $7.0 \times 10^4$. On the basis of this finding, it has been concluded that the protein moiety ($7.5 \times 10^5$ g/mole) of apolipoprotein B-containing lipoproteins consists of ten or eleven identical subunits.

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# APPENDIX A

## LIST OF ABBREVIATIONS AND SYMBOLS

### English Letter Symbols

| | |
|---|---|
| ApoA | Apolipoprotein A |
| ApoA-LP | Apolipoprotein A-containing lipoproteins |
| ApoB | Apolipoprotein B |
| ApoB-LP | Apolipoprotein B-containing lipoproteins |
| app | Apparent |
| CE | Cholesterol esters |
| d | Isolation density of lipoprotein fractions |
| $\bar{d}$ | Hydrated density of lipoprotein fractions |
| Dia. | Diameter |
| dil. | Dilution |
| F | Female |
| FC | Free cholesterol |
| HDL | High-density lipoproteins |
| hrs. or h. | Hours |
| LDL | Low-density lipoproteins |
| LP | Lipoproteins |
| M | Male |
| max. | Maximum |
| min. | Minutes |

LIST OF ABBREVIATIONS AND SYMBOLS--Continued

English Letter Symbols

| | |
|---|---|
| M. W. or M. | Molecular weight |
| obs. | Observed |
| PL | Phospholipid |
| PR | Protein |
| R | Gas constant |
| S | Observed sedimentation coefficient |
| $S^o$ | Observed sedimentation coefficient at infinite dilution |
| $S^o_{20,w}$ | Sedimentation coefficient at infinite dilution in water at 20°C |
| SDS | Sodium dodecyl sulfate |
| $S_f$ | Flotation coefficient in NaCl solution of density 1.063 g/ml or of other densities |
| $S^o_f$ | Flotation coefficient at infinite dilution in NaCl solution of density 1.063 g/ml at 26°C |
| Sved. | Svedberg unit ($10^{-13}$ second) |
| t | Time in seconds |
| t | Temperature in centigrade |
| T | Absolute temperature in °K |
| TCA | Trichloroacetic acid |
| TG | Triglyceride |
| $\bar{V}$ | Partial specific volume |
| VHDL | Very high-density lipoproteins |
| VLDL | Very low-density lipoproteins |

LIST OF ABBREVIATIONS AND SYMBOLS--Continued

Greek Letter Symbols

| | |
|---|---|
| αLP | Alpha-lipoproteins |
| βLP | Beta-lipoproteins |
| γ-globulin | Gamma-globulin |
| Δ | Difference |
| η | Viscosity of solvent at 25°C relative to that of water at 25°C |
| $\eta_t$ | Viscosity of solvent at t°C |
| $\eta_t^o$ | Viscosity of water at t°C |
| μ | Ionic strength |
| π | 3.1416 |
| ρ | Solvent density |
| σ | Standard deviation |
| Σ | Summation |
| ω | Angular velocity |

Miscellaneous Symbols

| | |
|---|---|
| ♀ | Female |
| ♂ | Male |
| ∞ | Infinite |

## THE DEFINITION AND ABBREVIATIONS FOR SERUM OR PLASMA LIPOPROTEINS

VLDL, very low-density lipoproteins, lipoproteins of d < 1.006 g/ml and $S_f$ > 20

LDL, low-density lipoproteins, lipoproteins of d 1.006-1.063 and $S_f$ 0-20; βLP, β-lipoproteins, lipoproteins displaying the mobility of β-globulin in free electrophoresis

ApoB-LP, lipoproteins containing a single protein moiety, apolipoprotein B

HDL, high-density lipoproteins, lipoproteins of d 1.063-1.210 g/ml; αLP, α-lipoproteins, lipoproteins displaying the mobility of $\alpha_1$-globulin in free electrophoresis

$HDL_2$, subfraction of high-density lipoproteins of d 1.063-1.125 g/ml

$HDL_3$, subfraction of high-density lipoproteins of d 1.125-1.210 g/ml

ApoA-LP, lipoproteins containing a single protein moiety, apolipoprotein A

VHDL, very high-density lipoproteins, lipoproteins of d > 1.210 g/ml

$VHDL_1$, very high-density lipoproteins, lipoproteins of d 1.210-1.250 g/ml

$VHDL_2$, very high-density lipoproteins, lipoproteins of d > 1.250 g/ml

# APPENDIX B

# COMPUTER PROGRAMS

Program for Logarithm of X for the Calculation of

Sedimentation and Flotation Coefficients

| Symbol | Meaning |
|---|---|
| K | Plate number |
| A | The distance between the sedimenting or flotating boundary and the reference hole in cm. |
| B | The distance between two reference holes in cm. |
| BA | Average of B. |
| ASR | Answer in ln X (X is the distance from boundary to the center of rotation in cm. at time t). |
| COMASR | Answer in log X. |

```
C       PROGRAM FOR LOGARITHM OF X FOR SEDIMENTATION
C       AND FLOTATION CALCULATIONS.
        DIMENSION A(100),B(100),ASR(100),COMASR(100)
        PRINT 98
   98 FORMAT(1H1,///////)
   99 READ 1, N,M,K
    1 FORMAT(3I5)
        PRINT 2, K
    2 FORMAT(6X,14HPLATE NUMBER =,I5/)
        READ 3, (A(J), J=1,N)
        READ 3, (B(J), J=1,M)
    3 FORMAT(8F8.3)
        PRINT 44,  (A(J),J=1,N)
        PRINT 44, (B(J), J=1,M)
   44 FORMAT(1X, 8F8.3/)
        BX=0.
        DO 10 J=1,M
   10 BX=BX+B(J)
        XM=M
        BA=BX/XM
        PRINT 4,BA
    4 FORMAT(/6X,12HB, AVERAGE =, F8.3/)
        DO 11 J=1,N
   11 ASR(J)=LOGF(1.6*A(J)/BA+5.72)
        PRINT 5, (ASR(J), J=1,N)
    5 FORMAT(/6X,21HANSWER IN NATURE LOG , 5F12.5)
        DO 12 J=1,N
   12 COMASR(J)=ASR(J)/2.30259
        PRINT 6, (COMASR(J), J=1,N)
    6 FORMAT(/6X,21HANSWER IN COMMON LOG , 5F12.5)
        PRINT 7
    7 FORMAT(//////)
        GO TO 99
        STOP
        END


C       SAMPLE INPUT

     5    5  301
  30.416  29.852  29.355  28.975  28.398
  33.934  33.902  33.935  33.952  33.932
```

Program for the Calculation of Diffusion Coefficient

| Symbol | Meaning |
|---|---|
| A | Area |
| NN | A test. NN < 1, program reads YF; NN > 1, program reads A. |
| N | Number of measurements of H. |
| IPN | Plate number. |
| IPICN | Picture number. |
| TM | Time in minutes recorded for each picture. |
| YI | Base line. |
| YF | Measurement of boundary on y axis. |
| TO | Time between the instant of boundary formation and the first picture taken. |
| DELTAX | Interval of measurements of boundary on x axis. |
| RPM | Centrifugal speed. |
| HMAX | H maximum. |
| F | Magnification factor. |
| DAPP | Apparent diffusion coefficient. |
| WSQ | $\omega^2$. |

```
C       PROGRAM OF DIFFUSION COEFFICIENT
        DIMENSION YF(100), H(100)
  100 READ 1, NN, N, IPN, IPICN
    1 FORMAT(4I4)
        READ 2, YI,TM,TO,DELTAX,RPM,HMAX,F
    2 FORMAT(7F10.3)
    3 FORMAT(F12.6/)
        READ 4, S
    4 FORMAT(E12.6)
        PRINT 5, IPN, IPICN, TM
    5 FORMAT(1H1//////3X,6HPLATE ,I3,3H - ,I3,4X,
     116HTIME INTERVAL = ,I3,6H  MIN.///)
        PRINT 6, HMAX
    6 FORMAT(3X, 7HHMAX = ,F10.6/)
        IF(NN-1)10,10,13
   10 READ 2, (YF(J),J=1,N)
        DO 11 J=1,N
   11 H(J)=ABSF(YF(J)-YI)
        A=0.
        DO 12 J=1,N
   12 A=A+H(J)*DELTAX
        GO TO 14
   13 READ 3, A
   14 CONTINUE
        RATIO=A/HMAX
        G=(RATIO/F)**2
        PRINT 101, G
  101 FORMAT(3X,16H(A/HMAX/F)**2 = , F9.7/)
        TIME=12.5664*(60.0*TM+TO)
        PRINT 102, A
  102 FORMAT(3X,7HAREA = ,F10.6/)
        PRINT 103,RATIO
  103 FORMAT(3X,9HA/HMAX = ,F9.6/)
        P=G/TIME
        WSQ=(.1047*RPM)**2
        DAPP=P*(1.-WSQ*S*(60.*TM+TO))
        TIMRVS=1./(60.0*TM+TO)
        PRINT 106, DAPP
  106 FORMAT(3X, 34HDIFFUSION COEFFICIENT, APPARENT = , E12.6/)
        PRINT 107, TIMRVS
  107 FORMAT(3X, 17H1/T    (1/SEC) = , E11.5/)
        GO TO 100
        STOP
        END
```

```
C      SAMPLE INPUT


    1  24 606   2
     21.807      4.000    492.000       .200 12590.000     10.487     21.395
  .176000E-12
     21.717     21.685     21.644     21.548     21.275     20.715     19.834
     18.991     17.533     15.617     13.627     11.895     11.320     11.828
     14.083     16.550     18.411     19.771     20.557     20.973     21.258
     21.473     21.604     21.699
```