Determination of apolipoprotein B by kinetic (rate) nephelometry

Heinrich Wieland, Peter Cremer, and Dietrich Seidel

Zentrum für Innere Medizin der Universität Göttingen, Abteilung für Klinische Chemie, Robert-Koch-Strasse 40, 3400 Göttingen, West Germany

Abstract Determination of apoB by rate nephelometry is a simple, rapid and precise method, which is also suitable for a routine laboratory. It can be standardized with lipoprotein-B or LDL fractions, but only if these fractions are diluted with fresh whole serum. The standardization is also valid for VLDL if the samples are diluted with hydroxypolyethoxydodecane (trade name: Thesit) in a concentration of 0.33 g/liter. Using rate nephelometry, a strong correlation between the contents of cholesterol and apoB of VLDL as well as of LDL can be demonstrated. Similar high correlations are achieved if apoB is determined chemically in isolated lipoprotein fractions. The ratio of apoB to cholesterol is constant but not the same in both VLDL and LDL. There is also a strong correlation between the concentration of apoB in whole serum with the LDL cholesterol values in a normotriglyceridemic population. Therefore, the assay of apoB in whole serum by rate nephelometry is proposed as a screening method for dyslipoproteinemia (increased concentrations of atherogenic lipoproteins at normal or elevated levels of cholesterol in plasma).—**Wieland, H., P. Cremer, and D. Seidel.** Determination of apolipoprotein B by kinetic (rate) nephelometry. *J. Lipid Res.* 1982. **23:** 893–902.

Supplementary key words lipoproteins • LDL-cholesterol • screening

It is well known that apoB-containing lipoproteins are associated with the development of atherosclerotic vessel disease (1, 2). Furthermore, recent reports have established that the concentration of apoB in plasma is closely connected with coronary heart disease (CHD), as diagnosed by coronary angiography (3–6). However the interpretation of apoB concentrations is difficult, as data from prospective studies are not yet available and there is no agreement on the normal range of concentrations. A wide range of values (75–129 mg/dl) has been reported (5, 7) and this may be due to *a*) the investigation of different populations, namely normolipemic males or a random population; *b*) the employment of different methods for the quantification of apoB, for example radioimmunoassay (RIA) (8–15), radial immunodiffusion (RID) (5, 16, 17), electroimmunoassay (EIA) (11, 18–20), and laser nephelometry (7, 21); *c*) differences in the antisera used with regard to the antigen, animal species, and specificity of the antibody; and *d*) differences in the standardization of the methods, in terms of the standard used and the chromogenicity factor for apoB. Each of these factors also influences the ratio of the concentration of apoB in whole serum to the corresponding low density lipoprotein (LDL) cholesterol concentration, for which values ranging from 1.3 (13) to 2.0 (10) have been reported. Provided that this is a constant ratio, a good correlation between apoB and LDL cholesterol would be expected, as long as both parameters are determined accurately and reproducibly. However, although good correlations have been obtained (8, 14), reports of relatively poor correlations have also appeared (10, 13) and, indeed, most investigators report only intermediate correlations (9, 21–23).

The present investigation of the correlation of apoB in whole serum and in LDL with LDL cholesterol was undertaken in order to determine whether there is a constant relationship between the major protein and lipid components of the same macromolecule. Such a relationship would serve as a useful criterion for the evaluation of any method for the measurement of apoB. Thus, methods with a good correlation of apoB to LDL cholesterol would yield comparable results and thereby enable a normal range of apoB concentrations to be defined.

This study presents a method for the determination of apoB by kinetic or rate nephelometry, by means of a commercially available instrument. Major emphasis was placed on the precision of the method, so that it fulfilled the rigid criteria of quality control for both within and between assay precision.

MATERIALS AND METHODS

Ultracentrifugation

A Beckman L 5-75 model ultracentrifuge was operated at 10°C. For preparative purposes, fresh plasma from healthy blood donors was ultracentrifuged for 24 hr at 50,000 rpm in a type 60 TI rotor. The fractions were

Abbreviations: RIA, radioimmunoassay; EIA, electroimmunoassay; RID, radial immunodiffusion; VLDL, very low density lipoprotein; LDL, low density lipoprotein; PAGE, polyacrylamide gel electrophoresis; Lp-B, lipoprotein B; TMU, tetramethylurea.

Downloaded from www.jlr.org by guest, on June 19, 2012

recovered by a tube-slicing technique. For analytical purposes, serum samples from patients were processed as follows. A Beckman type 25 rotor (100 positions for tubes with a capacity of 1 ml) was used for the isolation of very low density lipoprotein (VLDL) and LDL fractions, by centrifuging for 48 hr at 25,000 rpm only at the peripheral positions of the rotor. In order to isolate VLDL, 800 μl of serum was layered with 200 μl of 0.15 M NaCl and LDL was isolated after flotation of VLDL and LDL_1. The floating fractions were recovered by aspiration with a 2-ml syringe, equipped with a U-shaped needle to facilitate aspiration of the fraction from below. The LDL fractions were isolated between the densities 1.03 and 1.06 g/ml. The densities were adjusted with NaBr using a digital pycnometer (Heraeus-Paar, Graz, Austria). The concentration of LDL cholesterol was determined in the fractions obtained after flotation of VLDL. Precipitation of LDL was carried out using a commercially available combination of sodium phosphotungstate and $MgCl_2$ (HDL Cholesterol, Boehringer-Mannheim, Mannheim, F.R.G.) according to the described method.

Determination of cholesterol

Cholesterol was determined enzymatically with a test kit (CHOD-PAP, Boehringer-Mannheim, Mannheim, F.R.G.) and an automatic dilutor (model 5232, Eppendorf, Hamburg, F.R.G.). The absorption was measured at 546 nm in a photometer type 6118 (Eppendorf, Hamburg, F.R.G.).

Determination of protein

Protein was determined according to Lowry et al. (24) using human albumin from Behringwerke (Marburg/Lahn, F.R.G.) as a standard. In samples subjected to preparative isoelectric focusing, the modifications of Bensadoun and Weinstein (25) were applied.

Polyacrylamide gel electrophoresis (PAGE)

This was performed by analytical discontinuous PAGE, essentially as described by Davis (26) with equipment from Desaga (Heidelberg, F.R.G.). Separating gels of 7% polyacrylamide were used in a Tris-glycine buffer system in the presence of 4 M urea for the stacking gel and 8 M urea for the separating gel. The gels were removed and stained in a 0.12% solution of Coomassie Blue R-250 supplied by Serva (Heidelberg, F.R.G.).

Preparative isoelectric focusing

This was done in equipment from Pharmacia (Freiburg, F.R.G.) by mixing 20 ml of sample containing 5 g of protein per liter with 200 ml of 1% agarose (Agarose IEF, Pharmacia, Freiburg, F.R.G.), which had been previously solubilized by boiling in a water bath for 15 min and then cooling to 55°C. Then 2 ml of ampholytes pH 3–10 (Pharmalyte 3–10, Pharmacia, Freiburg, F.R.G.) was added to this mixture. After careful mixing, the solution was poured in the frame provided with the apparatus for flat bed focusing and allowed to congeal on a level surface. The mold was kept overnight at 4°C in a humid chamber. Small agarose strips (1 cm) were removed from either end of the plate and replaced by electrode strips soaked with the ampholytes. Isoelectric focusing was carried out for 15 volt-hr at constant 1000 W. Despite considerable electroendosmosis, the gel could be fractionated with a grid. Samples were recovered after grinding the agarose strips and removing the agarose by ultracentrifugation in a 50 TI rotor for 2 hr at 40,000 rpm.

Immunoelectrophoresis

This was performed according to the method of Scheidegger (27) using pre-cast agarose plates (Lipidophor, Immuno-Diagnostika, Heidelberg, F.R.G.).

Radial immunodiffusion (RID)

Radial immunodiffusion for quantification of apoB was performed with partigen plates (Nor-Partigen, Behringwerke, Marburg/Lahn, F.R.G.) according to the technique described by the supplier (incubation for 5 days at room temperature).

Lipoprotein electrophoresis

Qualitative and quantitative electrophoresis of lipoproteins was carried out using the Lipidophor system (from Immuno-Diagnostika, Heidelberg, F.R.G.) by the procedure suggested by the supplier. The day to day precision of the quantitative electrophoresis was monitored by means of the Lipidophor control serum.

Determination of apoB in VLDL and LDL

The soluble apoproteins of VLDL were extracted with isopropanol according to Holmquist and Carlson (28). The difference between total and isopropanol-soluble VLDL protein was considered to represent the amount of apoB present in VLDL. Before protein determination the LDL fractions (d 1.03–1.06 g/ml) were analyzed by PAGE after extraction with tetramethylurea (TMU) (29) and by immunoelectrophoresis against anti-human serum obtained from Behringwerke (Marburg/Lahn, F.R.G.). If no bands could be detected by PAGE after application of 0.3 mg of LDL protein per gel, and if only one precipitin arc appeared in immunoelectrophoresis after application of 0.02 mg LDL protein, the sample was considered to contain only apoB as protein and was employed in the study.

Preparation of the antigen

Lipoprotein B (Lp-B) was used as an antigen. It was isolated by preparative isoelectric focusing of the d $<$ 1.21 g/ml fraction of fresh plasma, obtained after

Downloaded from www.jlr.org by guest, on June 19, 2012

dialysis against phosphate-buffered 0.075 M NaCl, pH 7.3. The lipoproteins present at pH 4.86 yielded a single band on electrophoresis and fulfilled the criteria for Lp-B as outlined above. Ampholytes were removed from the sample by ultracentrifugation at density 1.06 g/ml for 24 hr.

Preparation of the antiserum

The antiserum was produced in sheep. After dialysis against 0.15 M NaCl, Lp-B in an amount equivalent to 5 mg of apoB was thoroughly mixed with complete Freund's adjuvant, and injected intradermally into two animals (axillae and inguinal folds). After 4 weeks a booster injection of Lp-B without Freund's adjuvant, in an amount equivalent to 0.5 mg of apoB, was administered in the same areas. The animals reacted with pronounced enlargement of the corresponding lymph nodes and were slaughtered 10 days following the booster injection. Blood was collected and the serum was recovered by low speed centrifugation. The specificity of the antiserum was tested by immunoelectrophoresis against human whole serum. It gave a single band, extending from the β to the pre-β position.

Determination of apoB by kinetic or rate nephelometry

The kinetics of the antigen–antibody reaction were followed using a nephelometer with a two-channel recorder (Beckman Immunochemistry System, Beckman Instruments, Munich, F.R.G.). One channel of the recorder indicates the scatter signal produced by the antigen–antibody reaction. The other channel shows the calculated first derivative of the scatter signal, which represents the velocity of the turbidity development. At constant concentration of antibody, the velocity of the antigen–antibody reaction is proportional to the concentration of the antigen. At maximum reaction velocity, the first derivative, or rate of the reaction, reaches its peak. Measurements made at this point are expressed as rate units per volume of sample. The reaction takes place under constant stirring in a cuvette containing polyethyleneglycol (PEG), molecular weight 6,000, 40 g/l (Serva, Heidelberg, F.R.G.) in 0.6 ml of 0.02 M phosphate buffer, pH 7.1. To this reaction mixture, 42 μl of the "sample dilution" and 42 μl of the "antiserum dilution" are added with a fixed volume Sherwood pipette provided by Beckman. As shown later, the determination of apoB in VLDL requires a detergent. All sample dilutions were therefore made with 0.02 M phosphate buffer, pH 7.1, containing 0.33 g/liter hydroxypolyethoxydodecane (30) supplied under the trade name of Thesit by Desitinwerke, Karl Klinke, Hamburg, F.R.G. This concentration was achieved by making a 15-fold dilution of a stock solution (5 g/l) of the detergent. The serum dilution of 1:36 was obtained by using twice the fixed ratio (1:6) diluter. The dilution of the

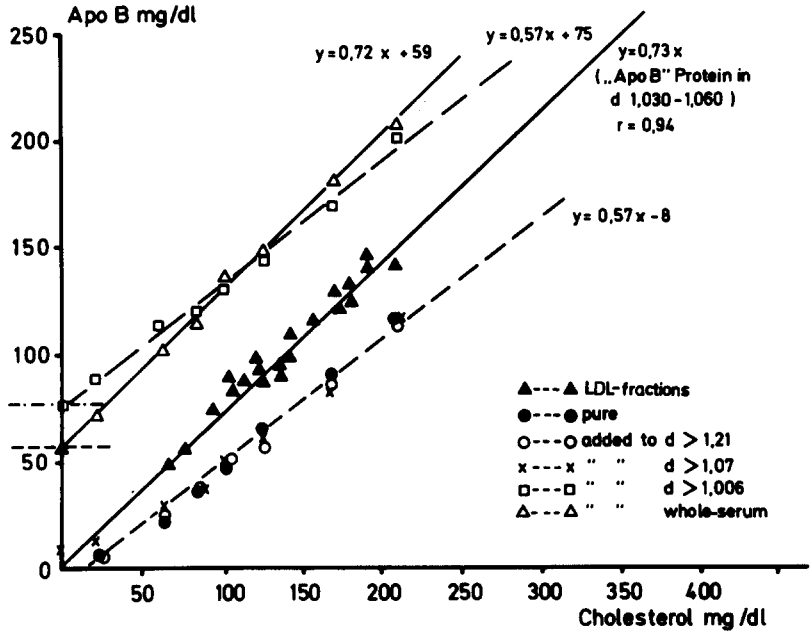

Fig. 1. Effect of the medium used for dilution on the dilution curves of Lp-B. The slope of the regression line of the protein vs. cholesterol content (y = 0.73x) of 21 different LDL fractions (d 1.03–1.06 g/ml) indicates how much apoB should be found by rate nephelometry in a certain amount of Lp-B, expressed in terms of cholesterol. If the Lp-B standard is diluted with 0.15 M NaCl, a lower slope (y = 0.57x) results. This also occurs if serum fractions obtained by ultracentrifugation are used for dilution. The slope of the regression line of protein vs. cholesterol in isolated LDL can only be reproduced if the Lp-B standard is diluted with whole serum.

Downloaded from www.jlr.org by guest, on June 19, 2012

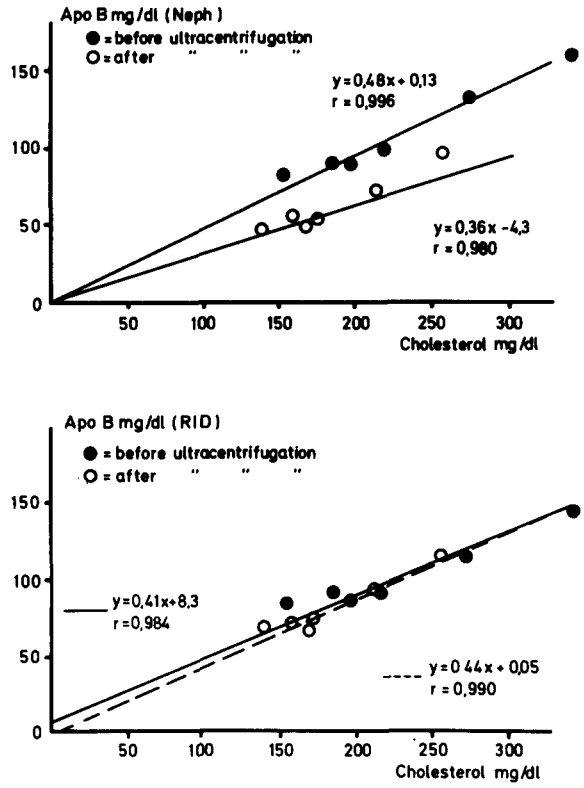

Fig. 2. Effect of ultracentrifugation on the velocity of the apoB anti-apoB reaction. ApoB and cholesterol are determined in whole serum before and after ultracentrifugation. The separation is abolished by shaking the tubes after ultracentrifugation. The ratio of apoB to cholesterol is considerably lower after ultracentrifugation when apoB is determined by rate nephelometry, but not when RID is used.

antiserum is 1:6 with the phosphate-PEG buffer also present in the reaction cell. Since PEG causes a slowly developing turbidity by precipitating some proteins (which can be removed by centrifugation and subsequent filtration through Whatman No. 1 filter paper), the diluted antiserum is allowed to stand for 24 hr before use or freezing. It is stable for at least 3 months at −20°C, and when deep-frozen at −90°C no loss of reactivity has been observed to date, up to 2 years. Nonimmune serum gave no reaction under the conditions described.

For standardization, isolated lipoprotein fractions were added to fresh whole serum. Both serum and lipoprotein fractions were diluted with the Thesit–phosphate buffer in half the usual ratio (1:18) and then mixed 1:1 (v/v). The instrument is set on manual mode and AB card M 33 is inserted. The cuvette, filled with phosphate–PEG buffer, is placed in the light path and the diluted sample is pipetted into the cell. A small peak appears, but baseline is reached after 10 sec. As soon as the diluted antiserum has been pipetted, the "option" button is pressed and the rate units appear on the display, usually reaching their peak within 1 min. Determinations were always made on duplicate samples. The within and between assay precision was determined using the Lipidophor control serum. The same serum in three different dilutions was used daily as a secondary standard.

RESULTS

Standardization

The assay system was standardized with Lp-B. The measured rate unit was proportional to the amount of Lp-B, expressed in terms of its cholesterol content. The slope of the standard dilution curve (rate unit/cholesterol) varied according to the medium used for dilution of the standard (**Fig. 1**). It was steepest when the standard was added to fresh whole serum. The same slope is obtained when the protein content of different undialyzed LDL fractions (d 1.03–1.06 g/ml) is plotted against their cholesterol content. In order to assay sample and standard under identical conditions, all determinations of apoB by rate nephelometry, either in ultracentrifugally isolated lipoprotein fractions from serum or in the Lp-B standard from plasma, were performed in a medium of fresh whole serum.

The effect of ultracentrifugation was examined by subjecting 1-ml fractions of six different sera to routine ultracentrifugation for the flotation of VLDL without layering with NaCl. The tubes were subsequently shaken to thoroughly mix the separated fractions. Cholesterol and apoB were determined before and after ultracentrifugation. **Fig. 2** illustrates that the ratio of apoB to cholesterol decreased by 25% after ultracentrifugation, when apoB was determined by rate nephelometry, but

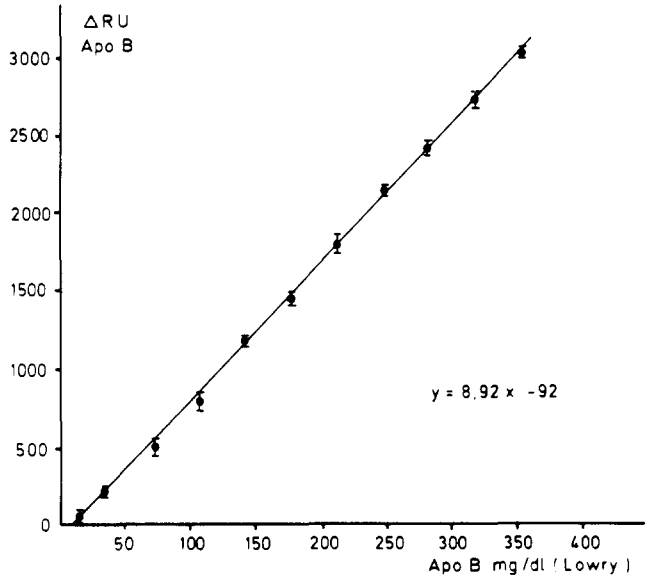

Fig. 3. Standard curve for Lp-B. Lp-B is added to fresh whole serum and determined by rate nephelometry. Each point is the mean of five determinations.

Downloaded from www.jlr.org by guest, on June 19, 2012

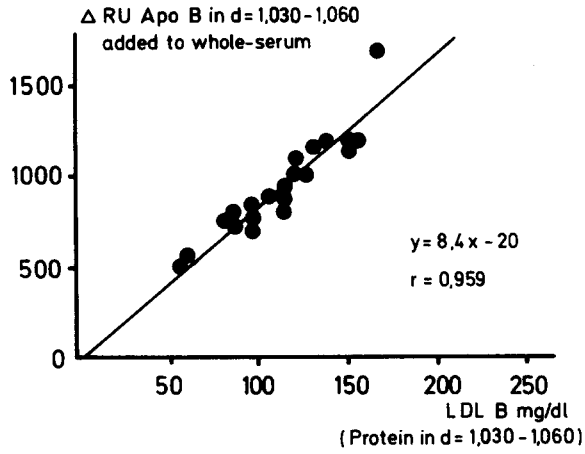

Fig. 4. Correlation of the increase in rate units in fresh whole serum brought about by addition of LDL fractions with the protein content of these fractions. The slope of this curve was used for standardization, since a good correlation was obtained with 21 different samples, all of which contained apoB as the almost exclusive protein component.

that there was no effect when RID was used to determine apoB.

The conversion of the changes in rate units (ΔRU) into mg apoB/dl is achieved by dividing ΔRU by 8.9 (**Fig. 3**). The method is linear for a concentration range of 0.043–0.600 mg of apoB in the assay system, which corresponds to a range of at least 25–345 mg apoB/dl plasma. The linearity of the assay even above 345 mg/dl indicates that most determinations of apoB are done under conditions considerably away from antigen excess. The chosen dilutions are optimal for the measurement of apoB in serum from normal subjects, as well as from patients with hyperlipoproteinemia. The sensitivity of the assay can be increased by using different dilutions and/or the AB card M 44.

The method was also standardized with isolated LDL fractions and the factor for conversion of rate units into apoB concentration was calculated to be 8.4 (**Fig. 4**). This compared favorably with the factor calculated for Lp-B. Thus it was decided to employ this factor for standardization throughout the study.

Influence of hypertriglyceridemia

In serum from patients with hypertriglyceridemia, which is free of chylomicrons, rate nephelometry yields higher results than RID (**Fig. 5**). It has been reported (21, 30) that laser nephelometry overestimates apoB, especially in hypertriglyceridemic samples, unless a detergent is included in the assay system. The test kits used for this procedure contain hydroxypolyethoxydodecane, and we also found an improvement in our results, as compared with RID, when this detergent was present in the assay system (**Fig. 6**). The reaction velocity is then reduced by approximately 50% and even more markedly in the case of hypertriglyceridemic samples. Since RID is considered to underestimate the content of apoB in

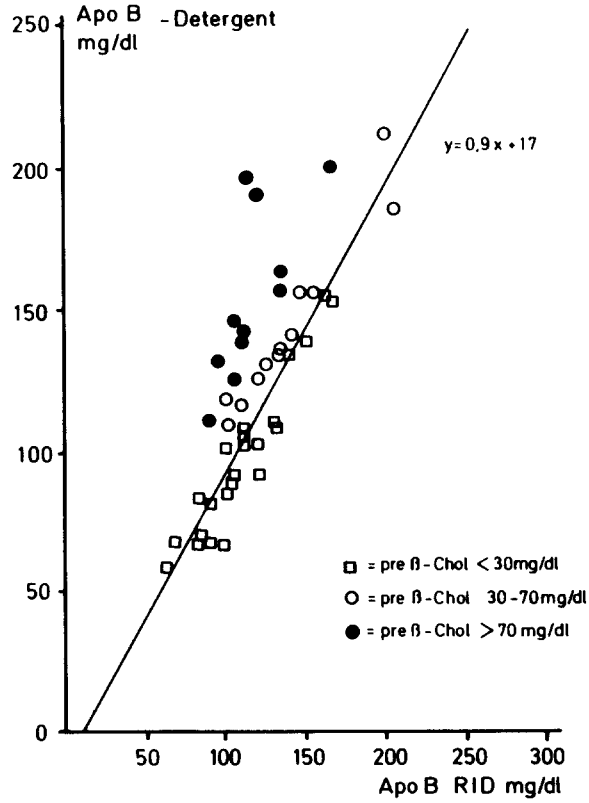

Fig. 5. Comparison of rate nephelometry with RID. ApoB was determined by rate nephelometry in the absence of detergent. This leads to an apparent overestimation of apoB in samples with abnormally high concentrations of pre-β-lipoproteins (triglycerides, 70–450 mg/dl).

VLDL (17), we compared the values obtained by rate nephelometry with those from quantitative electrophoresis and found a good correlation (**Fig. 7**).

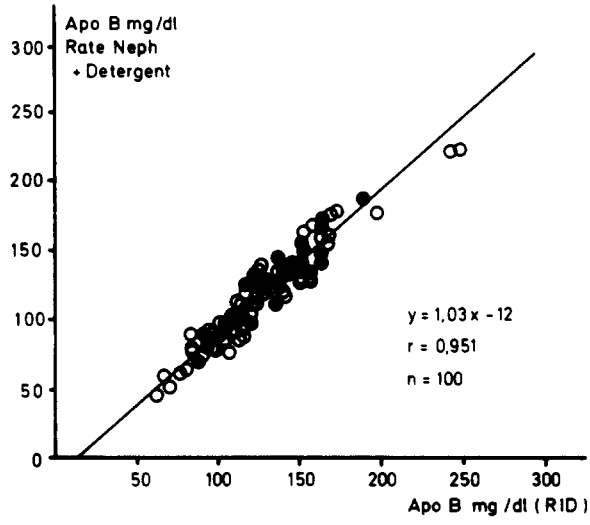

Fig. 6. Correlation of apoB concentrations determined by rate nephelometry and RID. For rate nephelometry, sample dilution was made with detergent (hydroxypolyethoxydodecane, Thesit). ApoB is not overestimated by rate nephelometry in hypertriglyceridemic samples (closed circles).

Downloaded from www.jlr.org by guest, on June 19, 2012

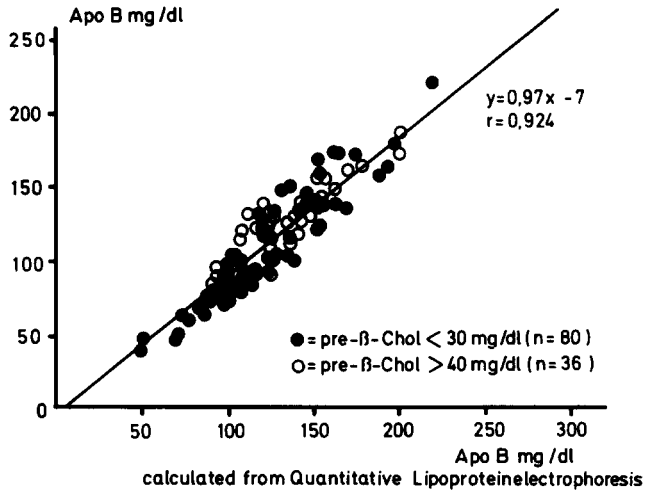

Fig. 7. Correlation of apoB concentrations determined by rate nephelometry with those calculated from quantitative lipoprotein electrophoresis. Rate nephelometry was performed after sample dilution with detergent. For calculation of apoB after electrophoresis, a ratio of apoB to cholesterol of 0.73 in β-lipoproteins (Fig. 1) and of 0.44 in pre-β-lipoproteins (Fig. 9) was predicted. Samples rich in pre-β-lipoproteins (open circles) are noted on both sides of the regression line. Therefore rate nephelometry does not appear to overestimate apoB in hypertriglyceridemic samples. On the other hand, detergent does not lead to underestimation of apoB. The good agreement with RID (Fig. 6) indicates that the latter also does not underestimate apoB in hypertriglyceridemic samples.

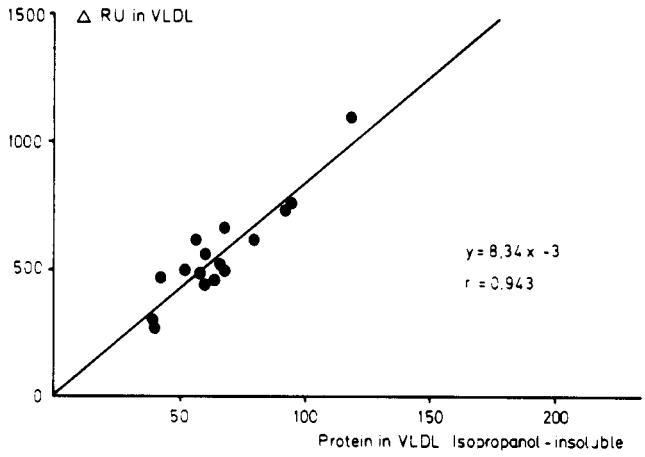

Fig. 8. Correlation of the increase in rate units produced by VLDL samples with their predicted content of apoB. Measurement of apoB by rate nephelometry was made in 16 different VLDL fractions added to fresh whole serum. The increase in rate units correlates well with the amount of protein, as determined after isopropanol extraction of the corresponding VLDL samples. The slope of the curve is very similar to that calculated in Fig. 4 (y = 8.4x), which was used for the standardization of apoB determination in whole serum. The similarity of the slopes for LDL and VLDL indicates that standardization with LDL fractions is also valid for VLDL, as long as apoB is determined by rate nephelometry and the sample is diluted with detergent.

The accuracy of the determination of the apoB content of VLDL by standardization of the method with LDL fractions was tested. The slope of the curve of ΔRU against isopropanol insoluble protein in VLDL fractions resulted in the calculation of a factor of 8.34 for the conversion of rate units into apoB concentration (**Fig. 8**). Since this factor is similar to that calculated from LDL fractions (8.4, see Fig. 4), it is concluded that apoB can be accurately determined in VLDL when the assay is standardized with LDL fractions.

Ratio of apoB to cholesterol in VLDL and LDL

Fig. 1 indicates that a constant relationship exists between protein and cholesterol in different LDL fractions. As shown in **Fig. 9** and **Fig. 10** this is also true for the isopropanol-insoluble protein and for apoB, as deter-

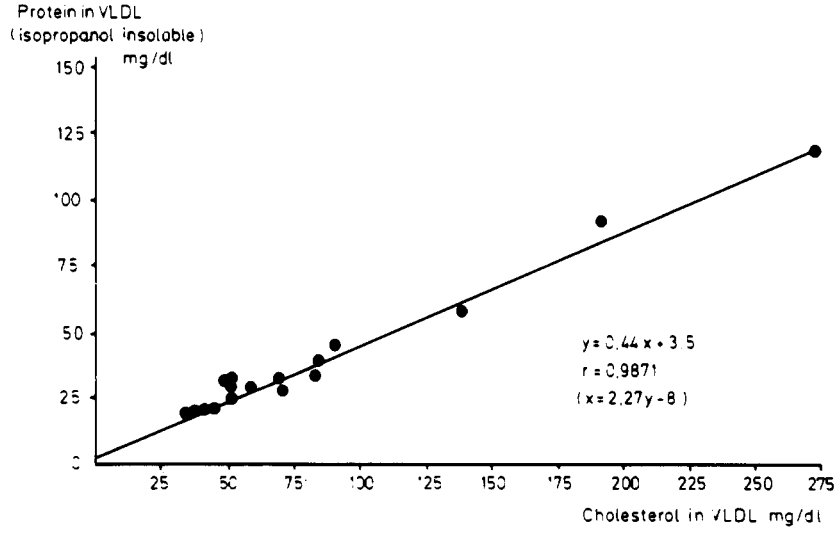

Fig. 9. Correlation of the content of isopropanol-insoluble protein in different VLDL fractions with their cholesterol content. The ratio of isopropanol insoluble protein to the cholesterol content of a VLDL fraction is 0.44 and very reproducible. This permits calculation of the apoB content of VLDL from the cholesterol content and also from the cholesterol content of pre-β-lipoproteins (Fig. 7).

Downloaded from www.jlr.org by guest, on June 19, 2012

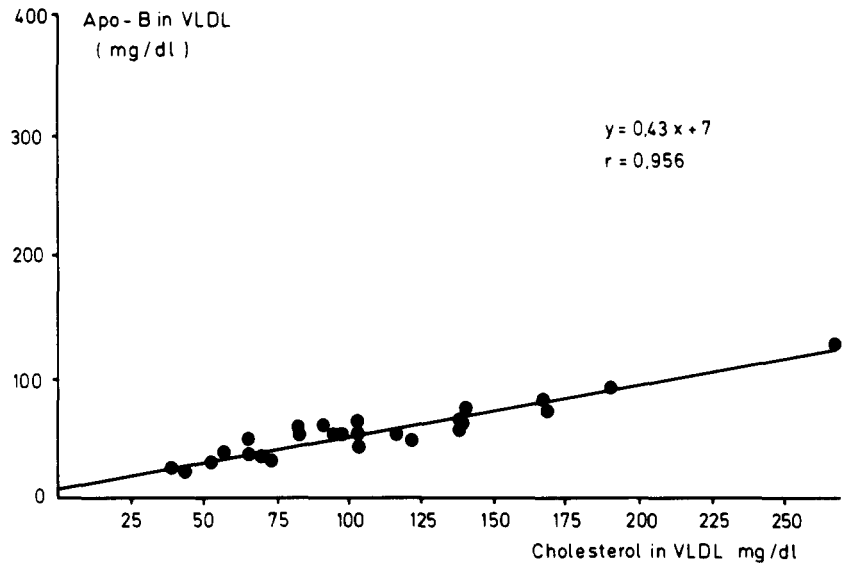

Fig. 10. Correlation of the apoB content of different VLDL samples from hypertriglyceridemic subjects with their cholesterol content. The apoB content was determined by rate nephelometry. The slope of the regression line is similar to that of Fig. 9. ApoB can therefore be determined accurately by rate nephelometry even in hypertriglyceridemic plasma. The good correlation of the two parameters strongly indicates a constant ratio of apoB to cholesterol in VLDL.

mined by rate nephelometry, and the cholesterol content of VLDL. The concentration of apoB in whole serum can be calculated by quantitative electrophoresis from the sum of (β-Lp cholesterol $\times$ 0.73) + (pre-β-Lp cholesterol $\times$ 0.44) according to Fig. 7. The relatively constant ratio of apoB to cholesterol in LDL explains the high correlation between the apoB concentration in the d 1.006 g/ml infranatant and the LDL cholesterol concentration in corresponding sera (**Fig. 11**). In a normotriglyceridemic population there is also a high correlation between the concentration of apoB in whole serum and LDL cholesterol (**Fig. 12**). Thus apoB, even when determined in whole serum, serves as a good estimate of the concentration of LDL cholesterol.

Precision

A coefficient of variance (CV) of 2.1% was calculated for the within-assay precision in a series of 20 determinations of apoB by rate nephelometry. A CV of 4.2% was calculated for the between-assay precision, as determined with a lyophilized control serum for a period of 200 days.

DISCUSSION

The major advantages of the described method are its rapidity, simplicity, and high precision. Since the antiserum can be diluted in advance, manipulations are confined to dilution of the sample, filling of the cuvette with buffer, and pipetting of the prepared antiserum and sample. The method can be standardized with a secondary standard, e.g., lyophilized human serum with intact remaining lipoproteins, and this can also be used for quality control. With the use of the ICS system, comparable results can be expected from different laboratories, especially if the same antiserum is used, for example anti-

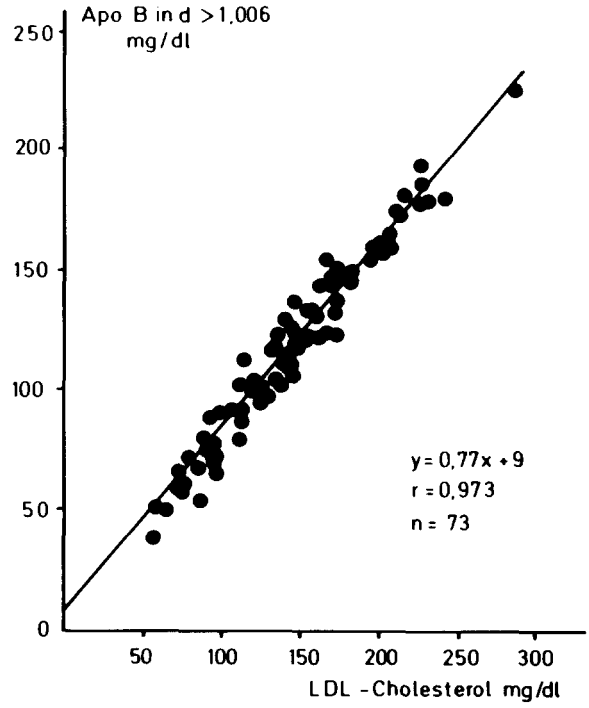

Fig. 11. Correlation of the apoB concentration in d 1.006 g/ml infranatants of 73 different sera with their cholesterol concentration. The good correlation suggests a fairly constant ratio of apoB to cholesterol also in LDL (16). The somewhat steeper slope as compared with that in Fig. 1 may be due to cholesterol-poor apoB-containing proteins (Lp-a) in that fraction.

Downloaded from www.jlr.org by guest, on June 19, 2012

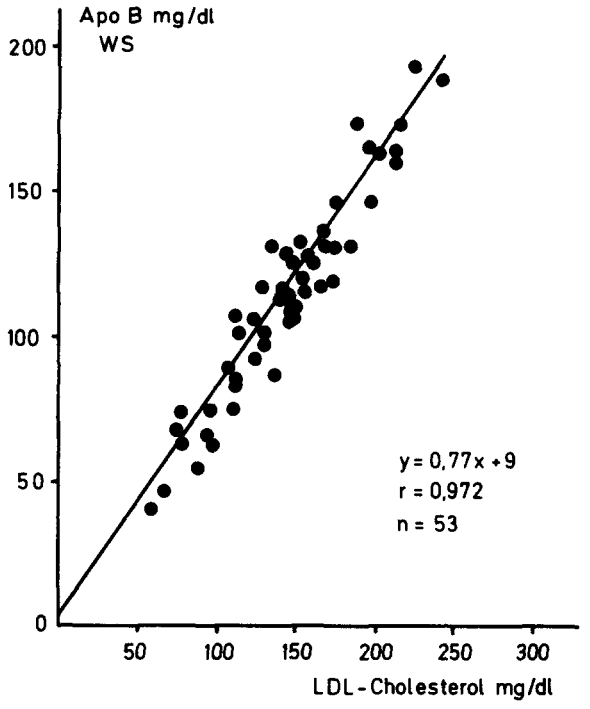

Fig. 12. Correlation of the apoB concentration in whole serum with LDL cholesterol in a normotriglyceridemic population. The good correlation observed between apoB and LDL cholesterol in VLDL free serum is not markedly diminished if VLDL apoB is incorporated, as long as the VLDL cholesterol concentration remains normal (below 30 mg/dl).

Lp-B serum from Behringwerke, which gave the same results as our own prepared anti-serum.

The immunological procedure of RID has also proved reliable for the determination of apoproteins. However the precision is considerably less than that of rate nephelometry, and results are not available for 5 days and this is not desirable for clinical purposes. Although electroimmunoassay (EIA) provides a means of investigating the antiserum, it seems more useful as a research tool than as a reliable routine method.

The usefulness of nephelometry may be criticized on the grounds that the immune complexes must be of constant size, so that the signals derived from light scattering are proportional to the concentration of antigen. Thus in the case of apoB, which is a component of lipoproteins of various sizes, this problem becomes evident in the different values obtained for the analysis of hypertriglyceridemic samples by rate nephelometry and RID. The light scattering increases when particles, which are able to scatter light by themselves, are incorporated in the immune complexes. It is likely that the action of the detergent is not entirely due to a decrease in the velocity of the antigen–antibody reaction, which is more noticeable if the antigen is a constituent of the triglyceride rich particles. It may also be due to disintegration of these particles, as the scatter of the whole antigen–antibody complex is reduced (30). The presence of detergent in the concentrations employed in this assay apparently leads to a correct recognition of apoB by the antibody, as shown by a comparison of both rate and laser nephelometry (30) with RID (Fig. 6). The RID procedure used in this study is assumed to give accurate results in hypertriglyceridemic samples, since it also compares well with enzyme immunoassay (30). Moreover the good agreement between rate nephelometry and RID, and between rate nephelometry and the apoB values calculated from quantitative electrophoresis of lipoproteins, provides evidence that RID does not underestimate apoB in serum rich in VLDL (Fig. 7), provided that the plates are developed for 5 days (30).

The usefulness of rate nephelometry for the accurate determination of the apoB content of VLDL and LDL was confirmed by determining the protein content of several lipoprotein fractions (Figs. 4, 8). Furthermore the determination of apoB in VLDL can be standardized with Lp-B, despite the fact that apoB in VLDL is a constituent of a much larger particle than Lp-B. The almost identical slopes support the accuracy of the chemical determination of apoB in VLDL as isopropanol insoluble protein. Usually TMU is used for delipidization (29). However it has been shown that isopropanol extracts 10% more protein from VLDL, but not from apoB (28). In this study the average percentage of isopropanol-insoluble protein of 17 samples was 45.8 ± 5.3%. This agrees well with Curry et al. (19), who determined apoB gravimetrically as insoluble protein according to Lee and Alaupovic (31) and found that it constituted 40 ± 7% of the d < 1.019 g/ml fractions of normolipemic subjects and 46 ± 10% of the corresponding fractions from hyperlipemic subjects. Investigations employing column chromatographic techniques and 0.1 M decylsulfate showed that apoB comprises only 29% of the total VLDL protein (10). This corresponds to the amount of apoB in VLDL detectable by RIA using antibodies to LDL. In a study with RIA, Albers, Cabana, and Hazzard (9) also found that only 50% of the TMU-insoluble protein was apoB. An apparent underestimation of apoB in VLDL is associated with RIA when antibodies to LDL are used. This was shown by Schonfeld et al. (32) who incubated VLDL with bovine milk lipase and observed an increase in the immunoreactivity of apoB, together with the loss of triglycerides from the particle. Hydroxypolyethoxydodecane may exert a lipase-like effect on VLDL, as the apoB content of these lipoproteins can only be determined accurately in its presence.

It was surprising that the signal for apoB of VLDL, LDL, and the d 1.006 g/ml infranatant was less after ultracentrifugation. However, mixing of the samples with whole serum compensates for this loss in immunoreactivity. A similar phenomenon may in part account for the observation of Albers et al. (9) that the differences between the concentration of apoB in whole serum and

Downloaded from www.jlr.org by guest, on June 19, 2012

in the corresponding d 1.006 g/ml infranatant, as determined by RIA, were always about 50% greater than the apoB concentrations in the corresponding VLDL fractions also determined by RIA. The decreased reactivity observed with anti-serum to Lp-B only occurs with nephelometry, as ultracentrifugation does not affect RID (Fig. 2). Underestimation of the apoB content of a standard would lead to a falsely high concentration range. However, it seems that this effect is not noted with other types of immunoassay, as the concentrations of apoB reported are fairly compatible with LDL cholesterol in these studies.

Variations in the reported concentration ranges for apoB are probably due to differences in the deviations of the color yield of apoB from that of the standards (bovine or human albumin) used for protein determination. Thus factors of 0.77 (10, 12, 33), 0.82 (9), and 1.0 (13, 14) have been used to calculate the protein content of LDL, when bovine albumin was used. In the case of human albumin, a factor of 0.9 has been suggested (19), although such a factor has also been considered unnecessary (11) owing to similarities in the amino acid composition of human albumin and LDL with respect to the aromatic amino acids (34). In this study human albumin was used without a correction factor.

These differences in chromogenicity factors may also account for the wide range of reported ratios of cholesterol to immunologically determined apoB in VLDL and LDL. In VLDL from normal subjects a ratio of 7.4 (13), 6.4 (10), and 2.2 (9) has been reported. According to Skipski (35), the ratio of total cholesterol to protein in VLDL is about 1.5. In agreement with Curry et al. (19), we found that the insoluble protein component of VLDL accounted for 40–50% of the total protein, resulting in a cholesterol to apoB ratio of 3.0–3.7. By chemical determination of apoB we found a ratio of 2.3 in the VLDL fractions from normal and type IV patients (Fig. 9). Similarly, rate nephelometry yielded a ratio of 2.37 in samples from hypertriglyceridemic subjects (Fig. 10). This ratio approximates the expected one most closely and is found independent of the degree of hyperlipoproteinemia. Furthermore we found excellent correlation between apoB and cholesterol in VLDL (r = 0.987 and 0.956) with both methods for determination of apoB in VLDL, isopropanol extraction and rate nephelometry. This is surprising in view of the poor correlations reported, for example, r = 0.25 in type IV patients (10) and r = 0.198–0.308 (8, 9). The reason for these discrepancies may lie in the variable recognition of apoB in VLDL by RIA (19, 32). Values ranging from 2.0 (10) or 1.7 (9) to 1.3 (13) for the ratio of cholesterol to apoB in LDL have been reported. The average ratio of cholesterol to protein in LDL is 1.4 (35), an estimate derived from similar data reported in seven different publications. We found a ratio of 1.37, as determined in isolated LDL fractions, and a ratio of 1.30 in the d 1.006 g/ml infranatants (Fig. 11). Lees, who was one of the first to determine apoB by RID (16), reported a good correlation (r = 0.89) between the concentration of apoB and LDL cholesterol in the d 1.006 g/ml infranatant. According to Karlin et al. (14) the apoB concentration in plasma, as determined by RIA, also correlates well with LDL cholesterol (r = 0.92). In the first determination of apoB in human plasma by RIA, however, Schonfeld et al. (10) obtained poor correlations of apoB in plasma or LDL with LDL cholesterol in samples from normal subjects (r = 0.22 and 0.29, respectively). Other correlations reported to date are r = 0.577–0.708 (9), r = 0.75 (8), and r = 0.57 (23). Our results (r = 0.973) are in good agreement with the RID results of Lees (Fig. 11) and of Karlin et al. (r = 0.972) (Fig. 12).

We consider that the possibility of measuring related lipoprotein parameters with different, independent methods is an important step toward the identification and characterization of plasma lipoproteins with abnormal composition. Divergent results obtained by two methods that usually agree well may indicate the presence of abnormal lipoproteins, such as β-VLDL and LP-X, which were described previously for type III hyperlipoproteinemia and cholestasis, respectively. This approach may prove helpful not only for basic research, but also for clinical chemistry as an aid for diagnosing and following the course of a disease.

In a study comparing the lipoprotein profile with the results of coronary angiography (36), we showed that of all lipoprotein parameters, the concentration of β-lipoproteins is most closely connected with coronary heart disease (CHD), that the concentration of α-lipoproteins is best evaluated in conjunction with the actual concentration of β-lipoproteins, and that a "risk" profile is frequently found at "normal" cholesterol concentrations in the plasma. This implies the need for lipoprotein quantification also at normal cholesterol concentrations, because an increase in the concentration of β-lipoprotein can be masked by a decrease in that of the α-lipoproteins. The number of retrospectively unnecessary lipoprotein quantifications might be considerably reduced by a screening procedure, designed to detect persons with borderline concentrations of β-lipoproteins (β-lipoprotein cholesterol 140 mg/dl (36)) for further analysis of their lipoprotein pattern. Because of the excellent correlation of apoB (determined by rate nephelometry) with LDL cholesterol, the described method may prove to be the screening method of choice.

This work was supported in part by grants from Deutsche Forschungsgemeinschaft SFB 89 and from the Verband der Deutschen Lebensversicherunganstalten.

Manuscript received 3 November 1981 and in revised form 1 April 1982.

Downloaded from www.jlr.org by guest, on June 19, 2012

REFERENCES

1. Gofman, J. W., W. Young, and R. Tandy. 1966. Ischemic atherosclerosis and longevity. *Circulation.* **34:** 679–697.
2. Barr, D. P., E. M. Russ, and H. A. Eder. 1951. Protein–lipid relationships in human plasma. II. In atherosclerosis and related conditions. *Am. J. Med.* **11:** 480–493.
3. Whayne, T. F., P. Alaupovic, M. D. Curry, E. T. Lee, P. S. Andersen, and E. Schechter. 1981. Plasma apolipoprotein B and VLDL-, LDL-, and HDL-cholesterol as risk factors in the development of coronary artery disease in male patients examined by angiography. *Atherosclerosis.* **39:** 411–424.
4. Kladetzky, H. G., G. Assmann, S. Walgenbach, and P. Tauchert. 1980. Lipid and apoprotein values in coronary angiography patients. *Artery.* **7:** 191–205.
5. Riesen, W. F., R. Mordasini, C. Salzmann, A. Theler, and H. P. Gurtner. 1980. Apoproteins and lipids as discriminators of severity of coronary heart disease. *Atherosclerosis.* **37:** 157–162.
6. Sniderman, A., S. Shapiro, D. Marpole, B. Skinner, B. Teng, and P. O. Kwiterovich, Jr. 1980. Association of coronary atherosclerosis with hyperbetalipoproteinemia (increased protein but normal cholesterol levels in human plasma low density (β) lipoproteins). *Proc. Natl. Acad. Sci. USA.* **77:** 604–608.
7. Dedonder-Decoopman, E., C. Fievet-Desreumaux, E. Campos, S. Moulin, P. Dewailly, G. Sezille, and J. Jaillard. 1980. Plasma levels of VLDL + LDL cholesterol, HDL cholesterol, triglycerides and apoproteins B and A-I in a healthy population. *Atherosclerosis.* **37:** 559–568.
8. Thompson, G. R., M. E. Birnbaumer, R. I. Levy, and A. M. Gotto. 1976. Solid phase radioimmunoassay of apolipoprotein B (apoB) in normal human plasma. *Atherosclerosis.* **24:** 107–118.
9. Albers, J. J., V. G. Cabana, and W. R. Hazzard. 1975. Immunoassay of human plasma apolipoprotein B. *Metabolism.* **24:** 1339–1351.
10. Schonfeld, G., R. S. Lees, P. K. George, and B. Pfleger. 1974. Assay of total plasma apolipoprotein B concentration in human subjects. *J. Clin. Invest.* **53:** 1458–1467.
11. Durrington, P. N., J. T. Whicher, C. Warren, C. H. Bolton, and M. Hartog. 1976. A comparison of methods for the immunoassay of serum apolipoprotein B in man. *Clin. Chim. Acta.* **71:** 95–108.
12. Bedford, D. K., J. Shepherd, and H. G. Morgan. 1976. Radioimmunoassay for human plasma apolipoprotein B. *Clin. Chim. Acta.* **70:** 267–276.
13. Bautovich, G. J., L. A. Simons, P. F. Williams, and J. R. Turtle. 1975. Radioimmunoassay of human plasma apolipoproteins. Part 1. Assay of apolipoprotein B. *Atherosclerosis.* **21:** 217–234.
14. Karlin, J. B., D. J. Juhn, A. Scanu, and A. R. Rubenstein. 1978. Measurement of serum apolipoprotein B by radioimmunoassay. *Eur. J. Clin. Invest.* **8:** 19–26.
15. Eaton, R. P., and D. M. Kipnis. 1969. Radioimmunoassay of betalipoprotein-protein of rat serum. *J. Clin. Invest.* **48:** 1387–1396.
16. Lees, R. S. 1970. Immunoassay of low-density lipoproteins. *Science.* **169:** 493–495.
17. Sniderman, A., B. Teng, and M. Jerry. 1975. Determination of B protein of low density lipoprotein directly in plasma. *J. Lipid Res.* **16:** 465–467.
18. Kahan, J., and L. Sundblad. 1969. Immunochemical determination of β-lipoproteins. *Scand. J. Clin. Lab. Invest.* **24:** 61–68.
19. Curry, M. D., A. Gustafson, P. Alaupovic, and W. J. McConathy. 1978. Electroimmunoassay, radioimmunoassay and radial immunodiffusion evaluated for quantification of human apolipoprotein B. *Clin. Chem.* **24:** 280–286.
20. Avogaro, P., G. Bittolo Bon, G. Cazzolato, and E. Rorai. 1980. Relationship between apolipoproteins and chemical components of lipoproteins in survivors of myocardial infarction. *Atherosclerosis.* **37:** 69–76.
21. Heuck, C. C., and G. Schlierf. 1979. Simplified immunonephelometric quantitation of apolipoprotein B in hyperlipoproteinemic serum. *Clin. Chem.* **25:** 782–785.
22. Nazir, D. J., P. L. Brown, and M. J. McQueen. 1978. Evaluation of an automated immunoassay procedure for apo-LDL for phenotyping type-II hyperlipoproteinemia. *Clin. Chim. Acta.* **88:** 31–36.
23. Vergani, C., G. Trovato, and M. Dioguardi. 1978. Serum total lipids, lipoproteins, cholesterol, apoproteins A and B in cardiovascular disease. *Clin. Chim. Acta.* **87:** 127–133.
24. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
25. Bensadoun, A., and D. Weinstein. 1976. Assay of proteins in the presence of interfering materials. *Anal. Biochem.* **70:** 241–250.
26. Davis, B. J. 1964. Disc-electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121:** 404–427.
27. Scheidegger, J. J. 1955. Une micro-méthode de l-immunoélectrophorèse. *Int. Arch. Allergy.* **7:** 103–110.
28. Holmquist, L., and K. Carlson. 1977. Selective extraction of human serum very low density apolipoproteins with organic solvents. *Biochim. Biophys. Acta.* **493:** 400–409.
29. Kane, J. P. 1973. A rapid electrophoretic technique for identification of subunit species of apoproteins in serum lipoproteins. *Anal. Biochem.* **53:** 350–364.
30. Becker, W., W. Schäfer, and W. Ziegenbein. 1980. Standardization of immunochemical quantitation of human apolipoproteins A and B. *Ric. Clin. Lab.* **10:** 203–212.
31. Lee, D. M., and P. Alaupovic. 1974. Composition and concentration of apolipoproteins in very low and low density lipoproteins of normal human plasma. *Atherosclerosis.* **19:** 501–520.
32. Schonfeld, G., W. Patsch, B. Pfleger, J. L. Witztum, and S. W. Weidmann. 1979. Lipolysis produces changes in the immunoreactivity and cell reactivity of very low density lipoproteins. *J. Clin. Invest.* **64:** 1288–1297.
33. Margolis, S., and R. G. Langdon. 1966. Studies on human serum β_1-lipoprotein. I. Amino acid composition. *J. Biol. Chem.* **241:** 469–476.
34. Oser, B. L., editor. 1965. Hawk's Physiological Chemistry. 14th ed. McGraw-Hill, New York. 132–133.
35. Skipski, V. 1972. Lipid composition of lipoproteins in normal and diseased states. *In* Blood Lipids and Lipoproteins. G. J. Nelson. editor. Wiley-Interscience, New York. 472–583.
36. Wieland, H., D. Seidel, V. Wiegand, and H. Kreuzer. 1980. Serum lipoproteins and coronary artery disease (CAD). Comparison of the lipoprotein profile with the results of coronary angiography. *Atherosclerosis.* **36:** 427–439.

Downloaded from www.jlr.org by guest, on June 19, 2012