# Immunochemical Quantification of Human Plasma Lp(a) Lipoprotein

**JOHN J. ALBERS**[1] and **WILLIAM R. HAZZARD**, The Northwest Lipid Research Clinic, Departments of Medicine and Biochemistry, University of Washington School of Medicine, Seattle, Washington 98104

## ABSTRACT

The Lp(a) lipoprotein was purified from human plasma by ultracentrifugation and gel filtration on 6% agarose. It contained 27% protein, 65% lipid, and 8% carbohydrate. Quantification of the Lp(a) lipoprotein was performed by radial immunodiffusion. Both within-assay and between-assay coefficients of variation were inversely concentration dependent, decreasing from 20% and 27%, respectively, at 3 mg/100 ml to 7% and 12%, respectively, at concentrations above 8 mg/100 ml. The lower limit of sensitivity of the assay was 1.5 mg/100 ml. Of 340 unrelated fasting subjects tested, 81% had levels of the Lp(a) lipoprotein exceeding this lower limit. The distribution of Lp(a) concentrations in this population was skewed with a mean of 14 mg/100 ml and a median of 8 mg/100 ml. Lp(a) lipoprotein was not significantly correlated with age, sex, or cholesterol or glyceride concentrations.

## INTRODUCTION

In recent years the focus of attention in plasma lipid research has shifted from the lipids themselves to their protein carriers and the lipoprotein complexes. Of the lipoproteins identified to date, the Lp(a) lipoprotein has remained especially enigmatic. This lipoprotein, which closely resembles the low density lipoprotein (LDL, *d* 1.019-1.063 g/ml) in its lipid composition (1), shares its major antigenic determinants with LDL (2); and it has been reported that 65% of its apoprotein is identical to the B or LDL apoprotein, 20% of its apoprotein is the "Lp(a) protein," and that albumin is a minor (<15%) but integral part of the Lp(a) apoprotein (3). Its density (*d* 1.050-1.12 g/ml) overlaps that of LDL and high density lipoprotein (HDL) (1,2), but its electrophoretic mobility on paper (4) or agarose (1,2,4) is pre-beta in contrast with the beta-mobility of LDL. In early reports Lp(a) was considered to be a qualitative genetic marker (5,6), but more recently it has been suggested to be a quantitative trait present in all individuals (7). Its physiological and genetic control, relationship to normal and abnormal states of lipid metabolism, and possible role in the atherosclerotic process remain unknown.

Definitive answers to these questions await the development of a highly sensitive, precise, and specific assay for the Lp(a) lipoprotein in physiological media. In the present study this problem has been approached by the development of an immunochemical assay utilizing radial immunodiffusion. The assay sensitivity and precision have been quantified and the technique applied to the measurement of the Lp(a) lipoprotein in an epidemiological survey of plasma lipid and lipoprotein concentrations in a population of 340 free-living adult employee volunteers.

## MATERIALS AND METHODS

### Blood Samples

For isolation of Lp(a) lipoprotein for immunization and preparation of standards, blood samples were drawn from healthy, fasting adults on ad libitum diets. In all instances, blood was drawn into tubes or bottles containing disodium ethylenediaminetetraacetate (EDTA) to give a final concentration of 1 mg/ml and the plasma promptly separated and stored at 4 C until analysis or ultracentrifugation. For determination of the distribution of Lp(a) lipoprotein, cholesterol, and glyceride levels among healthy, fasting adults, all employees of the Pacific Northwest Bell Telephone Company in Renton, Wa., were asked to volunteer for an epidemiological survey of lipid and lipoprotein levels. Ninety-two per cent (340) of this population from age 20-65 was sampled after an overnight (12-14 hr) fast.

### Preparation of Lipoproteins

Lipoproteins were isolated from sera of individual donors by sequential preparative ultracentrifugation (8). Specifically, the nonprotein solvent density of plasma from a normolipidemic fasting adult was adjusted to 1.060 g/ml with solid NaCl. Ultracentrifugation then was carried out ina 50 Ti rotor at 40,000 rpm at 10 C for 24 hr. The top 3 ml of each tube was removed with a tube slicer and the bottom fraction readjusted to 1.090 g/ml with

[1] Author to whom reprint requests should be addressed.

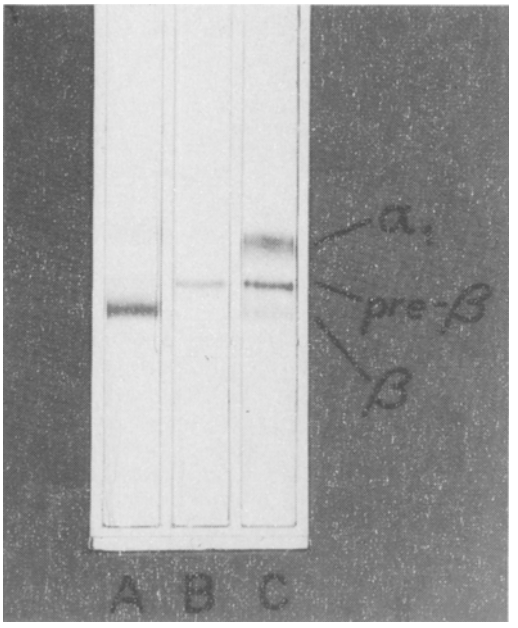


FIG. 1. Agarose electrophoresis of plasma and fractions thereof: (A) Whole plasma, (B) First major fraction or Lp(a) lipoprotein obtained from chromatography of 1.060-1.090 g/ml fraction on Bio-Gel A5m (see Fig. 2), and (C) 1.060-1.090 g/ml lipoprotein fraction.

solid NaCl and recentrifuged in a 50 Ti rotor at 45,000 rpm for 26 hr. The *d* 1.060-1.090 g/ml lipoproteins contained in the top 2.5 ml were subfractionated by ascending gel chromatography on 6% agarose gel (Bio-Gel A-5m, 200-400 mesh, Bio-Rad Laboratories, Richmond, Calif.) at 18 C utilizing a 2.6 x 90 cm column and a flow rate of 12 ml/hr (2). The sample volume was 4-6 ml and the eluting buffer 0.1 M Tris-HCl-0.15 M NaCl-0.001 M EDTA (pH 8.2).

Fractions were collected at 3 ml/tube. The absorbance of the eluates was measured with a Beckman model DU-2 ultraviolet spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) at 280 nm and the appropriate fractions pooled and dialyzed against 0.01 M Tris-HCl-0.001 M EDTA (pH 8.2) and then concentrated with Aquacide II (Calbiochem, La Jolla, Calif.). The lipoproteins subsequently were dialyzed against 0.15 M NaCl-0.001 M EDTA (pH 7.4) prior to use as standard or for preparation of antigen.

#### Electrophoresis

Polyacrylamide gel electrophoresis of whole lipoproteins was performed according to Narayan, et al., (9). The lipoproteins were prestained for lipid with Sudan Black B or, alternatively, poststained for protein with Amido-Schwartz in 7% acetic acid. Agarose electrophoresis was performed using the Bio-Gram A kit (Bio-Rad Laboratories).

#### Antigen and Antisera Preparation

The first fraction from the agarose column (see Fig. 1), constituting the Lp(a) lipoprotein, was pooled and concentrated with Aquacide II. Ca. 100 μg Lp(a) protein was placed on each 10-12 polyacryamide gel columns. The single, slowly migrating sudan positive band was cut out from each gel. The purified Lp(a) lipoprotein (ca. 1 mg) obtained from polyacrylamide gel electrophoresis along with the polyacrylamide gel was emulsified with an equal volume of Freund's complete adjuvant. Rabbits were immunized intramuscularly, subcutaneously, and intradermally, as described (10). A booster dose of ca. 1 mg Lp(a) protein in incomplete adjuvant was given at 1 month intervals. The rabbits were bled on the seventh day following the booster injection. Immunization of two rabbits with Lp(a) lipoprotein led to the production of precipitating antisera. These antisera were absorbed with LDL (*d* 1.030-1.040 g/ml) as follows: 0.5 mg LDL was added to ca. 20 ml each antiserum, incubated at 37 C for 30 min, then overnight at 4 C. This absorption procedure was repeated four-six times, until a precipitate no longer formed upon addition of LDL. This adsorbed antiserum was designated anti-Lp(a). Antiserum from one of the two rabbits was used in the present study.

Antisera against the high density lipoprotein polypeptides were those prepared and used previously (11).

#### Chemical Analysis

Lipids were extracted from the Lp(a) lipoproteins by the method of Folch, et al., (12). Cholesterol was determined by the method of Searcy, et al., (13); triglyceride by a modified procedure of Carlson (14) with triolein (Applied Science Laboratories, Inc., State College, Pa.) as standard; and phospholipid by the procedure of Bartlett (15). The factor 25 was used to convert phosphorus to phospholipid. It was assumed that 71.5% sterol in Lp(a) lipoprotein was sterol ester (1); the factor of 1.677, based upon cholesterol linoleate, was utilized to convert sterol ester to sterol mass.

For lipid analysis of whole plasma, samples were extracted with zeolite and isopropanol and analyzed for cholesterol and triglyceride with the Technicon Auto-Analyzer I, as outlined (16,17). During the course of this study the Northwest Lipid Research Clinic was in Phast III (Surveillance) of the Glyceride and Cholesterol Standardization Program of the

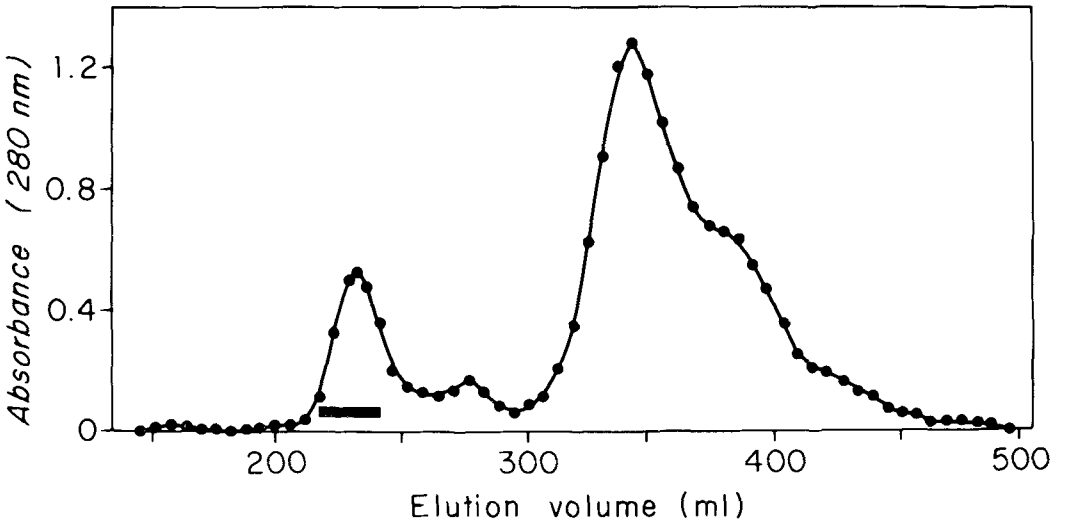


FIG. 2. Elution profile from Bio-Gel A-5m of a representative 1.060-1.090 g/ml lipoprotein fraction. ▬ = fraction constituting the Lp(a) lipoprotein.

Lipid Standardization Laboratory at the U.S. Center for Disease Control, Atlanta, Ga., and continuously met the standards for precision and accuracy specified by that program. Specifically, there was a coefficient of variation of less than 4% and accuracy within 3% of true value for cholesterol analysis and a coefficient of variation of less than 6% and accuracy within 3% for the glyceride analysis.

Protein was determined according to Lowry, et al., (18) with human serum albumin (HSA, Pentex) as standard. Protein nitrogen was determined by Kjeldahl digestion and Nesslerization (19); the factor 6.25 was used to convert protein nitrogen to protein. The Lp(a) lipoprotein preparations were diluted with 0.1% (w/v) sodium dodecyl sulfate prior to protein analysis. On the basis of Nessler nitrogen analyses, the Lp(a) lipoprotein protein, as determined by the Lowry method, was multiplied by 0.7 to convert to HSA protein equivalents. Hexose was determined by the anthrone method (20) with glucose as the standard.

#### Gel Diffusion

Immunodiffusion was carried out in 1% (w/v) agarose (Bio-Rad) in 0.15 M NaCl, 0.001 M EDTA, 0.05% (w/v) sodium azide, 0.02 M tris-HCl (pH 8.0), henceforth called RID buffer. An example of the micro Ouchterlony plate used for routine testing of human plasma has been shown previously (21). Anti-Lp(a) serum was diluted 1:5 (v/v) with RID buffer prior to use.

#### Quantitative Immunochemical Analysis

Quantitation of the Lp(a) lipoprotein was performed by the single radial immunodiffusion method of Mancini (22). To eliminate nonspecific precipitation reactions, only those plasmas which gave a positive reaction in double gel diffusion were considered positive in the quantitative method. Anti-Lp(a) serum was diluted 1:100 (v/v) with RID buffer containing 1% (w/v) bovine serum albumin (BSA) prior to use. Five ml of diluted anti-Lp(a) serum, heated to 55 C, was added to an equal volume of 2% (w/v) melted agarose solution cooled to 55 C. This antiserum-agarose solution was mixed thoroughly, avoiding bubbling, and immediately poured into prewarmed 100 x 100 x 15 mm phage typing dishes precoated with 1% silicone. The mixture was left to solidify on a level surface for 20-30 min. Thirty-six antigen wells of 2.2 mm diameter were punched out over the center of each grid (36 grids/dish) using a brass needle with a 2.2 mm bore. Three microliters of standard or plasma samples were added to each well. Unless otherwise indicated, each sample was added once to two different plates. Standards were added in duplicate wells chosen in different quadrants on each respective plate. The plates were placed in a humid chamber in a level position at 37 C. After 72-120 hr, the ring-shaped immunoprecipitates were measured in tenths of a millimeter using a micrometer lens.

The Lp(a) lipoprotein fraction, obtained from the 6% agarose column and constituting the Lp(a) lipoprotein standard, was diluted with RID buffer containing 3% (w/v) bovine serum albumin and kept at 4 C. Under these conditions the Lp(a) standard remained stable for at least two weeks, as judged by the lack of

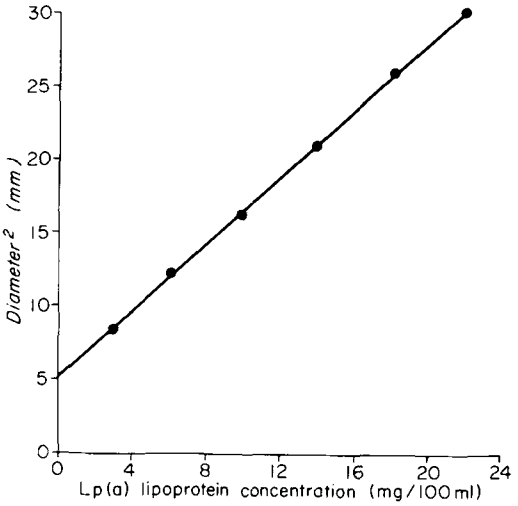


FIG. 3. A typical standard line showing the relationship between the square of the diameter of the immunoprecipitate and the Lp(a) lipoprotein concentration in mg/100 ml. Each point represents the mean of duplicate standards.

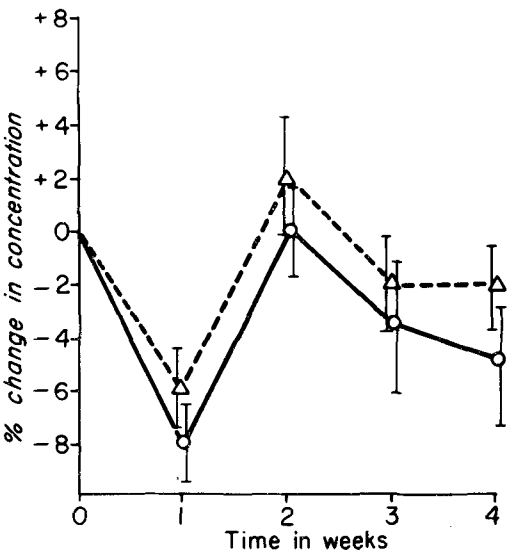


FIG. 4. Mean per cent change in concentration of Lp(a) lipoprotein at weekly intervals for samples stored unfrozen △ or frozen ○. Each point represents the average per cent change of six different samples. ⊢⊣ = standard error of the mean for each point.

change in the ring diameter of the immunoprecipitates. The Lp(a) lipoprotein standard ranged in concentration from 3-25 mg/100 ml.

## RESULTS

### Characterization of Lp(a) Lipoprotein Standards

The Lp(a) lipoprotein was isolated by separation of the *d* 1.060-1.090 g/ml lipoprotein fraction on Bio-Gel A-5m (6% agarose). The 1.060-1.090 g/ml lipoprotein fraction contained principally pre-beta and alpha$_1$ lipoproteins as shown in Figure 1C. This fraction was chosen, because it contains a small quantity of low density lipoproteins (see Figs. 1 and 5, ref [2]) and 77±8% of the total plasma Lp(a) lipoprotein, as determined by radial immunodiffusion Lp(a) quantification of five 1.060-1..090 g/ml lipoprotein preparations and their respective whole plasma. As indicated in Figure 2, the first major fraction obtained by agarose gel chromatography had an elution volume of ca. 227 ml and constituted the Lp(a) lipoprotein. It gave a single band with pre-beta mobility on agarose gel electrophoresis, as shown in Figure 1B. It also gave a single, slowly migrating, Sudan-positive band upon polyacrylamide gel electrophoresis (see Fig. 6, ref [2]) and reacted with anti-Lp(a) and anti-LDL sera, as shown previously (2). This Lp(a) lipoprotein fraction did not react with antihuman serum albumin or with antibodies against high density lipoprotein polypeptides R-Gln I (A-I) or R-Gln II (A-II).

The results of chemical analysis of Lp(a) are shown in Table I. Assuming that the sum of cholesterol, phospholipid, and glyceride represents total lipids and the sum of hexose, hexoseamine, and sialic acid represents total carbohydrate, this lipoprotein contained ca. 27% protein, 65% lipid, and 8% carbohydrate. The neutral lipids comprised 70% of the total lipids and the phospholipids 30%. Ca. 52% carbohydrate was hexose, the remainder consisting of hexoseamine and sialic acid (3).

### Specificity of Antisera

Immunization of a rabbit with Lp(a) lipoprotein produced an antiserum which, when absorbed with low density lipoprotein, reacted specifically with the Lp(a) lipoprotein. This antiserum, anti-Lp(a) serum, and anti-a-1, previously described (2), all gave identical precipitation reactions when tested against a panel of 50 human plasmas: 39 of these plasmas gave visible precipitates when tested against each of these three antisera. Each of these antisera gave a single precipitation band when tested against whole human plasma. Furthermore, these antisera did not react against a number of antigens when tested in double gel diffusion over a concentration range of 0.1-15 mg/ml. These antigens included high density lipoprotein-3 ($HDL_3$, *d* 1.125-1.21 g/ml); delipidated HDL and its constituent polypeptides R-Gln-I or R-Gln-II, isolated as described (11); very low density lipoprotein (VLDL) ($d<1.006$ g/ml); delipidated VLDL and its constituent polypep-

tides, isolated as described (23); low density lipoproteins of $d$ 1.030-1.040 g/ml; human serum albumin; and the plasma protein fraction of $d$>1.21 g/ml.

#### Assay Standard

The Lp(a) lipoprotein standards were calibrated by performing a Lowry protein. They were converted to HSA protein equivalents by multiplying by 0.7, then converted to total lipoprotein by multiplying by 3.7 (Lp(a) lipoprotein is assumed to contain 27% protein). The relationship between the square of the diameter of the precipitate ring and the amount of Lp(a) antigen was linear over the concentration range of 3-22 mg/100 ml, as shown in Figure 3. The diameter of the precipitates ranged from a minimum of 2.9 mm for the 3 mg/100 ml standard to a maximum of ca. 5.5 mm for the 22 mg/100 ml standard. The standards remained stable for at least two weeks while stored at 4 C as indicated by the lack of change in the ring diameter. Generally, most standards could be stored for four weeks or longer before a change of greater than 10% in the slope of the standard line or the diameter of precipitate was detected.

#### Stability of the Lp(a) Lipoprotein

An experiment was designed to test the effect of storage of both frozen and unfrozen plasma on Lp(a) concentration by immunoassay. Five 1 ml aliquots were taken from each of six different fresh plasmas. Four of the five aliquots from each plasma were frozen and stored at -20 C whereas the remaining aliquot was kept at 4 C. All samples contained .05% sodium azide. The Lp(a) lipoprotein concentration was determined on each of the fresh plasmas. At weekly intervals one of the frozen aliquots was thawed and the Lp(a) concentration determined on the thawed sample and on the original unfrozen sample. A comparison of samples stored at -20 C vs. those stored at 4 C is shown in Figure 4. The Lp(a) concentration for samples stored under either condition did not change significantly (less than 10%) over the 4 week period. Those samples stored at -20 C were consistently slightly lower on the average than those samples stored at 4 C. The test sample concnetrations ranged from 5-25 mg%. No attempt was made to assess the effects of storage as a function of initial concentration.

#### Assay Precision

An analysis of within-assay precision was performed, i.e. precision within the same radial immunodiffusion plate using the standards in duplicate. Twelve samples, whose concentra-

TABLE I

Chemical Composition of the Lp(a) Lipoprotein

| | Protein | Free Cholesterol plus cholesteryl ester | Phospholipid[a] | Triglyceride[a] | Hexose[b] | Hexoseamine[c] | Sialic acid[c] |
|---|---|---|---|---|---|---|---|
| μg | 1000 | 1542 ± 87 | 709 ± 32 | 138 ± 30 | 161 ± 12 | 84 | 66 |
| 1000 μg | 27.0 | 41.7 | 19.2 | 3.7 | 4.4 | 2.3 | 1.8 |
| Percent | | | | | | | |

[a]Four Lp(a) preparations were analyzed with each preparation extracted in duplicate and each extract analyzed in triplicate. Free cholesterol = 0.285 x total cholesterol; cholesteryl ester = 0.715 x 1.677 x total cholesterol.

[b]Two Lp(a) preparations were analyzed with each preparation analyzed in duplicate.

[c]See ref. 3.

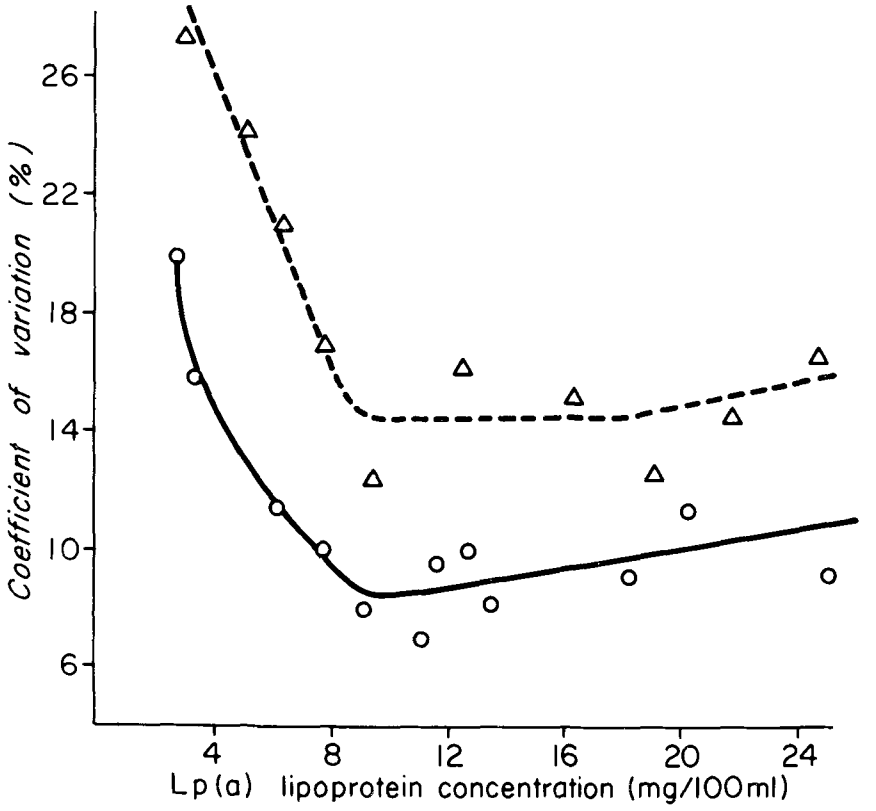


FIG. 5. Coefficient of variation as a function of Lp(a) lipoprotein concentration. o——o = within-plate variations, △----△ = between-assay variations.

tions of Lp(a) were pre-estimated and therefore known to span the entire standard range of 3-22 mg/100 ml, were analyzed on separate plates. Each sample was added to 12 wells randomly chosen within a plate. The standard deviation (S.D.) and the coefficient of variation (SD/mean x 100%) were calculated for each sample. Similarly, an analysis of between-assay reproducibility was performed, i.e. precision computed from the mean of six separate individual assays on 10 different samples performed at 2-4 week intervals utilizing different sets of standards. Each individual sample on a given day was added once to two different plates. As usual, each of the two plates contained the same set of standards in duplicate. As shown in Figure 5, the within-plate and between-assay variations were concentration dependent. The within-plate variation rose sharply at Lp(a) concentrations below 8 mg/100 ml to a maximum of 20% for sample concentrations of ca. 3 mg/100 ml. Similarly, the between-assay coefficient of variation rose steeply at Lp(a) concentrations below 8 mg/100 ml to a maximum of 27% for concentrations of ca. 3 mg/100 ml. The within-plate variation for samples between 8-25 mg/100 ml was nearly linear with an average variation of 9%, whereas the between-assay variation for this concentration range was ca. 15%.

#### Sensitivity of Assay

The double gel diffusion method could detect purified Lp(a) lipoprotein at a minimum concentration of 1.5 mg/100 ml (15 μg/ml). Of 340 unrelated fasting subjects tested, 275 or 81% had levels of the Lp(a) lipoprotein exceeding this lower limit. In the single radial diffusion method, the relationship between the square of the diameter of the precipitate and the amount of antigen was linear over the concentration range of 3-22 mg/100 ml, as shown in Figure 3. The quantitative immunodiffusion method, therefore, approaches the sensitivity of the qualitative gel diffusion method. As has been shown, however, the precision of the assay decreases sharply when analyzing samples with concentrations below 8 mg/100 ml. Furthermore, the immunoprecipitation rings with diameters of 2.9 mm or less, representing concentrations less than 3 mg/100 ml, were usually faint and difficult to read with

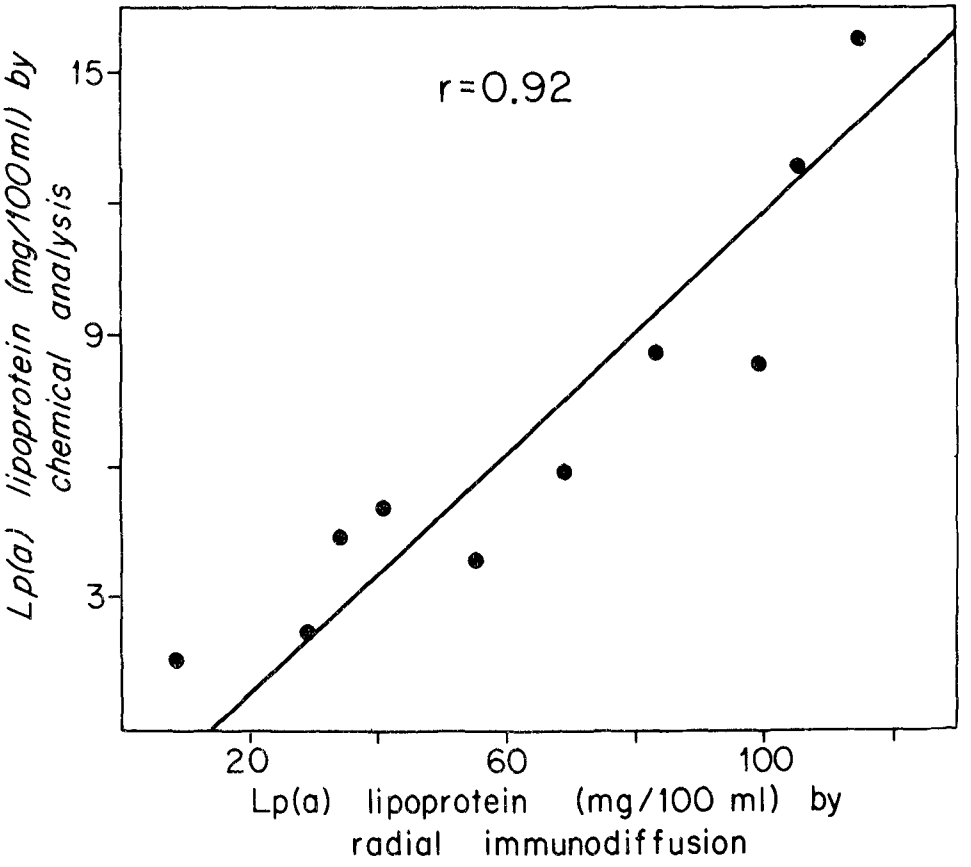


FIG. 6. Lp(a) lipoprotein levels: relationship of chemical analysis of first peak of 1.060-1.090 g/ml lipoprotein fraction from gel filtration column to the Lp(a) immunoassay of whole plasma.

a micrometer lens. Those samples which produced doubtful precipitin rings, but which were weakly positive by gel diffusion, were considered to have an Lp(a) concentration of 1.5 mg/100 ml. On the other hand, samples with concentrations of greater than 25 mg/100 ml and which gave strong positive precipitation reactions in double diffusion frequently give either faint or negative reactions upon radial immunodiffusion. These samples, therefore, were diluted until the plasma had a concentration within the standard range. When these plasmas with high concentrations were diluted serially, i.e. 1:2, 1:4, 1:8, etc., then the square of the immunoprecipitin diameter, plotted as a function of concentration, had the same slope as that produced by the purified standards.

Sixty-five individuals or 19% had undetectable Lp(a) lipoprotein concentrations in whole plasma. To determine if those plasmas lacked Lp(a) lipoprotein or merely contained levels below the threshold of detection by the gel diffusion method employed, the Lp(a) negative plasmas were concentrated ca. four-fold with Aquacide II and retested for Lp(a) lipoprotein after this concentration procedure. Thirty-nine of 65 negative plasmas had detectable Lp(a) lipoprotein after this concentration procedure. Thus, as many as 92% of the total population sampled were shown to have detectable levels of this lipoprotein.

#### Lp(a) Lipoprotein Levels: Relationship of Chemical Analysis to Immunoassay

Lp(a) lipoprotein was isolated from a series of 10 plasmas by agarose gel chromatography of the 1.060-1.090 g/ml lipoprotein fraction. Chemical analysis was performed on the first major peaks with an elution volume of ca. 227 ml by doing a Lowry protein analysis, correcting to HSA equivalents, and assuming 27% protein composition. The amount of Lp(a) lipoprotein obtained by this analysis was compared to the level of Lp(a) lipoprotein by immunoassay of whole plasmas. As indicated in Figure 6, Lp(a) lipoprotein by chemical analysis was highly correlated with the level of plasma Lp(a) lipoprotein by immunoassay (r = 0.92).

TABLE II

Age Distribution by Decades

| Age | Males | | Females | | Total | |
|---|---|---|---|---|---|---|
| | Number | Per cent | Number | Per cent | Number | Per cent |
| 20-29 | 32 | 20 | 83 | 47 | 115 | 34 |
| 30-39 | 43 | 26 | 46 | 26 | 89 | 26 |
| 40-49 | 57 | 35 | 32 | 18 | 89 | 26 |
| 50-59 | 30 | 18 | 14 | 8 | 44 | 13 |
| ≥60 | 2 | 1 | 1 | 1 | 3 | 1 |
| Total | 164 | 48 | 176 | 52 | 340 | 100 |

The yield of Lp(a) lipoprotein from whole plasma utilizing the above isolation procedure was 11.1 ± 3.4%.

**Lp(a) Lipoprotein Concentrations: Relationship with Age, Sex, and Cholesterol and Glyceride Concentrations**

Distribution of the population by age and sex is shown in Table II. The population contained ca. equal number of males and females with females having the younger age distribution. The distribution of Lp(a) concentrations for the 340 free-living, unrelated adult fasting subjects is shown in Figure 7. This distribution was highly skewed with a mean of 14.0 mg/100 ml and a median of 8.0 mg/100 ml. Males and females had similar distributions (Fig.8). The median test showed no sex difference in either mean or median Lp(a) levels, males having a mean of 14.1 mg/100 ml and females a mean of 13.9 mg/100 ml with a maximum value for both sexes of 76 mg/100 ml.

Figure 9 indicates that at the 5% level there was no statistically significant correlation of Lp(a) with either cholesterol and/or triglyceride concentrations. Analysis of the data using Spearman's nonparametric correlation in the significance test corroborated this conclusion.

As indicated in Figure 10, Lp(a) levels were found by Pearson's coefficient to be independent of age ($r = 0.052$; $0.5 < p < 0.6$). This conclusion was corroborated by Spearman's nonparametric coefficient. On the other hand, both cholesterol and triglyceride increased with age (Table III).

## DISCUSSION

This article describes the quantification of the Lp(a) lipoprotein by a single radial immunodiffusion assay. The Lp(a) lipoprotein, called LDL-a-1 in a previous publication (2) and elsewhere referred to as the "sinking pre-beta" lipoprotein (4), shares the major antigenic determinants of LDL (2) and is, therefore, assumed to share the major LDL protein. In the described technique, immunization of rabbits with purified human Lp(a) lipoprotein produces antisera which reacts with both LDL and Lp(a) lipoproteins. All precipitating antibodies reacting with LDL subsequently are removed by absorption, leaving antibodies which react with the Lp(a) lipoprotein but not with any other known plasma lipoprotein or lipoprotein polypeptide from VLDL, LDL, or HDL. The Lp(a) lipoprotein, found in the density range of 1.050-1.090 g/ml (and isolated from the *d* 1.060-1.090 g/ml range in this study), therefore, differs from the other low and high density lipoproteins present in this hydrated density class by the nature of the antigenic determinant(s) unique to this lipoprotein. Whether this determinant(s) is due to a unique polypeptide in the Lp(a) molecule or, altenatively, the carbohydrate moiety has not been ascertained; nor has the possibility that the lipid moiety

TABLE III

Correlation Coefficients for the Normal Population:Age vs. Plasma Lp(a) Lipoprotein and Lipid Levels

| Statistical test | Age vs. Lp(a) | Age vs. Cholesterol | Age vs. Glyceride |
|---|---|---|---|
| Pearson's correlation coefficient | 0.052 | 0.444[a] | 0.162[a] |
| Spearman's rank coefficient | 0.074 | 0.441[a] | 0.273[a] |

[a]Significant at the 0.002 level.

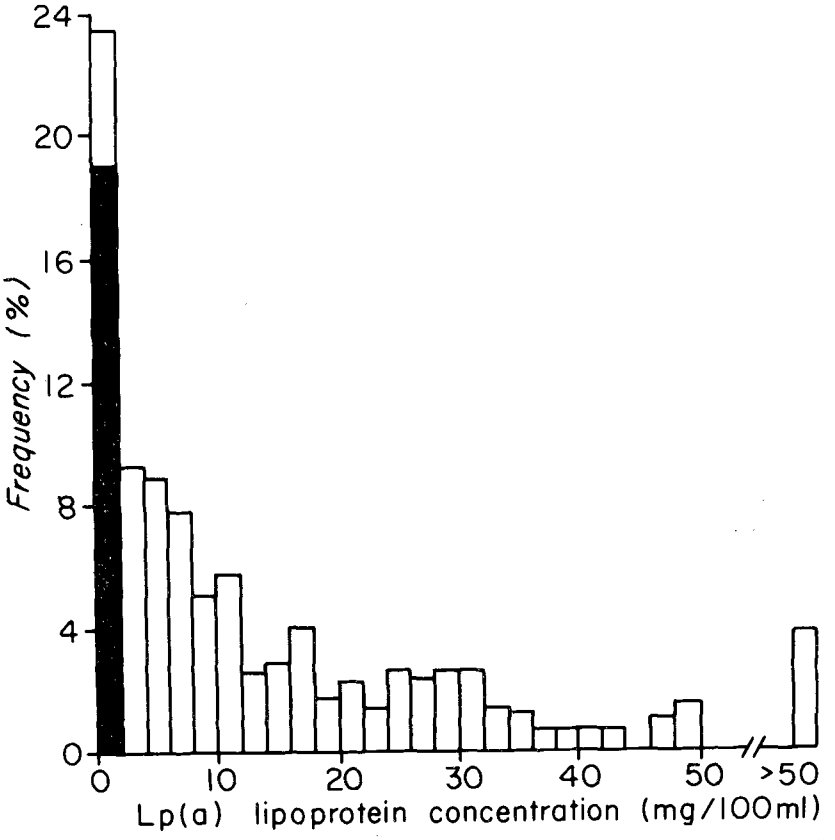


FIG. 7. Frequency distributions of plasma Lp(a) lipoprotein concentrations in 340 fasting adult subjects. ▬ = percentage of subjects with undetectable Lp(a) lipoprotein concentration in whole plasma.

could contribute to the formation or stability of this antigen been excluded.

The Lp(a) lipoprotein differs from low and high density lipoprotein, not only in immunological properties, but also in numerous chemical and physical-chemical properties. One of the distinguishing chemical characteristics of the Lp(a) lipoprotein is its high carbohydrate content; the hexoses and heoseamines are ca. three times and the sialic acid content six times as high in Lp(a) lipoprotein as in LDL. The lipid/protein ratio of Lp(a) was shown to be 2.4, considerably lesser than the value of 3.5 reported for LDL (24). On the other hand, the Lp(a) lipoprotein lipid composition was similar to that reported for LDL of *d* 1.010-1.050 g/ml (24). Other characteristics which distinguish the Lp(a) lipoprotein from LDL are its mol wt, ca. 5.4 x $10^6$, compared to 2.3 x $10^6$ for LDL; sedimentation coefficient at *d* 1.002 of 13.4 compared to 8.4 for LDL; and pre-beta mobility on agarose, compared to beta mobility for LDL (2).

The immunochemical quantification of lipo-

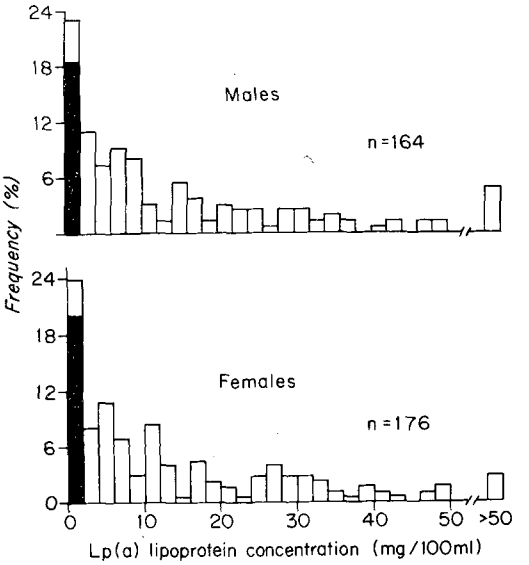


FIG. 8. Frequency distributions of plasma Lp(a) lipoprotein concentrations of male and female adult subjects.

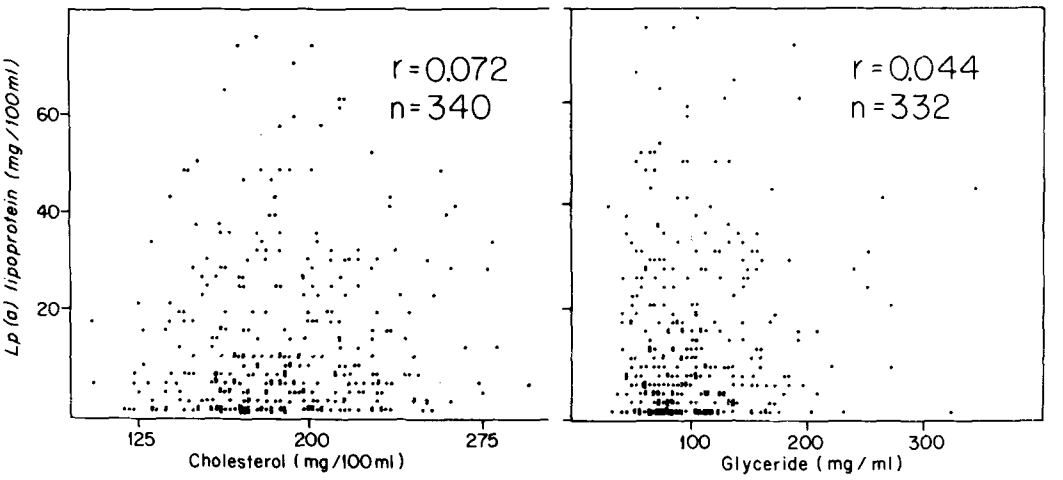


FIG. 9. The relationship between plasma Lp(a) lipoprotein and cholesterol on the left and plasma Lp(a) lipoprotein and glyceride on the right in a group of adult employee volunteers.

proteins has not been employed extensively, due to the lack of specificity and reproducibility in previous assays. Difficulties in producing monospecific antisera, failure to define precisely the specificity of each antiserum, the sharing of polypeptides among lipoproteins of different hydrated density classes, the masking of antigenic determinants by lipids, the instability of lipoprotein standards, and the lack of precision in the assay methods have all contributed to the lack of general acceptance and use of quantitative immunochemical methods among workers in the lipoprotein field. In the present study, the unique antigenic determinant(s) of the Lp(a) lipoprotein was exploited to develop a specific, reproducible, and sensitive method for the immunochemical quantification of the Lp(a) lipoprotein in human plasma.

The quantification of the Lp(a) lipoprotein by radial immunodiffusion was reasonably precise with a 15% between-assay variation for the concentration range of 8-25 mg/100 ml and somewhat less precise for the concentrations under 8 mg/100 ml.

Plasma samples with concentrations above 25 mg/100 ml were diluted and then assayed at 1:100 antiserum dilution. Alternatively the plasma could have been assayed undiluted with the antiserum at a lower dilution. On the other hand, for samples with low Lp(a) concentrations a higher dilution of antiserum would have not improved the sensitivity or the precision, because at antibody dilutions greater than 1:100 the precipitin rings were too faint to be resolved. It has been observed, however, that repeated filling of the wells with the test and standard samples increases sensitivity and precision for the samples with low Lp(a) concentrations.

The population of 340 free-living adult volunteers, representing 92% of the Pacific Northwest Bell Company employees in Renton, Wa., were predominantly white (327 of 340), middle-class residents of the suburban Seattle area. A fairly broad spectrum of socioeconomic and occupational groups was represented in the population. Additional epidemiological characteristics of this population will be reported subsequently in the description of the Northwest Lipid Research Clinic Pacific Northwest Bell Prevalence Survey. The Lp(a) lipoprotein distribution in this population was skewed dramatically (mean, 14 mg/100 ml; median 8 mg/100 ml), yielding a curve similar to a negative expoential function. Normal levels of the Lp(a) lipoprotein have not, as yet, been published; and no precise criteria have been established for the upper limits of normal for this plasma fraction. For plasma cholesterol and glyceride, the upper 5th percentile can be considered abnormally high. A similar 95th percentile upper cut-off for the normal Lp(a) concentration would be 48 mg/100 ml. However, since Lp(a) levels have yet to be correlated with any disease condition, such an arbitrary definition of an abnormal Lp(a) level currently would have no clinical utility.

The Lp(a) lipoprotein was detected in unconcentrated plasma in ca. 81% of all individuals tested, whereas in previous studies the Lp(a) lipoprotein was detected in only 30-45% plasmas tested (6). The explanation for this large difference in Lp(a)-positive individuals most likely lies in the increased sensitivity of the gel diffusion method used in the present

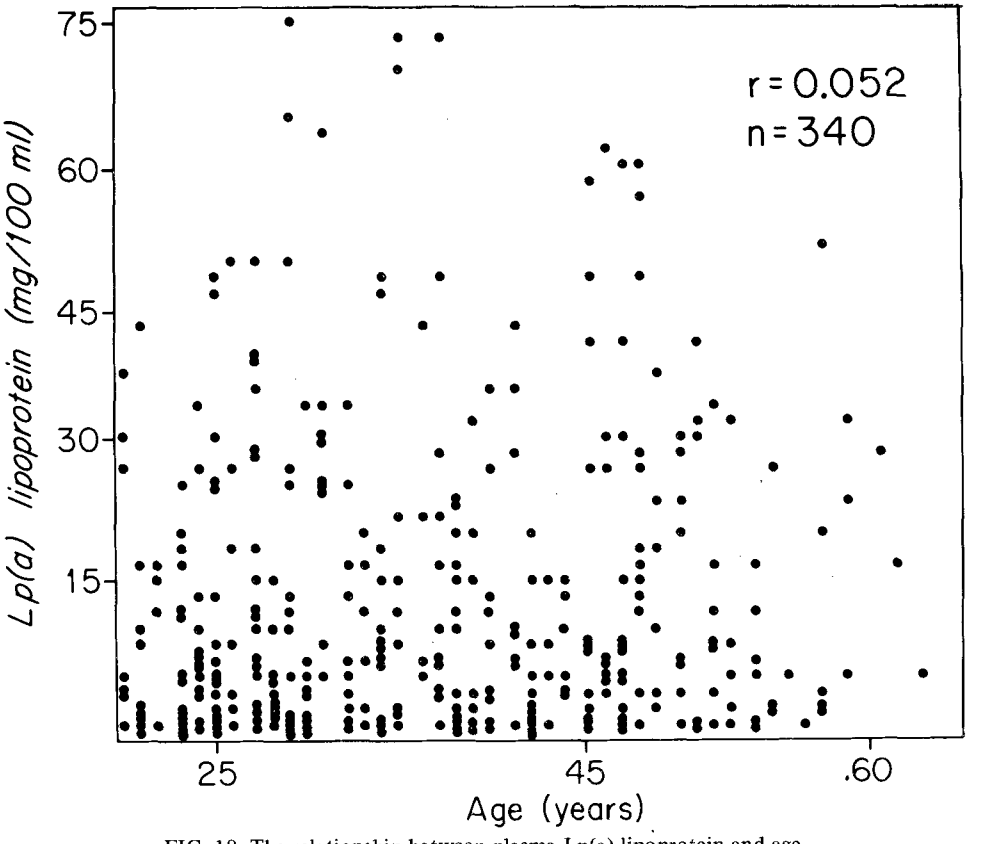


FIG. 10. The relationship between plasma Lp(a) lipoprotein and age.

studies rather than differences in the population studied. Even the presently described method is less than ideal, because of the common circumstance in which the concentration of Lp(a) in human plasma lies below its lower limit of sensitivity. Nevertheless, it appears to be the most sensitive quantitative procedure for the Lp(a) lipoprotein described thus far. Furthermore, when used in conjunction with gel diffusion studies, this assay procedure is ideal for screening of plasma samples for high Lp(a) levels.

The demonstration of detectable Lp(a) levels by only a fourfold concentration of plasma in an additional 11% of the population (92% therefore being Lp(a) positive) supports the concept that Lp(a) lipoprotein exists in all individuals. Previous investigations which suggested that Lp(a) may be a qualitative trait present in a minority of the population appear to have been based upon methods which were insufficiently sensitive to detect the lower levels measured by the present technique.

Even though 65% of the mass of the Lp(a) lipoprotein is lipid (of which 65% is cholesterol by wt), Lp(a) lipoprotein concentrations were not significantly correlated with total cholesterol levels in the population studied. This lack of correlation is not surprising, since the Lp(a) lipoprotein cholesterol generally represents only a small percentage (3%) of the total plasma cholesterol concentration, e.g. given a mean Lp(a) concentration of 14 mg% the Lp(a) lipoprotein would contribute only an average of 5.9 mg cholesterol/100 ml plasma.

Furthermore, the Lp(a) lipoprotein concentration was independent of age. This contrasted with the fact that total cholesterol significantly increased with age, and the fact that total cholesterol is highly correlated with LDL cholesterol (25) tends to suggest that the Lp(a) lipoprotein may be controlled metabolically independent of LDL despite the sharing of a common protein moiety.

ACKNOWLEDGMENTS

This work was supported in part by PHS contract NHLI-71-2157, PHS grant HL-15263-01, and American Heart Association grant 72-639 (supported in part by the Idaho Heart Association and the Northeastern Chapter of the Washington State Heart Association). Technical assistance was provided by F. Bigornia, L.

Suzuki, and W.R. Hazzard, Investigator of Howard Hughes Medical Institute. C. Ehnholm provided anti-Lp(a) serum.

## REFERENCES

1. Simons, K., C. Ehnholm, O. Renkonen and B. Bloth, Acta Pathol. Microbiol. Scand., Sec. B 78:459 (1970).
2. Albers, J.J., C. Chen and F. Aladjem, Biochemistry 11:57 (1972).
3. Ehnholm, C., H. Garoff, O. Renkonan and K. Simons, Ibid. 11:3229 (1972).
4. Rider, A.K., R.I. Levy, and D.S. Fredrickson, Circulation 42:III (1970).
5. Berg, K., Acta Pathol. Microbiol. Scand. 59:369 (1963).
6. Berg, K., Vox Sang. 11:419 (1966).
7. Harvie, N.R., and J.S. Schultz, Proc. Nat. Acad. Sci. 66:99 (1970).
8. Havel, R.J., H.A. Eder and J.H. Bragdon, J. Clin. Invest. 34:1345 (1955).
9. Narayan, K.A., H.L. Creinin and F.A. Kummerow, J. Lipid Res. 7:150 (1966).
10. Albers, J.J., and S. Dray, Biochem. Genet. 2:25 (1968).
11. Albers, J.J., and F. Aladjem, Biochemistry 10:3436 (1971).
12. Folch, J.M. Lees, and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
13. Searcy, R.L., L.M. Bergquist and R.C. Jung, J. Lipid Res. 1:349 (1960).
14. Carlson, L.A., Acta Med. Soc. Ups. 64:208 (1959).
15. Bartlett, G.R., J. Biol. Chem. 234:466 (1959).
16. Laboratory Methods Committee, "Manual of Laboratory Methods, Lipid Research Clinics Project," Vol. 2, 1972.
17. "AutoAnalyzer Manual," Technicon Instruments Corporation, Tarrytown, N.Y., 1970.
18. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193:265 (1951).
19. Chase, M.W., and C.A. Williams in "Methods of Immunology and Immunochemistry," Academic Press, New York, N.Y., 1968, p. 257-270.
20. Mokrasch, C., J. Biol. Chem. 208:55 (1954).
21. Albers, J.J., and S. Dray, J. Immunol. 103:155 (1969).
22. Mancini, G., A.O. Carbonara and J.F. Heremans, Int. J. Immunochem. 2:235 (1965).
23. Albers, J.J., and A.M. Scanu, Biochem. Biophys. Acta. 236:29 (1971).
24. Hatch, F.K., and R.S. Lees, Advan. Lipid Res. 6:1 (1968).
25. Schrott, H.G., J.L. Goldstein, W.R. Hazzard, M.M. McGoodwin and A.G. Motulsky, Ann. Intern. Med. 76:711 (1972).

[Revised manuscript
received October 26, 1973]