EVALUATION OF IMMUNONEPHELOMETRY AS A METHOD FOR THE QUANTITATION OF HUMAN SERUM APOPROTEIN B

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ABSTRACT

The technique of immunonephelometry was evaluated for the quantitation of serum apoprotein B. The light scattering characteristics of low density lipoprotein apoprotein B and anti-low density lipoprotein antibodies were evaluated, and appropriate working conditions for the immunoassay are described. Performance characteristics including, specificity, sensitivity, and intra- and interassay variability were assessed, and found to satisfactory for apoprotein B measurement. Parameters affecting the accuracy and sensitivity of the assay, such as the selection of a primary standard, are discussed. The quantitation of apoprotein B was optimized by the addition of lipoprotein-free serum to the low density lipoprotein standards. Under these conditions, the estimates of apoprotein B in the density >1.020 grams/ml serum fraction by immunonephelometric and radial immunodiffusion methods agreed quite well. The values found in whole serum by immunonephelometry were significantly higher than those found by the same method in fractionated serum, and by the reference method in whole serum. The factors contributing to these differences were evaluated, and are also discussed.

Résumé

L'éfficacité des techniques immunonéphélométriques face à la quantification de l'apoprotéine B du sérum a été évaluée. Les propriétés de dispersion de lumière des lipoprotéins légères (LDL), de l'apoprotéine B et de l'anticorps aux lipoprotéines LDL ont été estimées, et les conditions nécessaires au bon fonctionnement du immuno-titrage sont décrites. Les caractéristiques de fonctionnement telles que la spécificité, la sensibilité et la variabilité entre les titrages et à l'intérieur d'un même titrage ont été déterminées et jugées adéquates pour la quantification de l'apoprotéine B. Les paramètres pouvant influence la précision et la sensibilité du titrage tels que la sélection du standard primaire y sont discutés. La quantification de l'apoprotéine B a été maximisée par l'addition d'un sérum dépourvee de lipoprotéines aux standards des lipoprotéines LDL. Dans de telles conditions les calculs pour l'apoprotéine B du sérum faisant partie de la fraction de densité >1,020 grammes par millilitres obtenus tant par les techniques immunonéphélométriques que par la méthodes d'immunodiffusion radicale corespondaient avantageusement. Des valeurs obtenues par l'immunonéphélométrie celles du sérum entier étaient plus élevées que celles du sérum fractionné (de façon significative). De plus en ce qui a trait au sérum entil, la technique immunonéphélométrique fournie des valeurs plus élevées (de façon significative) que la méthode de référence. Les facteurs pouvant contribuer à ces différences ont été déterminés et sont présentés.

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Notice

The experiments described in this thesis were conducted between 1979-1981. Many of the findings reported were still relatively original at the time. Only a portion of the laboratory work actually conducted by the author is presented in this report.

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PART ONE

INTRODUCTION

Lipids form a major component of human plasma. Because of their insolubility in water and most biological fluids, plasma lipids do not circulate in their free form. Rather, these substances are transported in the form of lipid-protein complexes called lipoproteins (1-7). These water soluble, macromolecular complexes are composed of various kinds of lipids, chiefly triglyceride and cholesterol, and one or more proteins called apolipoproteins or simply apoproteins (8-9). The plasma lipoproteins were initially more intensively studied in terms of their lipid than protein moieties, because at the time, the available methodology for protein analysis was cumbersome, time consuming and applicable to a relatively small number of samples (10-13). Recently, simpler assays capable of measuring plasma apoproteins have become available (14-33). With the advent of specific immunoassays for detecting and quantifying apoproteins, the physiological roles of the lipoproteins in normal and abnormal lipid transport have become more fully elucidated (8,34-40).

The lipid-free protein components of lipoproteins are obtained by treating intact lipoproteins with organic solvents, detergents, or chaotropic agents. The apoproteins are usually classified according to the alphabetic (ABC etc.) nomenclature suggested by Alaupovic (41). At the present time, there are seven well-defined apoprotein species designated apo A-I, apo A-II, apo B, apo C-I, apo C-II, apo C-III and apo E. The apoproteins are widely distributed throughout the entire lipoprotein spectrum although each class of lipoproteins has a more or less typical apoprotein composition (table 1). Apoproteins play critical roles in

maintaining the structural integrity of lipoprotein particles, as well as containing the reactive groups required for interaction with enzymes and cellular receptors. These proteins therefore confer specificity to lipoprotein particles and, in effect, direct their metabolic fate within the circulation. In this regard, specific biological roles for some apoproteins have been established (8).

Interest in plasma lipoproteins stems from their physiological roles in normal lipid transport and their pathophysical roles in disorders of lipid transport, particularly atherosclerosis (1-2,40,42). The most obvious important function of lipoproteins is to transport triglyceride and cholesterol through plasma in a stable, colloidal form. Elevation of total plasma cholesterol and the major cholesterol carrying lipoprotein, low density lipoprotein (LDL), are associated with an increased risk for coronary artery disease (44-45). The clearest example of the causal relationship between cholesterol and lipoproteins in the pathogenesis of atherosclerosis is found in the hereditary lipoprotein disorder familial hypercholesterolemia (FH), where the levels of LDL cholesterol are above the 95th percentile for the population (46-47, 62). Aside from these cases, the concentration of total plasma and LDL cholesterol overlap considerably in persons with and without coronary artery disease. In fact, it is now recognized, that many individuals with documented coronary atherosclerosis have plasma cholesterol levels within the normal range and by fiducial norms, are normocholesterolemic (48-49). The disease in the vast majority of these patients, however, is being recognized through

the measurements of plasma apoprotein levels (49-56), particularly those associated with LDL (49, 53). Recent studies (49-56) have shown that many coronary patients with normal plasma and LDL cholesterol levels have elevated levels of LDL apo B - a lipoprotein pattern called hyperapobetalipoproteinemia by Sniderman (49). The data also indicated that the disorder is frequent among coronary patients and it is becoming recognized that plasma apo B rather than lipid levels are the more sensitive parameters for separating coronary from noncoronary patients (49-56).

The newly recognized association between plasma apo B concentrations and coronary artery disease is expected to lead to an enormous interest in quantitating apo B levels, particularly for clinical and epidemiological purposes. Immunoassays with a capacity to handle a large volume of samples will be required to facilitate these measurements. Because of its technical simplicity, use of simple, stable reagents, capacity for automation, and other features, immunonephelometric assay (INA) appears ideally suited for these applications (57-58). The technique, being one of the newer immunoassays however, is not yet fully established for this purpose. The aim of the present investigation therefore, was to evaluate INA as a method for the quantitation of serum apo B levels, particularly for its use in this laboratory.

PART TWO

REVIEW OF THE LITERATURE

I. Properties of apo B and the apo B Lipoproteins

Apo B is present in serum combined with lipids in the form of lipoproteins. The serum lipoproteins constitute a heterogeneous group of macromolecular complexes with varying physical properties and chemical compositions. Based on their flotation rates in salt solutions (hydrated density), the plasma lipoproteins are usually separated into four or five distinct classes called chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), LDL, and high density lipoproteins (HDL). Apo B is an obligatory structural component of chylomicrons, VLDL, IDL, and LDL, comprising respectively, approximately 20%, 40%, 60% and 90% of the total protein mass of these particles (table 1). It is not a normal constituent of HDL.

The physiochemical characteristics of apo B are still incompletely understood owing to technical difficulties involving dissociation and solubilization of the protein. Nevertheless, evidence for apo B heterogeneity has been emerging from analysis of the protein in both rat (59) and human serum (60). One series of proteins found in human plasma LDL, is represented by species of molecular weight 549,000 (B-100), 407,000 (B-74), and 126,000 (B-26) daltons (60). Based on their size and amino acid composition, the B-74 and B-26 subspecies appear derived from the predominant B-100 form. A distinct second type of apo B (B-48), that is a major component of chylomicrons, and which is not found in LDL, has a molecular weight of 265,000. This species is probably analogous to a 248,000 molecular weight protein

elaborated by rat intestine (59). The chylomicron and LDL species of apo B are probably under separate genetic control, and a disorder characterized by the selective absence of the B-48 form and chylomicrons has recently been described (38). The complete absence of apo B from serum is found in the rare autosomal recessive disorder known as abetalipoproteinemia (61). The two forms of apo B are believed to have distinct immunological properties (38). However, the B-100 and B-48 protein peptides appear to share common subunits as demonstrated by their partial immunological crossreactivity with antisera raised against chylomicron and LDL apo B (38).

The apo B content of VLDL, IDL and LDL appears to be constant and independent of particle weight and lipid and protein constituents (8). In contrast, the lipid components of these lipoproteins are quite variable (8). Apo B is evidently an obligatory structural component of chylomicrons and VLDL, being necessary for the synthesis and secretion of these lipoprotein species (61). To date, only the absorptive mucosal cells of the small intestine and the parenchymal cells (hepatocytes) of the liver have been demonstrated to synthesize apo B (2). These organs, therefore, appear to be the exclusive sources of nascent lipoproteins (1-2). In vivo, chylomicrons, containg the B-48 protein, are metabolized to form chylomicron 'remnants', which are then rapidly removed by the liver (167-168). VLDL, containing the B-100 protein, are released from the liver and metabolized to LDL through the metabolic cascade that first generates IDL (168-169). Apo B is not an exchangeable protein and remains as an obligatory structural component of triglyceride rich lipoprotein particles during

catabolism. In normal subjects the turnover of apo B in VLDL, IDL and LDL are virtually identical, implying that all LDL originates in VLDL, and both VLDL and IDL are quantitatively converted to LDL (168-169). The major role of LDL involves the delivery of cholesterol to a number of extra-hepatic cells (62). This function is accomplished through uptake of intact LDL particles by a receptor mediated process termed the LDL pathway (62). Apo B seems critical to the receptor mediated uptake of LDL, since chemical modification of its arginine or lysine residues abolishes binding and uptake by both fibroblasts (63) and hepatocytes (64) in vitro. In normal subjects, the serum level of apo B is ultimately determined by the balance between three sequential, multistep processes involving; (1) production of VLDL, (2) conversion of VLDL to LDL, and (3) clearance of LDL.

II. Immunological Quantitation of Serum apo B Concentrations

Although proteins can be quantitated colorimetrically and gravimetrically, for routine clinical measurement immunoassay is the preferred method. During the last 10-12 years, many different immunological techniques have been utilized for the development of immunoassays for specific apoproteins. The concentration of apo B in plasma and serum has been most often quantitated by (1) radioimmunoassay ({RIA}, 14-20), (2) radial immunodiffusion (RID) assay (21-23, 67-68), (3) electroimmunoassay ({EIA}, 24-26, 69-72), (4) enzyme-linked immunosorbent assay ({ELISA}, 27-29, 76) and (5) immunonephelometric assay ({INA}, 30-33, 57-58, 73-75).

Lees et al (22) in 1970 using Mancini's (171) RID technique, developed one of the first quantitative immunoassays for plasma apo B. Several investigators, beginning with Curry et al (25), subsequently used the rocket immunoelectrophoresis method of Laurell (77) to quantitate apo B by EIA. The double-antibody RIA procedure was initially applied by Eaton and Kipnis (78) to quantitate beta-lipoprotein in the serum of rats. Subsequently, RIA's for apo B in human serum were described separately by Schonfeld (14), Bautovitch (15) and Albers (17), in the mid 1970's. The ELISA technique has been successfully developed for quantitating apo B, but this method has so far received limited application (27-29). Newer assays for apo B, based on immunonephelometry, have been recently developed (30-33) as an extension of earlier attempts to quantify proteins by automated immunoprecipitan (AIP) methods (79-84). This method is becomming increasingly more popular, partic-

ularly in the clinical setting (58). Commercial plates for use in RID are also available (85) and one manufacturer is currently developing an assay kit for INA (86).

Total serum apo B concentration is the sum of the apo B present in the individual lipoprotein subfractions including chylomicrons, VLDL, IDL and LDL. By using fasting serum samples, the contribution of chylomicrons to the measurement is removed, since these particles are normally absent from serum after a 12-14 hour fast (87). Experiments comparing apo B concentrations before and after a fatty meal, however, have shown only a negligible difference in apo B levels in fasting and non-fasting serum (20, 33). The large size of these particles interferes with the performance of some immunoassays (15, 17), and for this reason, fasting serum is generally preferred for the analysis of serum apoproteins. The measurement of apo B in individual lipoprotein subfractions requires preliminary lipoprotein separation by preparative ultracentrifugation (88) or precipitation (89). The content of apo B can then be determined by immunoassay or colorimetrically (95) after preliminary extraction of insoluble protein by tetramethylurea (TMU) (90) or isopropanol (91-92). One assay (21) has been developed to selectively measure LDL apo B in whole serum. Recent data (67) suggests that this assay may be more sensitive to serum VLDL than shown originally by the authors.

Most assays utilize a narrow band of LDL (d=1.020-1.050) for preparing the LDL apo B assay standards and for the raising of anti-LDL anti-

serum. LDL in this density range consists almost exclusively of apo B, with only trace amounts of apo A and apo C (93-94). These trace quantities are insufficient to generate significant levels of antibody under the existing experimental conditions (93). With the exception of RIA, most methods use intact rather than delipidated LDL particles for preparation of the standards. The protein concentration in LDL is estimated colorimetrically, by the method of Lowry et al (95), using bovine serum albumin (BSA) as the protein standard. Some workers use a chromogenic correction factor (0.77 in 14, 16, 22, 29; 0.82 in 17; and 0.90 in 25 and 93), to correct for differences in the absorption of the two proteins. At the present time, there is no international standard for LDL apo B, and therefore no universally recognized reference value exists for the purpose of standardization of methods. Differences in the preparation of LDL standards and standardization procedures, is expected to result in some variation, perhaps 10-15%, in the levels of apo B quantitated by different methods (25).

A fundamental requirement of any immunoassay, is that the antigen occur with identical physical properties (eg., molecular size and charge) in the standards and samples (77, 97). The quantitation of apo B in plasma or serum is complicated by virtue of the presence of this protein in particles having a wide range of physical properties including size, charge, and composition (7). For practical reasons, isolated LDL is generally used as the primary standard for the quantitation of apo B in whole serum and isolated lipoprotein fractions, eg., VLDL and LDL. Because of differences in the physical properties of the various classes of apo

B lipoprotein it is feasible that the immunoreactivity of LDL and VLDL, for example, may not be identical. If these differences in lipoprotein immunoreactivity were quantitatively significant, then the measurement of apo B by certain immunological methods could be potentially unreliable.

The measurement of VLDL apo B levels both in isolated lipoprotein fractions and in whole serum, have been problematic for some immunoassays (17, 20). The quantitation of apo B in VLDL by chemical methods, for example, colorimetry and gravimetry after extraction of insoluble protein by TMU or isopropanolol, has been shown to produce similar results (90-94). These chemical methods may then be used for the evaluation of immunological techniques. Calvert et al (98), measured VLDL apo B by EIA and RIA and compared the values found by the immunoassays to those found by chemical extraction with TMU. The values found by the EIA and TMU methods were very similar, whereas the yield by RIA compared to TMU was about 50% lower. Albers et al (17), also found the RID and RIA estimates of d<1.006 g/ml apo B to be nearly 50% less than the TMU estimate. Curry et al (25), on the other hand, found the EIA, RIA and RID methods to compare well with the gravimetric determination of apo B in the d<1.019 lipoprotein fraction in normolipemic serum. In hypertriglyceridemic serum, where the content of apo B in this fraction is increased, the estimation of apo B by the RIA and RID methods were nearly 50% lower than EIA, which still compared well with the gravimetric estimate. Franchisini (99), found slightly higher levels of VLDL apo B by EIA compared to the TMU method. Taken together, these experiments show that the sensitivities of some assays for apo B in VLDL may

quite different.

Recent evidence has shown that the quantitative differences in VLDL apo B may be related to differences in the immunoreactivity of lipoprotein particles. Schonfeld (100), found differences to exist in the immunoreactivity of VLDL before and after in vitro lipolysis. In these experiments, the immunoreactivity of VLDL was assessed by RIA and by observing the interaction of VLDL with LDL receptors in cell culture. The hydrolysis of VLDL lipid was accompanied by increases in the cell reactivity and immunoreactivity of these particles. These changes were postulated to result from exposure of apo B antigenic sites on the surface of the particles, that were unmasked following the removal of lipid. Patton et al (101), however found the opposite effect, namely a loss of immunoreactivity of VLDL treated by lipases. Decklebaum (102-103), described a procedure for the in vitro production of LDL-like particles from VLDL using lipase enzymes. Reardon et al (104), applied this technique for the quantitation of VLDL, IDL and LDL apo B in a modified EIA. Before assay, the isolated lipoprotein particles were subjected to in vitro lipolysis. Treatment of VLDL and IDL particles resulted in a 100% yield in the apo B content quantitated by immunoassay compared to protein determined by TMU extraction procedures. In contrast, LDL apo B values were not significantly changed by the enzyme treatment. These findings indicate that the quantitation of triglyceride rich particles by conventional immunoassays including EIA, may well result in an underestimate of VLDL, IDL and hence total serum apo B values. Differences such as these may also contribute to

the variation in apo B levels found by different immunoassays (see next section). It is clear that factors such as the choice of the apo B standard and the treatment of the plasma samples are critical for the outcome of the assay. There is not yet a consensus of opinion as to which assay performs the best. The ideal immunological method should be capable of measuring the concentration of apo B in all lipoprotein particles relative to the actual mass of apo B protein present. Curry et al (25) have suggested that the EIA method most closely approaches this ideal at the present time.

III. Serum apo B Levels in Health and Disease

Tables 2 and 3 compare the mean levels of apo B measured in healthy populations of adult men and women by various immunological methods. The range of mean values is wide, varying between 70 and 130 mg/dl, although most of the means fall between 80 and 120 mg/dl. The standard deviations of most means is of the order of 15-30 mg/dl of apo B. Total serum apo B levels tend to be skewed to the right as is the distribution of total serum cholesterol (14, 17, 177). In general, the mean values are lower for those samples measured by RIA and RID, and higher for those measured by EIA and INA methods. At the present time, there are no universally accepted population norms for apo B analogous to those established by the LRC program for serum lipids. The sometimes wide variation in apo B levels as found by different methods, makes the interassay comparison of values more difficult. In lieu of an internationally accepted standard for LDL apo B, the relation between apo B values measured by the same method in different laboratories, and by different methods in the same and different laboratories, must be established for these comparisons to be meaningful. In not all instances have these relationships been clearly established. Factors contributing to the interassay variation in apo B values include differences related to the preparation of standards, and standardization procedures, differences in the sensitivities of the methods for quantitating apo B in the various lipoprotein fractions, and differences related to the characteristics of the populations studied (see below).

Table 4 shows the distribution of apo B among the various classes of serum lipoproteins in normolipemic serum. Most of the apo B is confined to the LDL (d=1.020-1.063 gm/ml) lipoprotein fraction, averaging about 90% of the total amount for the data considered in table 4. LDL apo B is highly correlated with serum and LDL cholesterol (14-15). Apo B in d≤1.020 gm/ml fraction accounts for between 5 and 15% of the total, with most of this being present in VLDL. As mentioned earlier, the apo B in this fraction tends to be underestimated by some methods. A few studies (15, 24-25), have shown a small amount of apo B in the HDL density fraction. This probably represents LDL with a higher than average density, and emphasizes the heterogeneity of the density distribution of these particles (181).

Most studies report slightly higher levels of total and LDL apo B in males than females; the differences usually amounting to between 5 and 15 mg/dl of apo B (17, 19, 24, 57, 73, 105). Some studies have found no differences (106), while others (17, 73), have found slightly higher levels in women, particularly when comparisons involve the later decades of life. Apo B levels are higher in older than younger subjects (105-106), some noting a continuous age related increase (17, 105), and others, a peak in the 5th or 6th decade followed by a decline thereafter (73). Albers et al (17), for example, found a 10 mg/dl difference between subjects in the 3rd and 7th decades, while Avogaro (105), found a difference of 20 mg/dl in a similar comparison. These comparisons are derived from cross sectional measurements, and longitudinal changes are less clear.

Apo B levels are low in the newborn child (20-30 mg/dl), but increase dramatically in the first week of life with the initiation of oral milk feedings (108-112). By the end of the first month of life, apo B levels are already 70-80% of comparative adult values (109-111). Further increases during the first year of life are small. One study (112) following apo B levels longitudinally, found no differences between the values measured at six months, and one and two years of life. The composition of the neonatal diet has been shown to influence the initial increase in apo B levels (111). Several studies have recently evaluated the use of apoproteins to screen for dyslipoproteinemia at birth (58, 113-114). A lipoprotein pattern resembling that of hyperapobetalipoproteinemia has been described in infancy (58). Studies of apo B levels in children and teenagers are still very scant (115).

The composition of the diet has been shown to have major effects on the serum levels of apo B. Vegetarians have lower levels of apo B, cholesterol and triglyceride, compared to individuals consuming a more typical North American diet (116). Substituting a vegetarian diet in a non-vegetarian, results in a lowering of LDL apo B and cholesterol levels. These changes are reversible when the ad lib diets are resumed (117-119). Ingestion of diets high in total fat (120), and cholesterol (120-122), or low in the ratio of polyunsaturated to saturated fats (120-122) are accompanied by increases in the levels of LDL apo B and cholesterol. The converse dietary changes produces the opposite effects (117-120). The effects of diet on the levels of LDL may have important implications for

the prevention of coronary artery disease (127).

The effects of other parameters commonly associated with daily living, for example, tobacco and alcohol consumption, exercise and drug ingestion, on the levels of apo B, are still largely unexplored. One study (125), recently reported a 7% higher level of apo B in smokers than non-smokers. The effects of alcohol consumption in individuals unaffected by liver disease are unclear. Both higher (123), and lower (124), levels of apo B have been reported in alcoholics compared to controls. The effects of moderate alcohol consumption are not known. Both acute (126), and chronic (127), exercise were not associated with any significant changes in apo B levels, in the two studies considering this parameter. The effects of estrogen-progesterone containing oral contraceptives on serum apo B levels has also produced conflicting findings. One study (128), showed lower levels of apo B in users of oral contraceptives compared to controls, while a second (129), showed significantly higher levels in users compared to former users and non-users. In the second study, the increase in LDL apo B was not accompanied by an increase in LDL cholesterol indicating a relative enrichment of LDL by apo B reminiscent of hyperapobetalipoproteinemia (49). Apo B levels have also found to fluctuate in women during the menstrual cycle (130). The effects of various physiological and drug related factors on the levels of apo B are important to document, and more studies are needed to establish these relationships.

The levels of apo B are elevated in a number of disorders of lipoprotein metabolism. The highest levels of apo B are found in the homozygous form of familial hypercholesterolemia (FH), where values in the 200-300 mg/dl range have been reported (15). This disease is due to a deficiency or defective function of LDL receptors, and is characterized biochemically by gross elevations in serum and LDL cholesterol, enrichment of LDL particles by cholesterol, an an increase in the LDL cholesterol to apo B (C/B) ratio (46-47). The levels of apo B are somewhat less elevated in the heterozygous form of FH (15), and in type IIb hyperlipoproteinemia (15, 24). The elevation of apo B is more variable in type IV hyperlipoproteinemia (endogenous hypertriglyceridemia) where VLDL are increased (14, 17, 24, 55). In this disorder, the apo B in VLDL may account for 20-50% of the total in moderate to severe hypertriglyceridemia. In some individuals with this disorder, LDL is also elevated, and these individuals may be more prone to atherosclerosis (55). Total apo B levels are increased in type III hyperlipoproteinemia (familial dysbetalipoproteinemia) owing to an increase in IDL (24-25). Several years ago, it was suggested that the cholesterol and protein content of LDL may vary independently of one another (22). A number of studies have now shown that apo B levels are increased in a significant number of patients with coronary artery disease (49-56), characterizing particularly, a subset of coronary patients with normal plasma and LDL cholesterol levels (49). In hyperapobetalipoproteinemia (49), LDL are enriched in apo B and the LDL C/B ratio is decreased. In most studies, apo B has discriminated the coronary from the non-coronary patients better

than most other parameters tested, and therefore apoprotein B appears to be an important new risk marker for atherosclerosis. Apo B levels are commonly elevated in poorly controlled diabetics, usually in proportion to the degree of hyperlipemia (131-132). Improvement in diabetic control is associated with a lowering of apo B levels (131-132). Various disorders of liver function including fatty liver, alcoholic hepatitis, acute infectious hepatitis and hepatocellular carcinoma are associated with high apo B levels (123, 133). Apo B levels are also increased in patients with the nephrotic syndrome (134). The lp(a) lipoprotein (65), which also contains apo B, is found in the HDL density range and only rarely contributes significantly to total serum apo B concentrations (66). Apo B levels are reduced in the uncommon disorder hypobetalipoproteinemia (1, 2), and absent from serum in the rare autosomal recessive disorder abetalipoproteinemia (61).

IV. Principles of Nephelometry and Immunonephelometric Assay

Nephelometry is defined as a method of detecting light energy scattered or reflected toward a detector which is not in the direct path of the transmitted light (135). A suspension of particles in solution can be regarded as a light scattering system. If this suspension behaves as an immunological system, then the principle of molecular light scatter can be utilized for the detection of antigens and antibodies. Both qualitative (presence or absence) and quantitative (concentration) determinations can be made.

The technique of laser nephelometry is based on the principle that a monospecific antiserum reacts to form immune complexes with a specific antigen (135). The formation of immunocomplexes in this system is measured by passing a collimated monochromatic beam of light through a solution containing the reactants. Antigen-antibody complexes in solution scatter light and the intensity of light scatter is measured by a photodetector (135). The output signal from the photodetector, corresponding to a change in voltage, is displayed as relative light scattering units (RLS) by the instrument (136-137). The intensity of light scatter is a function of both the number and size of the solute particles in solution, the wavelength of the incident light and other factors. These relations are defined by the equation (138):

$$C = f \cdot N \cdot \pi \cdot d^2,$$

where C is the intensity of scattered light, N is the number of small

scattering particles with a diameter d, and f is a factor of proportionality for the fixed conditions of the nephelometer. For mie[1] scatters such as immune complexes, the relation between the size of the particle and the intensity of light scattering is more complex (135). Because the scatter intensity of such particles increases considerably in the direction of the axis of the incident light, differences in intensity as a consequence of differences in particle size are even more pronounced for measurement at low angle (135). For hyperlipoproteinemic serum samples, additional effects caused by non-specific light scattering must also be taken into account (139). However, in the design of these instruments, the optimal angle for measuring light scatter produced by the antigen-antibody complexes has been determined and incorporated to maximize the effect of forward light scatter for the reactions and to selectively minimize forward light scatter produced by potentially interfering substances (135-137). Thus for the fixed conditions of nephelometry, and under appropriate immunological relationships, the photodetector output (intensity of light scatter) is proportional to the concentration of the antigen in the test solution (135). The antigen concentration for unknown samples is found by comparing RLS from a reference to that measured for the unknown.

Immunonephelometry has been successfully applied for determination of various serum proteins including immunoglobulins (141-143), rheumatoid factor (144), complement factors (142-143), and lysozyme (145). Many assay kits are available for the clinical application of this system.

From a practical point of view, the technique seems superior to other immunological methods for several reasons (135). Immunonephalometry is both specific and sensitive and its precision for quantitating most proteins is comparable to other methods. The procedures involved are technically quite simple and therefore less laborious. Incubation times are relatively short, results are rapidly available and the method is less time consuming. The reagents are inexpensive and there are no radioactive materials involved. Finally, the method is also capable of handling large numbers of samples and can be automated. These features of the assay make it especially suitable for clinical and epidemiologic applications.

The method has also been investigated for the quantitation of serum apoproteins including apo A (76, 146-148) and apo B (30-33). For this purpose however, the procedure is more complex than that involved for determination of other serum proteins. Apoproteins are present in serum in the form of lipoprotein complexes, and these complexes are distributed over various lipoprotein classes. The concentration of apoproteins vary among the lipoprotein classes, as does the size of the lipoprotein particles. These considerations are particularly relevant to the quantitation of apo B by this method, since apo B is present in most density classes of lipoproteins, and differences in the size of lipoprotein particles is maximal between VLDL and LDL. The larger VLDL particles present in whole serum or isolated lipoprotein fractions may cause non-specific light scattering (32). Such an effect would be expected to alter the proportionality between intensity of light scatter and concentration of antigen,

thereby potentially leading to an overestimate of serum apo B levels (32). This effect would be magnified in hyperlipoproteinemic serum samples (32-33, 74).

V. Quantitation of Serum apo B by INA

Analysis of lipoprotein concentration by light scattering techniques was first described by Stone et al (79), who used a micronephelometer to estimate the concentration of serum LDL and triglycerides. Subsequently Kahan et al (80-81), and Ritchie et al (82-84) described the use of an automated light scattering technique (AIP) to quantitate LDL protein immunochemically. These earlier attempts to measure apo B utilized instruments with a low intensity light source and measurements of protein concentration suffered from a lack of sensitivity. These problems resulted from a low serum to blank ratio caused by non-specific light scattering (84). In the last 5-6 years, a new generation of nephelometers has emerged containing a laser light source, and these instruments have greatly improved the sensitivity and precision of serum protein analysis. Consequently, many new studies have undertaken to evaluate this method for the quantitation of serum apo B (30-33).

Ballantyne et al (31), were one of the first to use laser nephelometry for the quantitation of apo B in serum. In this study, 87 normo- and hyperlipemic samples of plasma were analyzed by INA, AIP and RIA methods. The mean levels of apo B found by INA were significantly higher than those found by AIP and RIA (table 3). The correlation between the INA and AIP ($r=0.79$) and RIA ($r=0.70$) methods were only fair. The mean value found in a subset of 35 normolipemic subjects by the INA method was also more than twice that found by RIA (159 vs 71 mg/dl). Estimation

of apo B in the d>1.006 fraction resulted in a better correlation than found in plasma, indicating that the high plasma estimate may have been due to non-specific light scattering of the d<1.006 lipoproteins. However, the total apo B concentration found by combining the values found separately in the d<1.006 and d>1.006 fractions, agreed well with the whole plasma estimate. Further, the RIA and INA methods gave closely similar estimates for d<1.006 apo B, indicating that the non-specific light scattering effect may not have been excessive. The three methods also gave different estimates of apo B in two commercial LDL standards used for calibration. Again, INA produced higher, and RIA lower estimates, so that the sensitivities of the two assays for apo B appeared to be different, but consistently so. Ballantyne (149), recently reported a much lower mean value for a group of 24 normolipemic subjects (table 3). No modifications of the procedures used in the first study were described to explain the lower values found in the second.

Fievet-Desremaux et al (30), compared the levels of apo B measured by INA, EIA and enzyme immunoassay in 31 subjects, and found no significant differences in the values by the three methods. However, the mean values were very high (table 3), and it is unclear from the report exactly what kind of subjects were used for these comparisons. Dedonder-Decoopman et al (57), in a large series, reported mean apo B levels of 129 mg/dl and 120 mg/dl in 206 and 271 normolipemic male and female subjects respectively. These values tend to be somewhat higher than the total serum apo B levels found when similarly large groups have been

studied by other methods (table 2). Other investigators have described lower mean values for normolipemic samples analyzed by the INA method. Heuck et al (32), reported a mean apo B level of 82 mg/dl in 68 normolipemic subjects, with a correlation of 0.97 between this method and an RID assay. Heuck (75), recently updated his series reporting a mean value of 103 mg/dl for apo B in 432 normolipemic subjects. Rosseneu (33), compared the INA method with EIA under a number of experimental conditions. This study emphasized the need to have closely similar conditions present in the primary LDL standards and the serum samples, in order to eliminate the tendency for the method to overestimate apo B levels that may even occur in normolipemic serum samples. De Backer et al (56), recently compared apo B levels in 70 normolipemic survivors of myocardial infarction, and a similar number of normolipemic controls. The mean value found by INA in the ischemic patients was significantly higher than that found in the control subjects (143 vs 113 mg/dl). This finding illustrates that differences in the characteristics of the population may alone account for a significant amount of the variation in apo B levels observed for comparisons between this and other methods.

The INA method has also been used to measure the level of apo B in the serum of newborn infants. Van Biervliet et al (110), found a mean cord serum value of 24 mg/dl in 30 newborns, which agrees well with the values found by other methods (108-109). Since the concentration of VLDL is very low in newborn serum (150-152), non-specific light scattering is not a problem for measurements made on cord samples. Van Bier-

vliet (58), recently described a modification of the original INA technique, and this was applied to screen 1500 infants for dyslipoproteinemia at birth. The method involved the collection of blood obtained by heel prick, and adsorbed onto filter paper. The blood spots were then eluted by detergent, and the apo B was quantitated by INA. The apo B values found by the elution of blood spots correlated well with the values obtained by measurement in serum (58). Brewster et al (85), compared INA with EIA for the quantitation of beta-lipoprotein concentration in the cord serum of 232 newborn infants. The mean levels of apo B found by the two methods were 54 and 50 mg/dl respectively. The small difference between the values was not significant, but the mean levels are nearly twice as high as those found for newborns in most other studies (108-110). The difference was probably accounted for by the use of a commercial beta-lipoprotein standard for calibration of apo B. These commercial standards have resulted in higher estimates of apo B in other studies (113-114).

Immunonephelometry has been more problematic for the quantitation of apo B levels in hyperlipemic serum, particularly in the presence of hypertriglyceridemia, where the tendency for overestimate is much greater (32-33, 74). The overestimate in hyperlipemic serum is caused by non-specific light scattering of the larger VLDL particles (32, 74). The effect in hypertriglyceridemic serum is magnified because both the concentration (14, 17), and size (153) of the VLDL particles is increased. However, the problems created by VLDL in hyperlipemic serum have been largely sur-

mounted by the incubation of the serum samples in the presence of lipases and detergents (32-33, 74). Under these conditions, the assay has been fairly well standardized for the quantitation of apo B in these samples. Hyperlipoproteinemic serum has also been a problem for the quantitation of other apoproteins by this (75) and other (22, 77) methods as well.

In summary, many studies have recently evaluated the INA method for the quantitation of serum apo B. In some studies, the values found by INA have been significantly higher than those reported by most other methods. In other instances, the levels found by different methods have agreed quite well. In not all cases, were the working conditions of the assay described. It is therefore difficult to assess the extent to which procedural differences contribute to the variance of the findings. The heterogeneity of apo B levels in the population may partially explain some of the differences as well. The data indicates that the method potentially performs well for the analysis of apo B, although the exact conditions necessary for the the optimal performance of the assay are not entirely clear. It seems important, therefore, for each laboratory considering using the method, to evaluate the working conditions and establish the relationship for serum apo B quantitated by the new and established procedures.

PART THREE

EXPERIMENTAL

I. Introduction

The purpose of the present investigation was to evaluate immunonephalometry as a method for the quantitation of serum apo B. In the development of an immunoassay, there are four essential parameters to be evaluated. These include (1) specificity, (2) sensitivity, (3) precision, and (4) validation. As well, any potentially interfering variables must also be recognized and evaluated.

Firstly, the assay must be specific for the antigen in question. In immunoassays, this requirement is satisfied by raising a monospecific antiserum to the protein antigen. Secondly, the in vitro sensitivity of the assay must be adequate for detecting the in vivo concentrations of the antigen. If the antigen exists in mg/dl units of measurement, then an assay with a corresponding sensitivity level will be satisfactory for quantitation. Thirdly, the precision characteristics involve assessment of the intra- and interassay variability. If these are reasonably low, then the measurements should be reproducible and the method reliable for quantitating the antigen accurately. Fourthly, the assay must be standardized, ideally in the laboratory of eventual use, against a reference method established for the quantitation of the antigen. In the course of the validation procedure, interfering factors must be sought and appraised for their potential to affect the performance of the system.

The present method was evaluated with these principles in mind. To

validate the method, comparisons were made with an RID assay, which is the standard method used for quantitating apo B in this laboratory. The RID method, however, was not entirely satisfactory for this purpose as outlined in the text. Nevertheless, it proved to be adequate for establishing the fundamental principles of INA for the quantitation of serum apo B and in identifying those areas requiring further evaluation. In this sense, the present study represents a preliminary evaluation of immunonephalometry as an assay for serum apo B quantitation.

The research will be presented in the following form. In the initial section, the general procedures used throughout the study are described. Preliminary experiments dealing with the characterization of the precipitan curve and establishment of working conditions for the immunoassay are presented next. The performance characteristics of the assay are then described. The validation procedure is then detailed in four separate parts. Finally, a general discussion of the experimental work is presented, followed by a summary of the major findings and conclusions.

II. General Procedures

1. Laboratory Methods

(a) Isolation of LDL apo B (d=1.020-1.050 gm/ml)

A narrow band of LDL (d=1.020-1.050 gm/ml) was isolated from serum by conventional methods (88). This fraction has been shown to contain apo B virtually uncontaminated by other protein (93-94). Briefly, blood was collected in vacuum packed tubes by antecubital venipuncture from fasting (10-14 hours) healthy, normolipemic donors, and allowed to clot for 1-2 hours at room temperature. Serum was separated by low speed centrifugation (2500 g) for 20 minutes at 4°C. The serum was pooled and the density adjusted to 1.020 gm/ml by the addition of NaCl-KBr solution. The pooled serum was then fractionated by preparative ultracentrifugation for 20 hours at 105,000 g, 4°C in a Beckman model L2-65 untracentrifuge using a Ti-50 rotor. At the end of 20 hours, the top 2-3 ml cpntaining the d<1.020 lipoprotein fraction was removed by pasteur pipette and discarded. The infranatant, containing the d>1.020 serum fraction was removed by a clean pipette and transferred to a graduated cylinder. The volume of the 1.020 bottom fraction was measured and the density adjusted to 1.050 gm/ml by the addition of NaCl-KBr solution and ultracentrifuged for 24 hours at 105,000 g, 4°C. The supernatant containing the d=1.020-1.050 gm/ml lipoprotein fraction was removed and 'washed' using d=1.050 gm/ml NaCl-KBr by an additional ultracentrifugation for 24 hours. The top 1-2 ml containing

LDL, d=1.020-1.050 gm/ml, was removed by pipette and transferred to a clean container.

(b) Preparation of LDL apo B Reference Standards

After isolation, LDL (d=1.020-1.050 gm/ml) was dialyzed in the dark overnight vs normal saline (d=1.006 gm/ml, 0.195 M, pH=7.40) containing 1 mg/ml disodium EDTA as preservative. The dialyzed lipoproteins were then filtered through a 0.24 μm (average pore size) filter (Millipore Corporation, Bedford, Ma) into sterilized tubes. This constituted the 'stock' LDL apo B solution. Contamination of stock LDL by albumin or soluble apoproteins was ruled out by Oucherterlony immunodiffusion. The concentration of protein in the stock LDL was determined colorimetrically by the method of Lowry et al (95) using bovine serum albumin (BSA) as the protein standard. No chromogenic correction factor was used to correct LDL apo B values. Reference LDL standards were then prepared by serial dilution of stock LDL with filtered (0.24 μm, average pore size) salt solution (d=1.006 gm/ml, 0.195 M, pH=7.40). A range of standards were prepared (20-200 mg/dl apo B). The concentration of apo B protein in each standard so prepared was determined by the Lowry method. All LDL standards were stored at 4°C in sterilized test tubes until utilized.

(c) Preparation of Anti-LDL Antiserum

Antiserum to LDL apo B was prepared in rabbits. A total of 1.0 mg

of LDL (d=1.020-1.050 gm/ml) emulsified in Freunds' complete adjuvant 1:1 (v/v) was given subcutaneously to rabbits in five divided doses at weekly intervals. The animals were bled at six weeks. Antiserum was obtained by low speed centrifugation. The separated sera was filtered twice through a 0.24 μm Millipore filter and stored at -20°C with 0.02% sodium azide added as preservative. The specificity of the antiserum for LDL apo B was determined by the Ouchterlony immunodiffusion technique and immunoelectrophoresis. When examined by the Ouchterlony technique, there was no reaction of the antiserum against HDL, albumin or the d>1.050 infranate. Only a single precipitan arc was seen by immunoelectrophoresis against whole plasma and LDL.

(d) Preparation of Diluent

For all nephalometric procedures, dilutions of antigen and antiserum were made in phosphate-buffered saline ({PBS}, 0.01 M in phosphate, 0.15 M in NaCl, pH=7.40). The selection of this particular buffer is discussed elsewhere (154). The diluted buffer was twice filtered through a 0.24 μm Millipore filter and stored at room temperature. Periodic refiltering of the buffer was necessary to reduce extraneous light scattering caused by dust particles.

(e) Preparation of Lipoprotein-Free Serum (LFS)

Serum was collected from healthy, normolipemic donors. The density

of the pooled serum was adjusted to d=1.0250 by the addition of NaCl-KBr. The pooled serum was then fractionated by preparative ultracentrifugation for 24 hours at 105,000 g and 4°C. At the end of 24 hours, the top 3-4 ml containing the d<1.250 supernatant was removed by pasteur pipette. The infranatant, containing the d>1.250 gm/ml LFS was washed using d=1.250 gm/ml NaCl-KBr by an additional ultracentrifugation for 24 hours. After the second 24 hour run, the top 3-4 ml was removed. The infranatant was removed and dialyzed back to the original serum density (d=1.006 gm/ml) over three days against d=1.006 gm/ml NaCl containing 1 mg/ml EDTA, with a minimum of two changes of dialysate per day. The d=1.006 gm/ml LFS was then twice filtered through an 0.24 μm Millipore filter. The immunoreactivity of the LFS for apo B was checked by Oucherlony immunodiffusion and immunoelectrophoresis and found to be negative. The protein concentration of the LFS, determined by the Lowry method, was 6.0 gm/dl. The serum was stored in sterile plastic tubes with 0.02% sodium azide added as preservative and frozen at -20°C until needed.

(f) Preparation of LDL apo B Reference Standards in LFS

Stock LDL was serially diluted by pipette with LFS to prepare a range of LDL standards. The apo B concentration in each standard was then verified by radial immunodiffusion assay (21). All standards were stored in sterile tubes at 4°C until needed.

(g) Preparation of d<1.020 (top) and d>1.020 (bottom) Serum Samples

Serum was obtained from fasting, normolipemic subjects. Two mls of serum was placed in an 8.0 ml cellulose nitrate tube. The density of the serum was adjusted to 1.020 gm/ml by the addition of NaCl-KBr solution. The volume was then raised to 8.0 ml by the addition of d=1.020 gm/ml NaCl-KBr. Ultracentrifugation was performed at 105,000 g for 20 hours at 4°C. The 'top' fraction, containing d<1.020 gm/ml lipoprotein, was removed by pasteur pipette and transferred to a graduated test tube. The 'bottom' fraction, containing d>1.020 gm/ml serum, was similarly removed and transferred to a second graduated test tube. The volume of the top and bottom fractions were measured separately. The combined volume for all samples was always within 0.1 ml of the original volume (8.0 ml), so the loss of lipoproteins by this method was negligible. The lipoproteins were then transferred to sterilized test tubes and stored at 4°C until processing.

(h) Preparation of d<1.006 (top) and d>1.006 (bottom) Serum Samples

An aliquot of serum from fasting, normolipemic donors was used for this purpose. VLDL (d<1.006 gm/ml) was removed by preparative ultracentrifugation as follows. 2.0 ml of serum was placed into an 8.0 ml cellulose nitrate tube, overlayed with 6.0 ml of d=1.006 gm/ml NaCl (0.15 M), and ultracentrifuged at 105,00 g for 20 hours at 4°C. The top 2-3 ml containing d<1.006 gm/ml lipoproteins (VLDL) was removed by pasteur pip-

ette and transferred to a graduated test tube. The bottom fraction was similarly removed and the volume of each fraction was determined separately. The lipoproteins were then transferred to sterilized test tubes and stored at 4°C until processing.

(i) Quantitation of apo B in the d<1.020, d<1.006 (VLDL) and d=1.006-1.020 (IDL) Lipoprotein Fractions

Apo B in the d<1.020, d<1.006 (VLDL) and d=1.006-1.020 (IDL) lipoprotein fractions was estimated colorimetrically (95) following extraction of apo B according to the isopropanol technique of Holmquiest et al (91-92). The d<1.020 and d<1.006 gm/ml top lipoprotein fractions were isolated by ultracentrifugation as previously described. 0.5 ml of 1.020 or 1.006 top lipoprotein was mixed with 0.5 ml of isopropanol (Sigma Chemical Company), vortexed for one minute, and allowed to stand for 15 minutes at room temperature. The soluble protein was separated from insoluble, precipitated apo B by centrifugation for 10 minutes at 12,000 g. The clear supernatant containing the soluble protein was removed for quantitation. Protein determination was performed by the Lowry method. VLDL apo B was estimated by subtracting the quantity of soluble protein in the 1.006 top fraction from the total protein contained in the 1.006 top fraction. The difference, representing isopropanol insoluble protein, is an estimate of VLDL apo B. IDL (d=1.006-1.020 gm/ml) apo B was estimated by subtracting the apo B found in VLDL from the total apo B contained in the 1.020 top fraction.

2. Specimens

Blood samples for lipoprotein analysis were obtained from fasting, healthy, normolipemic donors, most of whom were medical students. The age of the subjects ranged between 18 and 40 years.

3. Instrumentation

Light scattering was analyzed using a Hyland laser nephalometer (Hyland Division, Travenol Laboratories, Costa Mesa, Ca) according to the manufacturers' recommended procedures (137).

4. Analysis of Data

Lipid and apoprotein measurements were analyzed using linear regression analysis. The statistical significance of mean data was determined using the paired, two-tailed Students' t-test.

III. Characterization of the Precipitan Curve for LDL apo B-Anti-LDL

1. Introduction

Of primary importance to the development of an immunoassay in aqueous solution, is the characterization of the precipitan curve for the antigen-antibody system in question. In these experiments, various concentrations of LDL apo B were allowed to react with different concentrations of anti-LDL apo B antibodies, and the resulting light scattering from the formation of immune complexes was analyzed with the nephelometer. The purpose of this study was to define appropriate conditions for the construction of a reference curve for the immunoassay.

2. Materials and Methods

The antigen (Ag) and antiserum (As) were prepared as previously described. Five different concentrations of Ag, corresponding to 32, 42, 57, 99, and 143 mg/dl of apo B, were selected for study. The choice of these particular concentrations of LDL standards was somewhat arbitrary, but tended to span the normal range of LDL apo B found in serum. In experiment A, 0.025 ml (25λ) of each concentration of Ag was allowed to react with four different dilutions of antiserum (1:20, 1:40, 1:80, and 1:100). In experiment B, 0.005 ml (5λ) of each concentration of Ag was added to the same dilutions of antiserum. The final dilution of the Ag in experiment A was ≅ 1:40 and ≅1:200 in experiment B. Antiserum was prepared by

dilution in PBS. For immunoassay, 25λ or 5λ of Ag was added to a 10 by 75 mm disposable glass tube. 1.0 ml of diluted antiserum was then added, and this constituted time zero. Tubes containing the Ag-antibody (Ab) mixtures were prepared in duplicate. Each tube was gently mixed by inversion and allowed to react at room temperature. The following 'blanks' were also prepared; (1) a buffer blank, containing 1.0 ml of PBS, which was used to calibrate the instrument; (2) an antiserum blank, which contained 1.0 ml of diluted antiserum; (3) an antigen blank, prepared by diluting 25λ or 5λ of each standard in 1.0 ml of buffer. All reagents were dispensed by fixed volume Eppendorf pipettes. Light scattering of the free and combined complexes was analyzed by nephelometry after equilibration had been reached for all reaction mixtures (≅ two hours). The selection of this endpoint for measurement is considered in the next section of the paper.

3. Results

(a) Calculation of *net* Relative Light Scattering (ΔRLS)

ΔRLS, is defined as the light scattering attributable to the formation of Ag-Ab immune complexes. This was calculated by subtracting the RLS produced by the background (Ag and As blanks) from the total RLS produced by the immune complexes and background combined:

$$\Delta RLS = \text{Total RLS} - \text{Background RLS}$$

$$\Delta RLS = RLS\ \{(Ag\text{-}Ab)+(Ag)+(As)\} - RLS\ \{(Ag)+(As)\}$$

where Ag-Ab, represents the immune complexes, Ag, the free antigen, and

As, the antiserum. The mean Ag-Ab value from two separate determinations was used for the calculation.

(b) Contribution of the Background to ΔRLS

Tables 5 and 6 show, respectively, the light scattering produced by the Ag and As in solution alone. Inspection of table 5 shows that the light scattering produced by 25λ of Ag is greater than that produced by 5λ of Ag for each concentration of LDL standard considered. Light scattering tends to decrease with decreasing concentration of LDL apo B. Table 6 shows the light scattering caused by the antiserum diluted in buffer. The light scattering is highest with the lowest dilution of antiserum, and decreases with increasing dilutions of antiserum. The total background was highest in the reaction mixtures containing high concentrations of Ag and low dilutions of As. For the conditions tested in these experiments, the As tended to contribute more to the total background count than the Ag, particularly where low concentrations of Ag were involved. However, the background in all nearly all the reaction mixtures was small in comparison to the light scattering produced by the immune complexes, usually amounting to between 2-4% of the total RLS. When the conditions were such that there were very low concentrations of Ag and low dilutions of As, for example, 5λ of 23 mg/dl of Ag and a 1:20 dilution of As, the background seemed to contribute disproportionately to the total RLS. This was due to the scant amount of light scattering produced by the immune complexes under this condition (table 7).

(c) Precipitan Curve for LDL apo B-Anti LDL

Table 7 shows the ΔRLS for each reaction mixture considered and figures 1 and 2 illustrate the data plotted graphically. In the figures, the ordinate represents the ΔRLS after equilibrium was achieved between the various concentrations of Ag mixed with various dilutions of As. The abscissa shows the concentration of the Ag (mg/dl) in the reaction mixture.

When incubation of 25λ of Ag was carried out in a 1:100 dilution of As, light scattering was observed to decrease with stepwise increases in Ag concentration (figure 1). With lower dilutions of As (1:80, 1:40), RLS increases, then decreases with increasing concentration of Ag. With a 1:40 dilution of antiserum, the peak RLS occurs at a higher Ag concentration than with the 1:80 mixture. Finally, at a 1:20 dilution of As, light scattering increases fairly linearly over the entire range of Ag concentrations.

In figure 2, the results of experiment B are illustrated. In this experiment 5λ of Ag was added to each reaction mixture instead of 25λ. The concentration of Ag in each reaction mixture was therefore five fold less in experiment B than in experiment A. Inspection of figure 2 shows that light scattering increases over the entire range of Ag concentrations for all dilutions of As used. The ΔRLS at comparable Ag concentrations is higher with each stepwise decrease in As dilution, although the differences in light scattering were greater at the higher than lower antigen

concentrations. There was a tendency for the lower end of the precipitan curves to fall in the mixtures containing low dilutions of As. Light scattering appears to be linearly related to Ag concentration for the reaction mixtures containing the highest dilutions of As.

4. Discussion

In these experiments, the immunoreactivity of LDL apo B-anti LDL was evaluated in a light scattering system. The aim of these experiments was to define the conditions necessary for the production of a linear precipitan curve. A linear relationship indicates that the Ag/Ab ratio is optimal for the range of reference concentrations considered. A wide range of Ag and Ab concentrations with varying Ag/Ab ratios was used for this purpose.

In experiment A, 25λ of Ag was used in each reaction mixture. Using a 1:100 dilution of As resulted in a decrease in light scattering with increasing Ag concentrations (figure 1). This indicates a state of Ag excess where the concentration of Ab is insufficient relative to the concentration of Ag (136). Antibody, in this case combines with Ag in such a way as to hinder the formation of a lattice network of insoluble Ag-Ab complexes (155). The Ag molecule with many binding sites combines with the Ab to form relatively small soluble complexes, inhibiting the formation of a lattice (155). These small soluble complexes may not be detected by nephalometry (136). Antigen excess can occur whenever the

concentration of Ag exceeds the capacity of the As to form an insoluble lattice. As the ratio of Ab/As is increased, accomplished in this experiment by decreasing the dilution of As, the point of equivalence shifts toward higher concentrations of Ag. All points up to the point of equivalence are in Ab excess (155). Finally, increasing the Ab/Ag ratio still further (1:20 dilution) shifts the highest Ag concentration to the Ab excess side of the curve. The Ab excess side is the analytical working range of the precipitan curve since light scattering under this condition is proportional to the concentration of Ag in the test solution (136).

In experiment B, the Ag concentration of each LDL standard added was five fold less than the concentration used in experiment A because of a five fold reduction in the volume of the Ag used (5λ vs 25λ). Under these conditions, light scattering was observed to increase with stepwise increases in LDL concentrations for all dilutions of As used. However, only the precipitan curves obtained using the higher dilutions of As (1:80 and 1:100), appeared to be linear (figure 2). Samples with low concentrations of Ag in the reaction mixture (high Ab/Ag ratios) may also produce soluble complexes which cannot be accurately quantitated by nephalometry (136). In this instance, the Ab may block all of the reactive sites on the Ag thereby preventing lattice formation (155). The tendency for the low end of the precipitan curve under conditions involving low dilutions of As to fall off (figure 3), may be reflecting this characteristic of the immunoprecipitan reaction.

Clearly, there is an optimal ratio of Ag and Ab for each range of LDL standards considered. This ratio can only be determined empirically from experiments such as these. For the conditions tested in these experiments, a 1:20 dilution of As for 25λ of Ag and a 1:100 or 1:80 dilution for 5λ of Ag seemed to represent appropriate relationships as judged by the linearity observed between light scattering and Ag concentration for the range of LDL standards used. In fact, the precipitan curves produced under the conditions involving 25λ, 1:20 and 5λ, 1:100 mixtures are virtually superimposible (figure 3). This finding is expected since the ratio of Ag/Ab in these two instances is identical. Thus, a five fold higher Ag concentration requires a five fold higher Ab concentration as one fifth the concentration of Ag and Ab. The response, of course, is a five fold increase in light scattering. This means, therefore, that between the two conditions shown here, appropriate Ag/Ab ratios should result in the production of linear precipitan curves.

The range of the standard curve considered for this system is predicated on a number of factors. Firstly, the range of the standards should correspond to the range of the Ag concentrations found in vivo to minimize the error associated with excess concentration or dilution of serum samples. Secondly, the dilution of the As should be as high as possible for maximal conservation of this reagent. Thirdly, the sensitivity of measurement in this system is to some extent dependent on the range of the standard curve. This is so because the number of RLS units is fixed for any given sensitivity setting of the instrument[2]. A narrow range

standard curve would theoretically increase the sensitivity of measurement by maximizing the ratio of RLS/Ag units. Lastly, the error of measurement associated with pipetting is potentially greater with 5λ than 25λ of sample given that the fixed error of the pipettes is the same.

A consideration of these factors resulted in the following decisions concerning the construction of the standard curve. A range of LDL standards between approximately 20 and 100 mg/dl of apo B was selected. The volume of Ag in the standards and samples would be 5λ. These conditions seemed to be a reasonable compromise of all the factors. This would allow for maximal conservation of As and a 1:1 dilution of the serum samples prior to immunoassay would ensure that all but the extremes of serum Ag concentrations, would fall on the standard curve. Antigen concentrations beyond the upper limit of the standard curve (i.e. greater than 200 mg/dl), are easily detectable during immunoassay (137). The higher Ag concentrations would be further diluted. Moreover, the majority of the points would tend to fall on the mid portion of the curve where the error of measurement is the least. A narrow range of standards would also aim at keeping the sensitivity of measurement high. Finally, in direct testing, the pipetting error using 25λ of Ag was comparable to that of 5λ, and in both cases represented a negligible source of error (data not shown).

Although light scattering produced by Ag-Ab complexes has been shown to conform to a third order polynomial equation (156), it has also been demonstrated that data analyzed by manual and polynomial curve fitting

correlate highly (156). It therefore seemed reasonable to use the simpler hand drawn reference curve for the analysis of data.

The background light scatter produced by the free Ag and As particles was small relative to that produced by the immune complexes. However, it is the usual practice in end-point nephalometry (136) to measure the light scatter from both the serum and antiserum, each diluted to the final working conditions, to establish a background value. The blank values are then subtracted from the total light scattering of the reacted samples to yield the value of light scattering by the Ag-Ab complexes (ΔRLS). This practice was followed for the calculation of ΔRLS in all subsequent experiments.

It should be noted that because the Ab titer tends to vary from batch to batch of antiserum, each new batch of antiserum was tested to determine the appropriate dilution for use in the immunoassay. The dilution of As used throughout these experiments ranged between 1:80-1:100.

The protocol for routine immunoassay of serum samples formulated by the principles established in these experiments is shown in table 8. Unless otherwise stated, this procedure was followed for immunoassay in all subsequent experiments.

IV. Time Course of LDL apo B-Anti-LDL Immunoreactivity

1. Introduction

This experiment was undertaken to characterize immunoreactivity of LDL apo B-anti-LDL over time. In particular, the aim of the study was to determine an end-point in time for measurement of light scattering.

2. Materials and Methods

The Ag and As were prepared as previously described. Six different LDL standards corresponding to 20, 40, 61, 80, 91 and 104 mg/dl apo B were selected for study. Three serum samples corresponding to high, medium and low apo B concentrations, relative to those of the standards, were also evaluated. The concentration of apo B in the serum samples was determined by a RID assay as previously described (21). Standards and samples were immunoassayed according to the protocol outlined in table 8. Light scattering was measured at 15 minute intervals for the first two hours of reaction, and at 30 minute intervals for an additional two hours. ΔRLS was calculated as previously described.

3. Results

The results of these experiments are shown in figures 4 through 6.

Figure 4 shows the light scattering for the six LDL standards over time. The rate of change of light scattering (dC/dt), is most rapid during the first 15 minutes of reaction, particularly for the LDL standards containing high concentrations of Ag. For the reaction mixtures containing lower Ag concentrations, the initial dC/dt is less. Light scattering tends to become constant after about 45 minutes for the higher Ag concentrations, and 75-90 minutes for the lower Ag concentrations. The steady state is then maintained over the next 2-3 hours. There does not appear to be more variability in light scattering for the higher or lower, compared to the middle Ag concentrations.

The characteristics of the light scattering over time for the three serum samples is essentially the same as that seen for the LDL standards (figure 5).

4. Discussion

Quantitative measurements of specific proteins by light scattering techniques generally involves two methods; rate or kinetic, and end-point or equilibrium nephelometry (172). In the kinetic model, the initial rate of Ag-Ab complex formation in solution is monitored for the measurement of Ag concentrations. For various reasons that are discussed elsewhere (165, 172), this method was not employed here. The end-point method involves a single measurement when the dC/dt is small, that is, when the steady state is reached. The aim of this experiment, therefore, was to

determine an end point in time for the measurement of light scattering.

Under the Ag-Ab conditions tested here, the reaction mixtures containing higher concentrations of Ag demonstrated a higher dC/dt and appeared to reach equilibrium sooner than those mixtures containing lower concentrations of Ag. The difference in the initial rate of immune complex formation is presumably a function of the Ag concentration since the dilution of antiserum was held constant for all the reactions. Indeed, at a constant Ab concentration, the rate of increase in complex number and size is directly proportional to the Ag concentration throughout a range of moderate Ab excess (172). Thus with high concentrations of Ab, the random collisions between the solute particles are more frequent and this results in an enhanced rate of immune complex formation. As the combining sites on the Ag and Ab molecules become saturated, the formation of immune complexes slows although light scattering continues to increase mainly due to increases in the size of the complexes (172). At equilibrium, the dC/dt is very small as any further increase in the size of the complexes is small. In this system, equilibrium was reached for all the reaction mixtures after approximately 75-90 minutes; thereafter, the steady state condition was maintained for at least another 2-3 hours. This was assessed by the visual inspection of the data, and by observing that the equations for the regression lines calculated at the various time intervals were virtually the same between 90 minutes and four hours (data not shown).

The immunoreactivity of the serum samples and LDL standards over

time appeared to be very similar. As well, there was no significant change in the Ag or As blanks over time. The regression equations for the precipitan curves became constant after 75 minutes. The concentration of apo B in the serum samples tested calculated from the standard curves showed only minor variation after 75 minutes (data not shown). Hence, the light scattering properties of the immune complexes and the conditions of the immunoassay appear to be relatively constant after about 75 minutes remaining so for at least another 2-3 hours. Therefore two hours was selected as the end-point for measurement of light scattering. This allowed for a relatively short incubation period, but at the same time permitted considerable leeway in the actual time of measurement without any effect on the accuracy of quantitation. An example of the precipitan curve constructed after two hours of reaction is shown in figure 6.

The major advantage of end-point nephelometry is its simplicity. However, with a single-point measurement, the Ag and As blanks must be measured as well to correct for non-specific light scattering. Further, a small error in the light scattering measurement of 2-3% can be expected due to variation in the disposable tubes used. This aspect is considered further in the next section.

V. Performance Characteristics

1. Specificity

The specificity of the immunoassay for apo B was guaranteed by raising a monospecific antiserum to the Ag. When examined by the Ouchterlony technique (170), there was no reaction of the antisera against HDL, albumin, or the d>1.050 infranatant. Rabbit anti-LDL serum produced only a single precipitan line against whole serum or LDL on immunoelectrophoresis in agarose gel (175).

2. Sensitivity

The conditions of the immunoassay were selected for the measurement of serum levels of apo B. The lower limit of detectable Ag concentration in direct testing was 5-10 mg/dl (data not shown), which was well in excess of that required to measure apo B in serum. The minimal detectable Ag concentration attainable was not determined, but the assay was probably more sensitive than reported here. Indeed, the system has been used to measure as little as 5-10 µg/ml of Ag (172). In a separate experiment (see section IX), in which 11.5 mg/dl of LDL apo B was added to a series of d1.02 bottom serum samples, the recovery of the added apo B was high, averaging 97% of the expected value. As well, the correlation between the observed and expected values was high, r=0.98 (see table 30 and figure 18). Thus, the assay was extremely accurate for discriminating 10 mg/dl

increments of apo B.

3. Precision

Within-run and between-run precision was evaluated by analyzing a control specimen of serum containing normal concentrations of apo B and lipids.

(a) Materials and Methods

The within-run precision was determined using a serum sample with a mid range concentration of apo B relative to that of the reference curve. The apo B concentration of the serum sample (90 mg/dl), was determined by the RID assay as described elsewhere (21). Samples were diluted 1:1 in buffer, prepared in duplicate and assayed according to the protocol established for the immunoassay. A total of 16 paired aliquots were prepared for analysis. Light scattering was measured after two hours of reaction.

To evaluate the day to day precision, the same control serum specimen was assayed in duplicate in 14 separate runs over a two week period.

(b) Results

Table 9 shows the mean apo B values of paired sera for the 16 al-

iquots of serum determined during the same assay. The mean values range between 139 and 152 mg/dl with an overall mean of 146 mg/dl. The intra-assay coefficient of variation was 4%.

Table 10 shows the mean apo B values of paired sera determined by 14 separate immunoassays. The values range between 140 and 149 mg/dl with an overall mean of 144 mg/dl. The interassay coefficient of variation was 3 %.

(c) Discussion

The within-run coefficient of variation was slightly higher than the between-run coefficient of variation although both were within the range of those reported by others using the same (86) and other (17, 20, 25) methods for apo B analysis. The combined intra- and interassay coefficient of variation was about 7% which is lower than that found by most other methods (16-17, 20, 25). Rosseneu (33), recently reported a combined coefficient of variation of 6-7% for her INA method. These results indicate that measurements of apo B by this method are highly reproducible, and the method therefore appears reliable for this purpose.

4. Quality Control

The control serum was run in duplicate with each assay. The mean value should be within ±7% of the mean interassay value to be acceptable.

VI. Quantitation of Serum apo B by INA --Part 1

1. Introduction

The next series of experiments represents an initial evaluation of INA as a method for the quantitation of serum apo B. To validate the assay, serum apo B levels quantitated by INA were compared to those measured by a reference method, RID. Since light scattering is dependent on particle size as well as the concentration of the Ag, it was anticipated that the larger VLDL particles in serum might interefere with the performance of the immunoassay. Thus, in addition to whole serum measurements, serum in which VLDL was first removed by ultracentrifugation was also analyzed and compared.

2. Materials and Methods

Blood was collected from 12 fasting, healthy volunteers by antecubital venipuncture and serum was obtained by low speed centrifugation. An aliquot of serum was fractionated by preparative ultracentrifugation to remove VLDL, as previously described. The concentration of apo B in the d1.006 top lipoprotein fraction was estimated colorimetrically after extraction of the insoluble protein by the isopropanol method (91-92) as described earlier. INA of whole serum and d1.006 bottom serum fraction was performed according to the established protocol. The d1.006 bottom samples were assayed without prior dilution. RID assay of the serum

samples was performed as described elsewhere (21). All samples were stored at 4°C and all assays were performed within two weeks of the collection of samples.

3. Results

The results of these experiments are shown in tables 11 and 12, and figures 7 through 9.

Table 11 compares the results of the immunoassays performed on the 12 serum samples. The data presented is the mean values for two separate determinations. The mean total serum apo B concentration for the 12 samples estimated by INA was 156 mg/dl. This value was significantly higher ($p<.005$) than the value found by RID assay of whole serum (108 mg/dl) and INA of the d1.006 bottom serum fraction (130 mg/dl). The mean value found by INA on the d1.006 bottom fraction was also significantly higher ($p<.005$) than the mean value found on whole serum by RID assay.

The correlation between the serum values by the two methods was good ($r=0.90$), as was the correlation between the d1.006 bottom serum samples assayed by INA, and the whole serum samples measured by RID ($r= 0.82$). The whole serum and d1.006 bottom samples assayed by INA correlated less well ($r=0.63$), figures 7-9.

Table 12 shows the individual and mean values for VLDL apo B esti-

mated on a subset of 9 samples. The mean VLDL apo B level was 7.6 mg/dl.

4. Discussion

These experiments were undertaken as an initial evaluation of immunonephelometry as a method for the quantitation of serum apo B concentrations. For the purpose of standardization, an RID assay was used as the reference method for comparisons. The mean level of apo B was significantly higher using INA, although the two methods appeared to correlate fairly well. There are several explanations for these findings. Firstly, the RID method as developed and routinely used in this laboratory, is fairly specific for apo B in the d>1.020 serum fraction (LDL). It is much less accurate in quantitating apo B in the d<1.020 fraction and is relatively insensitive to apo B contained in VLDL (d<1.006), (21). On the other hand, INA measures the total serum apo B concentration (VLDL+IDL+LDL). Although most of the apo B in serum is contained in LDL (table 4), a significant but variable amount is also contained in the IDL and VLDL fractions. In normolipemic subjects, the amount of apo B in the d<1.020 lipoprotein fraction represents between 5-15% of the total or 5-15 mg/dl of apo B. However, the concentration of apo B in this fraction may increase considerably particularly in hypertriglyceridemia (VLDL), (14, 17, 24, 55), and type III hyperlipoproteinemia (IDL), (24). Although serum lipid measurements were not made in these subjects, all were young and healthy individuals, and it is extremely unlikely than any of them would have displayed significant hyperlipemia. For these reasons, the

estimates of serum apo B levels by the two methods would be expected to differ by perhaps 10-20% or an amount equivalent to the concentration of apo B present in the d<1.020 lipoprotein fraction. However the concentration of apo B measured by INA was found to be nearly 50% higher than the value found by the RID method. This suggests that other, and possibly more important factors, were responsible for the differences observed.

Because the size of IDL and particularly VLDL are much larger than LDL particles (79, 153), the larger VLDL particles in serum may have caused non-specific light scattering. This effect would tend to falsely increase the estimate of apo B by the INA method. That this effect may have contributed to the higher serum values, is suggested by the significantly lower mean value observed for the d1.006 bottom serum fraction of the same samples, where the effect of VLDL was removed by ultracentrifugation. However, even with the removal of VLDL, The mean value found for the d1.006 bottom samples was still significantly higher than the mean serum estimate found by RID assay. To determine the relative contribution of VLDL apo B versus VLDL particle size to the large (26 mg/dl) difference existing between the fractionated and unfractionated serum estimates by INA, the content of apo B was quantitated in a subset of 9 samples. The mean apo B value found in these samples was 7.6 mg/dl, which agrees well with the estimate of apo B found in this fraction in other studies (table 4). Adding the amount of apo B contained in VLDL to the amount present in the d1.006 bottom serum fraction, gives an approximation of the total apo B content in serum. For the nine samples considered, this amounted

to 136 mg/dl (table 12). Table 12 also shows the whole serum estimate for the same samples measured by the INA method (156 mg/dl). The difference between the two values, amounting to 20 mg/dl, was significant ($p<.01$). The higher estimate found in whole serum may to some extent be explained by the non-specific light scattering caused by the larger VLDL particles present in the serum samples. The experimental design, however, did not allow for further exploration of this effect.

Still other factors were thought to be responsible for the higher serum values found by INA as exemplified by the unexpected large difference in mean values observed between the serum samples measured by the RID assay, and the d1.006 bottom samples measured by the INA method. The difference between these two values was 22 mg/dl, which is similar to that observed between the whole serum and d1.006 bottom values. In theory, one would expect the difference between the serum RID and d1.006 bottom INA values to be much smaller if the contribution of IDL to these estimates were significant, since both the concentration of apo B and the size of the particles are less than that of VLDL (93, 178). One explanation for the large discrepancy between the values, and serving to explain the previous difference as well, may have been related to a difference existing between the LDL standards and the serum samples. The LDL standards used for calibration of the INA precipitan curve were prepared by dilution of stock LDL in serum-free salt solution, which is the usual practice involved for preparation of the standards used in the RID assay. One wonders if the absence of serum in the LDL standards might have led to differences

in the formation and light scattering of the immune complexes in these solutions. For example, if the LDL standards produced less light scattering than the serum samples, for equivalent concentrations of apo B, then the level of apo B would tend to be overestimated if calculated from such a standard curve. The differences in immune complex formation and light scattering is presumably related to a difference in the protein concentration, which was nearly an order of a magnitude less in the standards compared to the samples. The effect of protein, through protein-protein interactions, may be more important to the formation of immune complexes in aqueous solution compared to semi-solid media. The necessity of having standards and samples under closely similar conditions has been emphasized by others (20, 77, 176).

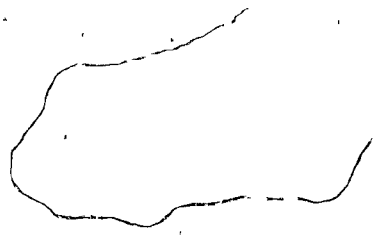
Another factor contributing to the differences seen, may have been related to protein losses encountered during the processing of samples, particularly with ultracentrifugation. In previous experiments (49), using similar techniques, the recovery of apo B in the d1.006 top and bottom fractions, compared to apo B contained in the unfractionated serum was 95% or greater. Thus handling losses, which in these experiments, was not directly estimated, might have been expected to account for a 5-6 mg/dl difference in values. The lower correlation found between the serum and d1.006 bottom values by the RID and INA methods respectively, compared to the whole serum estimates by RID and INA, suggests that the technical error may have been significant, since the expected finding would be an improvement in the correlation with the removal of VLDL.

On the other hand, a significant loss of protein from the d1.006 bottom samples would have had the effect of reducing the difference between the RID and INA values. The fact that the difference between these two means was so large, argues against significant protein loss through handling. Although the effect of handling losses to these differences are not clear, they are probably too small to be of major importance.

Finally, consideration must be given to the methods themselves, since they rely on different principles for the quantitation of protein. INA depends on the random interaction of Ag and Ab mixtures in aqueous solution, whereas RID involves the diffusion of Ag from a well into agarose gel impregnated with Ab. The formation of the immune complexes are then detected by different techniques in the two methods. In the aqueous system, the immunocomplexes scatter light, which is detectable in a matter of minutes. In the gel medium, the formation of immune complexes leads to a visible precipitan ring only after several hours. It is not known, for example, if the antigenic sites of LDL or its immunoreactivity are the same or different for the conditions of the two assays. Differences in the behavior of the reactants at the molecular level may lead to different quantitative estimates of apo B for an equivalent mass of protein. If the relation between the two methods for the quantitation of apo B was significantly different from 1:1, then such an effect would also serve to explain the differences found here.

In summary, the mean serum levels of apo B were significantly high-

er when estimated by the INA compared to the RID method. The estimates of apo B in the d1.006 bottom fraction was also significantly higher than the serum estimate found by RID, and significantly lower than the whole serum estimate found by INA. Even though the sample number was small due to logistical constraints, the differences that existed seemed clear. What was unclear, however, was the exact reason for these differences. The various factors that may have been involved were discussed. It is probable that a combination of factors are operative. The experimental design did not allow for a specific analysis of these factors. The extent to which some of these variables were involved was therefore examined in the next series of experiments.



VII. Quantitation of Serum apo B by INA - Part 2

1. Introduction

In the previous experiments, various factors were postulated to account for the higher levels of apo B found by INA compared to the RID method. These parameters included; (1) differences existing between the LDL standards and the serum samples, specifically related to the absence of serum in the LDL standards, (2) the presence of apo B in the $d \leq 1.020$ fraction of serum, (3) non-specific light scattering caused by the larger VLDL particles in serum, (4) excessive error associated with the the handling of the samples, and (5) inherent differences in the sensitivities of the two methods for the detection of apo B. The following experiments were designed to further evaluate the contribution of these factors.

2. Materials and Methods

Serum and plasma samples were collected from 15 healthy, fasting normolipemic volunteers and processed according to the following protocol:

A. Plasma samples

(a) apo B quantitation

i) INA

B. Serum samples

(a) Lipid analysis

i) cholesterol levels
ii) triglyceride levels

(b) apo B quantitation

1. Whole serum

i) INA
ii) RID

2. d1.020 bottom serum fraction

i) INA
ii) RID

3. d1.020 top lipoprotein fraction

i) Colorimetric protein determination

4. d1.006 bottom serum fraction

i) INA
ii) RID

5. d1.006 top lipoprotein fraction

i) Colorimetric protein determination

Serum cholesterol levels were estimated according to the method of Abell et al (157) with the color reagent of Zak et al (158). Serum triglyceride levels were estimated according to Carlson (159). Lipoprotein-free serum was prepared as previously described. Details of the serum d1.020 and d1.006 lipoprotein fractionation were also described previously. The concentration of apo B in the d1.020 and d1.006 top fractions was determined colorimetrically after extraction of insoluble protein with isopropanol as described earlier. INA of the serum samples was perform-

ed according to the protocol outlined previously. The d1.006 and d1.020 bottom samples were assayed without prior dilution. Estimation of apo B by RID assay was performed as described elsewhere (21). All samples were stored at 4°C until used, and all assays were performed within two weeks of the collection of samples.

3. Results

The results of this series of experiments is shown in tables 13 through 27 and figures 10 through 17.

In table 13, the individual and mean cholesterol and triglyceride levels are presented for the 15 subjects. The mean cholesterol and triglyceride levels were, respectively, 197 mg/dl and 106 mg/dl. All the individual values were below the 95th percentile (177), although in one subject (number 5), the serum triglyceride approached this level.

Table 14 shows the light scattering for comparable sets of LDL standards prepared separately in LFS and serum-free salt solution. Light scattering is significantly higher ($p<0.005$) for each of the standards prepared in LFS compared to serum-free salt. The increase in light scattering was greater for the lower than the higher concentrations of apo B. Figure 10 shows the standard curves produced after plotting the data. Although the two curves are linear and nearly parallel, the precipitan curve for the LFS-LDL standards is shifted considerably to the left of

the curve for the serum-free LDL standards. This means that for any RLS value, the calculated apo B concentration would be lower when derived from the LFS-LDL standard curve.

In tables 15 and 16, the individual and mean apo B levels are shown for the d1.020 bottom samples assayed by the INA and RID methods. In table 15, the values shown represent the actual apo B concentration in the sample. For the INA method, the individual and mean apo B levels are significantly lower ($p<0.005$) when calculations are made using the LFS-LDL standard curve (62 vs 80 mg/dl). The mean value found by the INA method using the LFS-LDL standard curve is very close to the value found by RID assay (62 vs 60 mg/dl, $p>0.05$). The values found by the two methods are also highly correlated ($r=0.97$, figure 11). The corresponding serum values for apo B in the d1.020 bottom fraction measured by RID and INA using the LFS-LDL standard curve for calculation, are also very similar (112 vs 115 mg/dl, $p>0.05$) and well correlated, $r=0.97$ (see table 16 and figure 12).

Tables 17 and 18 compare the individual and mean concentrations of apo B in the d1.006 bottom fraction measured separately by the two methods[3]. The values obtained by the INA method are significantly higher ($p<0.005$), but correlate well (figure 13).

Table 19 and figure 14 compare the individual and mean levels of apo B measured in whole serum by the two methods. The mean value for the INA method is significantly higher ($p<0.005$), although the values tended to

correlate well (r=0.95).

Table 20 compares the estimates of apo B in serum and plasma found by the INA method. The serum value was slightly higher than the plasma value (138 vs 135 mg/dl), although the difference was not significant. The correlation between the values was high (r=0.99).

In table 21, the individual and mean apo B values found in the d1.020 and d1.006 top lipoprotein fractions is presented. The mean apo B value found in the d1.020 top fraction was 13.5 mg/dl. The mean content of apo B in the d1.006 top fraction was 8.3 mg/dl. Subtraction of the d1.006 from the d1.020 top values, gives an estimate of apo B in the d=1.006-1.020 lipoprotein fraction (IDL). The mean value found in the present study was 5.2 mg/dl. The correlation between VLDL apo B and total serum triglyceride was good (r=0.82, figure 15 and table 27).

Noteworthy of the sample population studied, was the finding of hyperapobetalipoproteinemia in 5 (33.3%) of the subjects.

4. Discussion

In the previous study, the estimate of apo B found by the INA method was significantly higher than that found by the RID assay. Five factors were discussed as possibly accounting for these differences. These parameters included; (1) differences existing between the LDL standards and

the serum samples, (2) apo B present in the d1.020 top fraction, (3) non-specific light scattering caused by the larger VLDL particles, (4) error associated with the processing of the samples, and (5) inherent differences in the sensitivities of the two assays for quantitating apo B. The present series of experiments was therefore designed to shed some light on the relative importance of some of these variables.

(a) The effect of LFS-LDL standards on the quantitation of apo B

The first issue to be resolved, was whether differences existing between the LDL standards and the serum samples contributed to the higher estimate of apo B found by the INA method. The data presented in table 14 and figure 10 appears to answer this question. Table 14 shows that light scattering was greater for comparable concentrations of standards prepared in LFS compared to serum-free salt solution. A difference in the concentration of comparable LDL standards was not responsible for these findings since the estimate of apo B in each standard was checked by RID assay and found to be identical. As well, the LFS was verified to be free of apo B by testing in the RID, Ouchterlony, and INA methods. For these reasons, the difference in light scattering was therefore presumed to be related to the presence of serum in the LFS-LDL standards.

Figure 10 shows the standard curves produced by manually plotting the data. Both curves are linear over the range of concentrations used. The two curves are almost parallel to one another, although the LFS-LDL

standard curve is shifted to the left. In table 15, the apo B values for the d1.020 bottom samples calculated from the two standard curves are compared. The apo B values are significantly lower when calculated from the LFS-LDL standard curve. In the same table, the apo B values for the same samples quantitated by the RID assay are shown. The values found by INA using the LFS-LDL standards, and the RID method are very similar and highly correlated (figure 11). The d1.020 bottom fraction was initially used to compare the two methods, since this fraction contains a single class of lipoprotein containing apo B, LDL. These comparisons are therefore free from the influence of other factors such as differences in the size of particles, and the presence of apo B in lipoproteins that are poorly measured by the RID method. The close agreement of the apo B values found under these conditions by the two methods leads to the following conclusions.

Firstly, preparation of the LDL standards in LFS would appear to be a necessary condition for the standardization of the assay. This requirement is not surprising, since of fundamental importance to an immunoassay, is for the antigen in the standards and samples to exist under similar conditions (77). The addition of LFS to the LDL standards ensures that this requirement is satisfied. A mixture of LDL plus LFS appears therefore as a suitable standard for apo B quantitation by this technique, as its composition and behavior closely resemble those of native serum. The reason for the increase in light scattering with the addition of serum is unclear. The difference is presumably related to the effect of

protein. The antigen and antibody particles in solution are strongly dependent upon protein-protein interaction, and the increased protein concentration may enhance the formation and/or increase the size of the immune complexes. In these experiments, the concentration of protein in the LFS was 6 gm/dl and this approximates the normal serum protein concentration of the samples. The close agreement of the d1.020 values by the two methods suggests that the concentration of protein added to the LDL standards is optimal for the performance of the immunoassay. Nevertheless, it would be of interest to establish the relationship between the concentration of serum protein in the LDL standards and the resultant light scattering. A differential effect of protein concentration could potentially affect the quantitation of apo B. Clearly, this relation needs to be examined further.

The comparable mean values for the d1.020 bottom serum samples found by the two methods also suggests that the sensitivities of the two assays for the quantitation of LDL apo B are the same and roughly 1:1. However, the sensitivities of the two methods for quantitating d<1.020 apo B are quite different (see later).

The data presented here also suggests that the protein-protein interaction, seemingly essential for the performance of the light scattering method, is of only minor consequence to the quantitation of apo B by the RID assay. This point is illustrated by the fact that concentrations of apo B were identical for comparable LFS-LDL and serum-free LDL

standards calibrated by the RID method. The performance of other methods for example, EIA, have been shown to be dependent on the presence of protein in the LDL standards (33, 77), suggesting that the RID method be more closely examined as well.

In summary, the use of LFS-LDL standards appears necessary for the standardization of the immunonephalometric assay. Indeed, a recent report by Rosseneu et al (33), who also compared the effects of LFS-LDL and serum-free LDL standards on the quantitation of serum apo B, showed similar findings. In the present study, using the LFS-LDL standards resulted in a better agreement of apo B values by the two methods. The large difference in the values by the two methods in the previous study are explained to some extent, by the use of different LDL standards to calibrate the reference curve. As detailed below, there are other reasons for these differences as well. However, because of the improvement in the standardization of the assay with the LFS-LDL standards, all subsequent assays used these standards for calibration of the precipitan curve.

(b) Effect of handling losses

In the previous study, the question of handling losses was raised as as a factor contributing to the higher estimates of apo B found by the INA method. It was therefore important to determine if this variable was involved or not in the present study. That there was no excessive error associated with the processing of the samples in the present ex-

periments, was verified by comparing the estimates of apo B by RID assay, in whole serum, and the serum d1.006 and 1.020 bottom samples. Inspection of table 22, shows only minor differences in the mean values found for the fractionated and unfractionated samples. With a significant loss of lipoprotein through ultracentrifugation, the values in the fractionated samples would be expected to be low compared to the unfractionated value. The close agreement of the values suggests that the recovery of apo B in the fractionated samples was nearly complete. In addition, the data also suggests that the RID assay does not accurately quantitate apo B in the d<1.020 serum fraction, with the assumption that there is a significant amount of protein present in this fraction for detection. In this study, estimation of apo B in this fraction by an independent method, revealed that there was a significant amount of protein present (table 21), suggesting that the RID assay was insensitive for quantitating d<1.020 apo B protein. Indeed, this was an intentional feature of this assay as it was originally developed (21). In view of the relative insensitivity of the RID assay for d<1.020 apo B, coupled with the close agreement between the fractionated and unfractionated serum values, further proves that the recovery of apo B in the fractionated samples was high. Therefore excessive handling losses secondary to the processing of samples, would not appear to explain any differences found in the present study.

(c) The effect of other factors on the quantitation of apo B

Immunoassay of the d1.006 bottom serum samples produced somewhat

different findings as compared to the immunoassays performed on the d1.020 bottom samples. The values obtained by the INA method were significantly higher than those found by the RID assay, although the correlation between the two methods remained high (tables 17 and 18, and figure 13). The difference in the mean values found by the two methods amounted to 10 mg/dl of apo B. However, this is considerably less than the 22 mg/dl difference found in the previous study (table 11) and probably reflects the different curves used to calibrate the assays. There are three possible explanations for the difference found in this study; (1) the presence of apo B in the d=1.006-1.020 lipoprotein (IDL) fraction, (2) insensitivity of the RID method for the quantitation of d<1.020 apo B, and (3) non-specific light scattering caused by the larger IDL particles. All three factors were thought to play a role to a variable degree.

Firstly, inspection of table 22, which compares the apo B values found by RID assay on the unfractionated and fractionated serum samples, shows that the values agree very closely. With the understanding that the loss of lipoprotein through the processing of samples was negligible, then the expected finding should have been a stepwise increase in the concentration of apo B through the d1.020 bottom, d1.006 bottom and whole serum fractions. This is anticipated from the knowledge that each of these fractions contains successively more lipoprotein containing apo B. This was not the result however, indicating that the RID method did not accurately quantitate apo B in the d1.020 top fraction. For this reason alone,

the mean d1.006 value quantitated by the two methods, should differ by an amount, equal to the concentration of apo B actually present in the IDL fraction. The higher value is therefore partly explained by IDL apo B, presumably better measured by the INA method.

The estimate of apo B in the IDL fraction was determined by an independent chemical method. In this study, however, the d=1.006-1.020 lipoprotein fraction was not isolated directly. Rather, the apo B in IDL was estimated indirectly by subtracting the amount present in the d1.020 top from the d1.006 top fraction. The difference in these calculations, is an estimate of IDL apo B, and should only be considered as an approximation because of the indirectness of the methods used for determining this value. Table 21 shows the estimates of apo B found in the d1.020 top, d1.006 top, and d=1.006-1.020 lipoprotein fractions. The mean IDL apo B concentration was 5.3 mg/dl. When the apo B found in IDL is added to the amount measured in the d1.006 bottom fraction by the RID method, the combined value compares well with the d1.006 bottom estimate found by INA (table 23). The small difference remaining between the values was not significant ($p>0.05$). Thus, much of the difference existing between the d1.006 bottom RID and INA values is explained by the presence of of IDL apo B, which was not accurately quantitated by the RID method. On the other hand, the INA method does appear to be more accurate for the quantitation of IDL apo B, and this point is illustrated by the findings presented in table 24. This table shows that the combined d1.020 bottom (INA) and IDL estimate of apo B compares well with

the estimate found by INA in the d1.006 bottom fraction. The small difference between the two values is not significant, and therefore, most of the difference between the values by the INA method on the d1.020 and d1.006 bottom samples, is due to LDL apo B which is included in the d1.006 bottom measurement. The small remaining difference is within the range of methodological variability, but may also be partly explained by non-specific light scattering caused by the larger IDL particles.

In these experiments, the light scattering characteristics of the different classes of lipoprotein particles was not directly compared. The larger VLDL and IDL particles, however, are potential sources of non-specific light scattering. If this effect was significant, it would tend to affect the quantitation of serum apo B in the direction of an overestimate. Non-specific light scattering may provide an explanation for the findings presented in table 25. In this table, the apo B values determined in the d1.020 top fraction are added to the corresponding values found in the d1.020 bottom fraction. The combined values are an estimate of the total serum apo B concentrations. The mean value found for the samples in this study was 128 mg/dl and this may be compared with the mean whole serum value of 143 mg/dl found by the INA method. The difference between the two values, amounting to 15 mg/dl, is significant ($p<0.005$). It is plausible to attribute at least part of this difference to non-specific light scattering caused by the larger VLDL and IDL particles present in serum. The effect of VLDL particle size

is further illustrated by the findings presented in table 26. In this case, the apo B estimated in the d1.006 top fraction by chemical determination is added to the apo B found in the d1.006 bottom fraction by INA. The mean level of VLDL apo B found for the samples in this study was 8.3 mg/dl, which is very close to the value found in the previous study (table 12). The combined mean total apo B value is 133 mg/dl. The total serum apo B level found by the INA method is also significantly higher ($p<0.025$) than this value. Once again, the most plausible explanation for this difference is the non-specific light scattering caused by the larger VLDL particles. Comparison of tables 25 and 26 also suggests that the contribution of VLDL to this effect is at least twice that of IDL, which is consistent with the differences in the relative size of these particles (178) as well as their concentrations, as found in this study (table 21). Two additional findings point toward the effect of VLDL. Firstly, the whole serum estimates found by INA, were higher than the combined d1.006 top and bottom values in all but four of the 13 samples, and those with the higher VLDL apo B levels, tended to have larger differences. Moreover, the VLDL apo B levels correlated positively ($r=0.82$) with the serum triglyceride levels (figure 15), which in turn, are related to the size of the VLDL particles (153). It is therefore reasonable to suggest that the larger VLDL particles, through an effect of non-specific light scattering, were responsible for the small (8-10%) overestimate of serum apo B values associated with the INA method. It should be mentioned, however, that because the sample number was relatively small, a relatively large effect produced in a few samples

would contribute disproportionately to the overall effect seen for the whole group. The mean apo B value found on whole serum by the INA method may be higher than the combined estimate, for this reason alone. With a larger sample number, a relatively large effect observed in a few sera, would much less affect the whole group result. Clearly, more work is needed to clarify these relationships.

(d) Summary

To summarize, the INA method under optimal working conditions compares well with the RID assay for the measurement of apo B in the d1.020 bottom serum fraction. The addition of LFS to the LDL standards seems necessary for the performance of the immunoassay, although the relationship between the concentration of serum protein added and light scattering is not established. The estimate of apo B in the d1.006 bottom serum fraction by the INA method agreed well with the combined d1.020 bottom (INA) and IDL (chemical) estimates. The estimates of apo B in the d1.006 bottom serum fraction and in whole serum were significantly higher by the INA compared to the RID method. A major reason for these differences was related to the presence of apo B in the d1.020 top lipoprotein fraction, which was not accurately quantitated by the RID method. However, the whole serum apo B estimate was also significantly higher than the d1.006 bottom serum estimate using INA for quantitation. A part of this difference may be explained by the non-specific light scattering caused by the VLDL particles in serum. The magnitude of this

effect was obscured by the small sample number involved.

VIII. Comparison of VLDL and LDL Light Scattering Characteristics

1. Introduction

In the previous experiments, the higher estimate of apo B found in whole serum by the INA method, compared to the estimate based on the combination of immunoassay and colorimetric methods, was thought to be a consequence of non-specific light scattering caused by the larger VLDL particles present in serum. The purpose of these experiments was to compare the light scattering characteristics of isolated VLDL and LDL, and, if found to be different, to determine the extent that non-specific light scattering might have contributed to the overestimate of serum apo B levels found in the previous study.

2. Materials and Methods

VLDL was isolated from serum by preparative ultracentrifugation as previously outlined. An aliquot of VLDL from each of the 13 samples was used to prepare a pooled VLDL fraction. The concentration of apo B in the pooled VLDL estimated by the Lowry method, was 11 mg/dl. An LDL standard was prepared to approximate the concentration of apo B in the pooled VLDL fraction, and this corresponded to 11.5 mg/dl of LDL apo B. In the first experiment, the light scattering of isolated VLDL and LDL were compared over time by incubating, in triplicate, solutions containing 25λ of VLDL or LDL in a 1:30 dilution of antiserum. In a second

experiment, 5λ of VLDL containing 11 mg/dl apo B was added to an equal volume each dI.020 bottom serum samples at the time of assay by INA. A second phase of this experiment involved the addition of 5λ of LDL (11.5 mg/dl) to an equal volume of each of the 13 d1.020 bottom serum samples. Immunoassay was then performed as previously outlined.

3. Results

The results of the present series of experiments are shown in tables 28 through 30, and figures 16 through 18.

Table 28 compares the light scattering resulting from the VLDL and LDL Ag blanks. The VLDL blanks produce considerably more light scattering than the LDL blanks, but even the higher VLDL values do not significantly affect the calculation of ΔRLS.

Figure 16 illustrates the light scattering characteristics of VLDL and LDL over time. The figure shows that the initial dC/dt is less for the reaction mixtures containing VLDL than LDL. The VLDL reaction also requires more time to achieve equilibrium. After equilibrium is reached, (≅90 minutes), VLDL produces approximately 2.5 times as much light scattering as LDL for equivalent concentrations of apo B. Doubling the concentration of LDL, results in roughly 90% as much light scattering as one half the concentration of VLDL. Conversely, halving the concentration of VLDL results in slightly more light scatter than double the concentration

of LDL.

Table 29 presents the data for the individual and mean estimates of apo B in the d1.020 serum samples before and after the addition of VLDL. The mean concentration of apo B for the 13 samples was 62 mg/dl. The mean value after the addition of VLDL was 79 mg/dl or about 6 mg/dl or 8% higher than the expected mean value of 73 mg/dl.

In table 30, the individual and mean data for apo B before and after the addition of LDL is shown. The mean value found after the addition of 11.5 mg/dl LDL was 71 mg/dl, slightly less than the expected mean value of 73.5 mg/dl. The recovery of the added LDL was about 97%. The observed and expected values correlated well (r=0.98, figure 18).

4. Discussion

This series of experiments was specifically designed to compare VLDL and LDL light scattering properties. Because they were found to be so different, an attempt was made to estimate the magnitude of the non-specific light scattering effect in the serum samples.

In the first experiment, the light scattering of VLDL and LDL were compared over time. The VLDL particles scattered more than twice as much light as the LDL particles, for equivalent concentrations of apo B. The proportionality between light scattering and particle size was roughly

maintained when the concentration of VLDL was halved or LDL doubled, indicating a fairly constant relationship between particle size and light scattering under these conditions. The greater light scattering by VLDL was expected since the diameter of these particles is more than twice as large as LDL (178), and the surface area of the immunocomplexes so formed, would be larger as well. Thus, under conditions involving equivalent concentrations of apo B, the non-specific light scattering caused by the larger VLDL particles is substantial. The light scattering is non-specific, in the sense that it is disproportional to the light scattering produced by LDL apo B. The effect of this light scatter would be to cause an overestimate of apo B levels. Since the relative concentrations of VLDL and LDL in normal serum are substantially different from the 1:1 ratio examined here, the effect produced by VLDL in serum would presumably be smaller.

The lower initial dC/dt and the longer time required to reach equilibrium for VLDL compared to LDL, confirms a previous finding (32). These characteristics may be reflecting the lower immunoreactivity of the particles as shown in other studies (100).

The data presented in table 29, which summarizes the results of the second experiment, suggests that the non-specific light scattering of VLDL particles may well result in an overestimate of serum apo B values. This theme was explored by adding a fixed concentration of VLDL to the d1.020 bottom serum samples. The observed values were significantly

higher ($p<0.005$) than the expected values, calculated on the basis of the concentration of apo B added. The difference in the two values, representing an overestimate of the actual concentration, was small, amounting to 8%. This difference is most likely explained by non-specific light scattering resulting from the larger VLDL particle size. Since the observed and expected values for LDL correlated so well (table 30, figure 18), the difference seen for VLDL is unlikely to be explained by methodological variables.

The extent of the overestimate produced by the non-specific light scattering effect of VLDL, is probably proportional to the ratio of VLDL to LDL found in serum. When isolated VLDL and LDL were compared at a 1:1 ratio, the effect was substantial, and non-specific light scattering caused a two fold overestimate of apo B. In the second experiment, where the relative concentration of VLDL and LDL averaged about 1:6, the resulting overestimate was much less, and under this condition amounted to about 8%. In the previous experiment the relative ratio of VLDL/LDL in the serum samples averaged 1:14. The overestimate found there also amounted to 8% so it is evident that the proportionality between VLDL and LDL concentration, is but one of several variables involved. Another factor to consider is the size of the VLDL particles which are much less homogeneous in their size than LDL. It should be stressed, however, that the differences observed in the previous study, were produced by relatively few sera where the effect was large. The overall effect observed for the group may have been exaggerated for this reason alone. Nevertheless, the

findings presented here indicate that non-specific light scattering by VLDL is a bona fide effect that may well contribute to an overestimate of the serum concentration of apo B. The magnitude of the effect in normolipemic serum samples is not clear, but it is probably not large. The effect would presumably be greater in hyperlipemic serum where both the concentration (14, 17) and size (153) of VLDL are increased.

In summary, these experiments show that for equivalent concentrations of apo B, VLDL particles scatter significantly more light than LDL particles. The non-specific light scattering caused by the VLDL particles lead to a small, but significant overestimate of apo B concentrations for the conditions examined in this study. The overestimate of serum apo B levels seen in the previous study may be explained by a similar effect, although the relationship in that case is less clear. One approach to reducing the non-specific light scattering of VLDL particles is considered in the last phase of this research.

IX. Reduction of Non-specific Light Scattering of VLDL by Detergents

1. Introduction

In the previous experiment, the light scattering characteristics of VLDL and LDL were shown to be different. More specifically, VLDL scattered more than twice as much light as LDL, for comparable concentrations of apo B. The non-specific light scattering effect of VLDL, resulted in an overestimate of serum apo B levels. In the present study, the light scattering properties of VLDL and LDL are compared in the presence of detergents. It was hypothesized that treatment with detergents would prove to be effective for reducing the non-specific light scattering caused by the larger VLDL particles.

2. Materials and Methods

VLDL and LDL containing respectively, 11 and 11.5 mg/dl apo B, were allowed to react with antiserum in solutions containing various concentrations of detergents (0.0001-1.0%). The following detergents were tested; (1) Tween 20 (Sigma Chemical Company), (2) Triton X-100 (Sigma Chemical Company), and (3) Octadecenylamine-polyoxyethylene (ODA-POE), generously supplied by BASF company, Montreal. Detergents were prepared by dilution in filtered PBS. Antiserum was added to PBS-detergent solutions, to achieve a final antiserum dilution of 1:30. All reaction mixtures were prepared in triplicate. Immunoassay was performed according to the proto-

col outlined previously with the modifications described in the previous experiment for isolated VLDL and LDL. Light scattering was measured at 30 minute intervals over a three hour period.

3. Results.

The results of these experiments are presented in table 31 and figures 19 through 21.

Figure 19 is representative of the effect of the detergents on the light scattering of the free VLDL and LDL particles. For VLDL, the light scattering tends to decrease with increasing detergent concentration, while for LDL, no comparable change is observed.

Figure 20 shows the immunoreactivity of VLDL and LDL in the presence of detergent containing solutions over time. The time course observed in the presence of detergents is similar to that seen previously for the isolated lipoproteins (figure 16).

Table 31 and figure 21 summarize the effects of the three detergents on the light scattering of VLDL and LDL. The data in the table represents the % of the corresponding RLS value found for the VLDL and LDL reaction occurring in buffer alone. Inspection of table 30 and figure 21 reveals that the 0.001% concentration of tween 20 and triton X-100 had no effect on the light scattering of VLDL and LDL. A 0.001% concentration of

ODA-POE also had no effect on the light scattering of LDL, but decreased the light scattering of VLDL by about 45%. Incubation in an 0.01% concentration of all three detergents reduced VLDL light scattering considerably, with that of ODA-POE showing the greatest effect. A 0.01% concentration of both tween 20 and triton X-100 had no effect on LDL but the same concentration of ODA-POE decreased the light scattering of LDL by about 10%. The reduction in light scattering of VLDL in the presence of a 0.1% concentration of the detergents ranged between 75% to 85%. At this concentration of detergent, the reduction in the light scattering of LDL was also significant, with the greatest effect seen for triton X-100. The effect of light scattering of a 0.0001% concentration of detergent was no different than the effects produced by a 0.001% concentration (data not shown), with the exception of a 10% reduction of VLDL for ODA-POE. However, using a 1.0% concentration of detergent caused a further decrease in light scattering for both VLDL and LDL, beyond that seen with a 0.1% concentration.

4. Discussion

These experiments were designed to analyze the light scattering of the isolated VLDL and LDL particles in the presence of detergents. In these experiments, the isolated lipoproteins were allowed to react with antiserum in solutions containing various concentrations of detergents (0.0001-1.0%). The range of detergent concentrations used was wide and corresponded to a 10,000 fold change. Light scattering in the presence

of detergents was compared to the light scattering of lipoproteins in the absence of detergent.

At low concentrations of detergents (0.0001-0.001%), the light scattering of LDL was unchanged. Mixtures containing tween 20 and triton X-100 at these concentrations, also had no effect on the light scattering of VLDL. In the 0.0001% and 0.001% solutions containing ODA-POE, the light scattering was reduced by about 10% and 45% respectively. Thus for ODA-POE at these concentrations, there appears to be a differential effect on the light scattering of VLDL and LDL particles. This suggests that the detergent is somewhat more selective for VLDL. With a 10 fold increase in detergent concentration to 0.01%, the effect of ODA-POE is augmented, but its specificity for VLDL may be lost as judged by the small (10%) reduction in the light scattering of LDL. At this concentration, the tween and triton detergents also decreased the light scattering of VLDL with minimal effect on LDL. With a further 10 fold increase in detergent concentration to 0.1%, the light scattering of LDL now decreases significantly. At a 1.0% concentration, the light scattering of VLDL and LDL undergo a further small reduction. The major effects of these detergents on VLDL and LDL are therefore seen over a 100 fold concentration range, corresponding to 0.001-0.1%.

The three non-ionic detergents examined here, are all derivatives of polyoxyethylene (160). The differences among these are related to the polar groups of the molecules. The polar moieties in tween, triton, and

ODA-POE are, respectively, sorbital esters, p-t-octyl phenol, and alkylamines (160). The detergent properties of these surfactant molecules are related to their ability to solubilize lipid. In solutions containing lipoproteins, these molecules presumably cause a dispersion of lipid from the lipoprotein particle which is then solubilized by the formation of mixed micelles (160-161). The effect produced in different lipoprotein particles, is probably related to various physical properties such as, lipid content and composition, and the molecular stability of the particle. In vivo (7) and in vitro (102) treatment of triglyceride rich partivles with lipolytic enzymes results in the removal of lipid, accompanied by changes in the physical characteristics of the particles including size and electrophoretic mobility. The treatment of lipoproteins with detergents may produce similar changes. Indeed, earlier experiments involving the treatment of lipoproteins in vitro with tween and triton detergents, resulted in significant alterations in the physical properties of the particles as evidenced by the finding of significant changes in the ultracentrifugal flotation pattern and electrophoretic mobility of the particles so treated (161-163). At low concentrations, non-ionic detergents do not seem to cause desaturation or induction of conformational changes in proteins that would lead to loss of biological activity, including immunoreactivity (164). At higher concentrations, the entire particle may become structurally unstable, resulting in loss of biological activity (164). Because of differences in the surface membrane composition of VLDL and LDL, the affinities of the detergents for these particles may also differ. Such a specificity is likely imparted through

the polar group of the detergent molecule interacting with a specific configuration of membrane components (32, 68).

The properties of the detergents and their actions on the lipoproteins outlined above, may provide a plausible explanation for the data observed in this study. There are three basic ways that the detergents might have produced a reduction in light scattering; (1) by a reduction in the size of the particles and the immunocomplexes formed, (2) by altering (decreasing) the immunoreactivity of the Ag, or (3) by a combination of these mechanisms. Since LDL was less likely to undergo a change in particle size without a concomitant loss of immunoreactivity, any significant change in the light scattering of LDL was interpreted as evidence for loss of immunoreactivity. Therefore any change in VLDL light scattering without an accompanying loss of LDL light scattering under the same conditions, was interpreted as a reduction in particle size. The VLDL particles with a larger size, higher lipid content, and lower structural stability, relative to LDL, was affected by the detergents at lower concentrations. Moreover, at the lower concentrations, the detergents and in particular, ODA-POE, seemed to have a selective effect. The selectivity of this effect may have been related to the phospholipid composition as shown by Heuck (32). As the concentration of the detergents is increased, The selectively of action for VLDL is lost as the light scattering of LDL becomes significantly decreased. The resulting loss of immunoreactivity likely results from a change in protein conformation or surface antigenic components, secondary to structural alterations

of the particle. Such effects are known consequences of detergent action (164). Although the changes in light scattering were speculated to result from changes in particle size and immunoreactivity, these effects could be specifically tested through various devices, including electron microscopy of the lipoprotein particles and by observing the light scattering of a control immunoreaction, for example, IgG-anti-IgG where the reactants possess a homogeneous size. These experiments, however, were beyond the scope of the present study.

The important observation in these experiments was the relative effect of the detergents on the light scattering of VLDL and LDL. In these experiments, the concentration of apo B in VLDL and LDL were the same. The previous experiment demonstrated, that for equal concentrations of apo B, VLDL scattered more than twice as much light as LDL. The difference in light scattering is non-specific, in the sense that it is unrelated to LDL apo B. This light scattering is important, however, since it tends to falsely increase the estimate of apo B. The aim of these experiments was to identify those conditions that would reduce the light scattering of VLDL relative to LDL, by about 50%. This would then eliminate or at least reduce non-specific light scattering of VLDL and minimize the tendency of this effect to cause an overestimate of apo B. In the present experiments, the conditions approaching this situation involved a 0.01% concentration of tween 20 or triton X-100, or better, a 0.001-0.01 concentration of ODA-POE. These concentrations would represent guidelines for the testing of detergents in whole serum.

Various techniques have been tested for their ability to reduce the size of VLDL particles and produce particles having a uniform size. Decklebaum (102), described a method for the production of LDL-like particles from VLDL by in vitro incubation of lipoproteins in the presence of lipase enzymes. Reardon (104), then applied this principle to develop a modified EIA, as described previously. Non-specific light scattering is recognized as problematic for the estimation of serum apo B levels by the INA method (32-33, 74). Heuck (32) recently described a series of experiments, which showed the effectiveness of lipase enzymes for decreasing the non-specific light scattering of VLDL. Detergents also appear to be similarly effective (32, 74). In a second report (74), involving simplified procedures, Heuck demonstrated the effectiveness of ODA-POE in reducing the overestimate observed in hyperlipemic samples. In this regard, the detergent was effective in the 0.005-0.2% concentration range which compares well with the results of this study. Similarly, Rosseneu et al (33), tested the effects of various detergents including tween 20, triton X-100, ODA-POE and Apovax. Both Apovax and ODA-POE at a concentration of 0.01% produced a better agreement of serum apo B values measured by INA and EIA methods, particularly in the setting of hyperlipemic serum samples.

In summary, incubation of VLDL in the presence of detergents in the 0.001-0.01% concentration range was effective in reducing the non-specific light scattering of these particles. The results of these experiments should serve as guidelines for the evaluation of detergent effects in

serum samples. It is anticipated, that at appropriate concentrations, the overestimate of apo B levels that were observed using the INA method for quantitation, will be minimized.

PART FOUR

GENERAL DISCUSSION

The present study was undertaken as a preliminary evaluation of immunonephelometry as a method for the quantitation of serum apo B. The principle of nephelometry involves the detection of light scattering of immune complexes formed between antigens and antibodies in solution. For the fixed conditions of nephelometry, and under appropriate immunological relationships, the intensity of light scatter is proportional to the concentration of the antigen, and therefore, the antigen is quantifiable. The method, a priori, appeared suitable for the quantitation of apo B. For this purpose however, the procedure is potentially more complex than that involved for the determination of other serum proteins, since the antigen, apo B, is present in serum in the form of lipoprotein particles. In so far as the size of the lipoprotein complexes containing apo B is variable, and since the intensity of light scatter is further related to the size of the antigen and immune complexes formed, the effect of disproportionate light scatter caused by the larger lipoprotein particles present in serum, was of special concern to the performance of the immunoassay.

In the development of an immunoassay, there are four important parameters to be evaluated. These include; specificity, sensitivity, precision, and validation. As well, any potentially interfering factors must also be recognized and evaluated. The present assay, based on the technique of immunonephelometry, was developed with these considerations in mind.

The initial experiments were undertaken to establish the basic work-

ing conditions for the assay. The specificity of the immunoassay for apo B was guaranteed by raising a monospecific antiserum to the antigen. The characteristics of light scattering for the LDL apo B-anti-LDL system were demonstrated in preliminary experiments by allowing various concentrations of LDL apo B to react with various dilutions of antiserum. Under appropriate conditions, a precipitan curve was constructed which was linear over a wide range of LDL apo B concentrations. An end-point for the measurement of immunocomplex light scattering, was established by following the immunoreactivity of the reactants over time. The relatively short incubation period required (two hours) is a timesaving feature of this method. The in vitro sensitivity of the assay for the antigen corresponded to the levels of apo B found in serum, and therefore, only a minimal dilution of the samples was necessary for immunoassay. The lower limit of apo B detection was 5-10 mg/dl, and this is well in excess of that required to measure apo B in serum (tables 2 and 3). The minimal detectable antigen concentration attainable was not determined but it is probably more sensitive than reported here (172). The assay was also shown to be very sensitive to 10 mg/dl differences in apo B concentration. The precision of the assay for measuring apo B was studied, and the combined intra- and interassay coefficient of variation was about 7%. This value tends to be lower than that reported by most other methods (16-17, 20, 25), and the reliability of the assay for apo B measurement was judged to be high.

With the working conditions and performance characteristics of the

assay established, the next phase of development involved the validation procedures. The validation of the assay was somewhat hindered due to the lack of a reference method at the time, for measuring total serum apo B levels. The immunoassay used for standardization purposes, was a RID method, used routinely in this laboratory for the quantitation of apo B. The RID assay was designed to selectively measure LDL apo B in plasma, and is therefore not accurate for the quantitation of apo B in the d<1.020 lipoprotein fraction (21). This finding was also confirmed in the present study. For this reason, the apo B in the d<1.020 lipoprotein fraction was estimated by an independent method. Thus the reference method for measuring total serum apo B concentrations involved combining the separate estimates for d<1.020 and d>1.020 apo B by the chemical and immunoassays. The fact that an alternative immunoassay for total apo B was unavailable, made the validation procedure more tedious. At the same time, however, it fostered a more thorough analysis of the light scattering method. Indeed, one of the purposes for developing this method, was to have available an assay for total apo B.

Since light scattering is dependent on the size as well as the concentration of the antigen (138), and because apo B is present in lipoprotein particles of various sizes, the quantitation of apo B by this method was potentially more complex. It was anticipated that the larger VLDL particles might interfere with the performance of the immunoassay, by causing disproportionate light scattering. This effect potentially cause an overestimate of serum apo B levels.

In the initial experiments dealing with the validation of the procedure, significantly higher serum apo B levels were found with the INA compared to the RID method. Three major parameters were postulated to account for this difference; (1) differences existing between the LDL standards and the serum samples, (2) apo B contained in the d<1.020 lipoprotein fraction inaccurately measured by the RID assay, and (3) nonspecific light scattering caused by the larger VLDL particles present in serum. The next series of experiments therefore evaluated the contribution of these factors.

A fundamental requirement of an immunoassay, is that the antigen in the standards and the samples exist under closely similar conditions. This means, for example, that the properties of the antigen, such as molecular size and surface charge, be similar. Surface charge seems to be an important consideration for EIA techniques (77). Size differences seem to be equally important variables for the performance of other methods (17, 21-22, 32, 104). Of equal importance, is for the standard and sample antigens to be present in a similar background medium as well. For the initial standardization procedure, the LDL standards were prepared in serum-free salt solution, since this has been the procedure for preparation of the LDL standards used for the RID method. Because the overestimate of apo B values by the INA method seemed excessive, one wondered if a fundamental difference existing between the samples and the standards was partly responsible. The subsequent comparison of the light scattering of the LFS-LDL and serum-free LDL standards, and the resultant

differences in the levels of apo B calculated from the two standard curves, seemed to demonstrate the requirement for serum in the LDL standard solutions. By adding LFS to the LDL standards, the conditions for the antigen in the standards and the samples became more similar and the standardization of the method was improved as illustrated by the close agreement of apo B values in the d>1.020 serum samples measured by the INA and RID assays. In contrast, this requirement did not seem particularly crucial for the performance of the RID method as alluded to earlier. Rosseneu (33), has recently observed effects similar to those shown here. In that study, the levels of apo B measured by the INA method using serum-free LDL standards were significantly higher than those found by EIA (123 vs 71 mg/dl). Addition of LFS to the LDL standards resulted in a better agreement of the values (95 vs 85 mg/dl). It is also of interest to note that the addition of LFS produced opposite effects in the two assay systems. The concentration of serum protein used in the study by Rosseneu, was 4.7 gm/dl compared to the 6 gm/dl used in these experiments. These values are both within the normal range of serum protein concentrations. Although the standardization of the assays in these experiments seemed optimized by the concentrations of serum protein used, it is still of importance to these methods to clarify the relationship between serum protein concentration and the light scattering of LDL.

— In these experiments, the reference method for determining total serum apo B levels, involved combining the separate estimates found in the d<1.020 fraction by colorimetric assay and d>1.020 fraction by RID

assay. The mean value found by this method was 128 mg/dl. The mean value found by the RID method was 110 mg/dl. The difference between the two values was due to the presence of d<1.020 apo B poorly quantitated by the RID method. The apo B level found in whole serum by the INA method was 143 mg/dl. The difference between the estimates of total apo B found by the reference and INA methods was partly accounted for by the non-specific light scattering effect of the VLDL particles in serum. Whereas the extent of the effect was somewhat unclear, the existence of the effect and its potential for causing an overestimate, were proved through the following observations. Firstly, when the light scattering characteristics of isolated VLDL and LDL particles were compared, the larger VLDL particles scattered more than twice as much light as the LDL particles, for equivalent concentrations of apo B. This finding is in agreement with Heuck et al (32), who also compared the light scattering of isolated lipoprotein particles. Secondly, when VLDL was added to the d>1.020 serum samples, the observed apo B value was significantly higher than the expected apo B value. It seemed clear, that the difference in the values resulted from the non-specific light scattering of the VLDL particles. Although the overestimate was small (8%), and quantitatively similar to the difference found for apo B measured in serum by the reference and INA methods, it seemed plausible that the latter overestimate may have resulted from a similar effect as well. However, as mentioned earlier, the small sample number may have contributed to to there being a greater difference than actually present.

Immunonephelometric assays have recently been developed in a number of different laboratories for the quantitation of serum apo B (table 3). Ballantyne et al (31), were one of the first to evaluate the laser method for this purpose. The mean value found by the method in a group of 35 normolipemic subjects was 157 mg/dl. This value was twice as high as that found by RIA. Unfortunately, the calibration of their standard curve was not described, and it is therefore not possible to compare the working conditions of the two methods. In a more recent report (149), Ballantyne observed a mean apo B level of 100 mg/dl by this method in a group of 24 normolipemic subjects. Once again, it is not clear if methodological factors are responsible for this difference. Further, the small sample numbers used in these studies may not be reflective of actual population norms. Dedonder-Decoopman et al (57), in a series involving a large number of subjects, reported mean apo B values of 129 and 120 mg/dl in 206 and 271 male and female normolipemic subjects. These values tend to be higher than those found in similarly large groups of subjects by other methods (table 2). On the other hand, others using this method have found lower mean apo B values, that tend to agree better with those found by conventional methods. Heuck et al (75) very recently updated his series and reported a mean apo B value of 103 mg/dl for quantitations made in 432 normolipemic subjects. Rosseneu et al (33) also reported values that were favourable to other estimates. Rosseneu also showed the conditions necessary for these measurements as discussed earlier. Debacker (56), recently compared the levels of apo B measured by INA in 70 normolipemic post myocardial subjects and a similar number of normo-

lipemic controls. The values found in the coronary patients were significantly higher than those found in the controls (143 vs 113 mg/dl), and this shows that heterogeneity of the population studied may alone account for a significant amount of variation seen for apo B levels. The levels of apo B measured in cord serum by INA (58, 110-111), also compare well to those found by other methods (108-109). These studies show that the method tends to be fairly well standardized for the quantitation of apo B in normolipemic serum, although the exact conditions under which the standardization was accomplished, is not clear in all cases. The tendency for the method to overestimate the level of apo B in normolipemic samples may not be great, and this corroborates the observations of the present study. The higher values found in some cases may as well be a reflection of the heterogeneity of the population studied.

The standardization of the assay for the purpose of quantitating apo B levels in hyperlipemic serum seems to require some specific procedures (31, 33, 74). Without these procedures, the levels of apo B tend to be significantly overestimated (33). The overestimate found in hyperlipemic serum is caused by the non-specific light scattering of VLDL as shown by Heuck et al (32, 74). The effect in hypertirglyceridemic serum is magnified owing to an increase in both the size (153) and concentration of the particles (14, 17). Short of removal of these particles by ultracentrifugation, there are two possible ways of reducing light dispersion caused by larger lipoprotein particles; (1) by hydrolosis of lipid of intact lipoproteins with lipase enzymes, or (2)

by adding detergents. Heuck et al (32), showed that the non-specific light scattering of VLDL could be eliminated by allowing the particles to react in the presence of triglyceride lipases. When the effects observed in the isolated lipoproteins were tested in whole serum, the estimates of apo B were found to be lower and the results correlated better with those found for the control method. Subsequently, certain detergents were shown to produce similar effects in isolated lipoproteins and hypertriglyceridemic serum samples (32, 74). Solutions containing ODA-POE in the 0.005%-0.02% concentration range were shown to be particularly effective (74). Rosseneu et al (33), also compared the effects of various detergents including ODA-POE and Apovax and found both useful, although Apovax at a concentration of 0.01% was superior at reducing non-specific light scattering in type IIb serum samples. The present study also demonstrated the efficacy of detergents, including ODA-POE, for reducing non-specific light dispersion of isolated VLDL, but fell short of extending the findings to whole serum samples. Since the use of appropriate detergents appear to produce comparable results to those seen for enzymes, but on the whole involve simpler procedures, detergents appear to be the method of choice for the standardization of the assay where hyperlipemic samples are involved. It would be of interest to also establish the mechanisms by which detergents produce these effects. Non-specific light scatter has also been a problem for the quantitation of other proteins by this method (75, 146).

Many different immunological methods are now available for the quant-

itation of serum apo B. These include, RIA (14-20), RID (21-23), EIA (24-26) and enzyme immunoassay (27-29). The most recent addition, involves the method of immunonephelometry. There are certain features that make this technique attractive, particularly for clinical and epidemiological purposes. These characteristics were discussed earlier. To be reliable, however, the method must prove itself accurate for its particular measurement. These properties can only be established through experiments similar to those considered here.

PART FIVE

SUMMARY OF THE MAJOR FINDINGS

The main findings of this study may be summarized as follows:

1. The light scattering characteristics of LDL apo B-anti-LDL were evaluated under conditions involving different Ag/Ab ratios.
2. Appropriate conditions were described for the construction of a linear precipitan curve.
3. The time course of LDL apo B-anti-LDL was evaluated to determine an end-point in time for measurement (two hours).
4. Performance characteristics of the assay including specificity, sensitivity, and intra- and interassay variability were evaluated.
5. Light scattering of the LDL standards prepared in serum-free salt solutions and LFS were shown to be different. In particular, the light scattering of comparable concentrations of LDL standards was greater in LFS.
6. The standardization of the method was improved by the addition of LFS to the LDL standards. This was assessed by comparing the estimates of apo B in the d>1.020 serum fraction using serum-free and LFS-LDL standards. The measurements found by the INA method using the LFS-LDL standards agreed well with those found by RID assay.
7. The estimates of apo B found by INA in the d>1.006 serum fraction compared well to those found by combined IDL (chemical) and d>1.020 (INA) estimates.
8. The estimate of apo B found in whole serum and in the d>1.006 serum fraction were significantly higher than those found by RID assay.
9. A major reason for these differences was related to the presence of a significant amount of apo B in the d<1.020 lipoprotein fraction, that was not quantitated by the RID method.

10. The whole serum estimate of apo B was significantly higher than the d>1.006 and d>1.020 serum estimates. A significant difference remained after d<1.006 and d<1.020 lipoprotein were added to these fractions. These differences may be partly explained by non-specific light scattering caused by the larger VLDL particles.

11. For comparable concentrations of apo B, VLDL particles scatter more than twice as much light as LDL particles.

12. When VLDL was added to the d>1.020 serum samples, the observed apo B values were significantly higher than the expected values. The difference in the values resulted from the non-specific light scattering of the larger VLDL particles.

13. In the appropriate concentration range (0.001%-0.01%), tween 20, triton X-100, and ODA-POE were all effective in reducing the non-specific light scattering of isolated VLDL particles.

14. The mean level of apo B found in plasma and serum samples of the same subjects was not significantly different.

PART SIX

CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER STUDY

In conclusion, immunonephelometry appears to be a promising new method for the quantitation of serum apo B. In view of the newly recognized association between serum apo B levels and coronary heart disease, the measurement of serum apo B concentrations is going to become ever so increased in the future. Methods with both diagnostic and epidemiologic capabilities, such as INA, will be invaluable for facilitating these measurements. Further work is needed, however, before the assay can be considered fully validated. In my opinion, more work is needed in the following areas:

1. The relationship between the serum protein concentration added to the LDL standards and the light scattering of LDL needs to be clarified, since differences found here could potentially affect the quantitation of apo B.
2. Ideally, the standardization of the assay should be approached by using a recognized reference standard for LDL apo B. Because such a standard is not likely to be forthcoming in the immediate future, an alternate reference method capable of measuring total apo B levels, should be used for comparison studies. EIA would probably represent a suitable method, since it appears to be reasonably accurate for quantitating total apo B. This method has also been used by others for the standardization of INA, and therefore the results forthcomming from the different studies may be more comparable. The relation between EIA, RIA and INA would need to be established in a preliminary experiment.
3. A much larger number of samples, probably involving a hundred or so, should used for these comparisons.
4. The oversetimate of apo B by the INA method in normolipemic samples needs to be confirmed. This tendency may become smaller, or disap-

pear when a larger sample number is tested.

5. The extent of the non-specific light scattering effect in hyperlipemic serum samples needs to be established.

6. The effectiveness of detergents, for example, Apovax and ODA-POE, for reducing non-specific light scattering, needs to be demonstrated in whole serum. Detergents seem preferable for this purpose, because of the simplicity of the procedures involved. The mechanism of the detergent action responsible for these effects, would be interesting to examine as well.

These would seem to be the minimal additional requirements necessary to complete the validation of the method.

APPENDIX I - TABLES

Table 1. Chemical Composition of Human Plasma Lipoproteins.

	LIPID				PROTEIN							
	core		surface									
	TG	CE	FC	PL	A-I	A-II	B	C-I	C-II	C-III	D	E
Chylomicrons	88	3	2	7	<7	5	19	11	15	41	-	?
VLDL	60	13	8	19	<1	-	36	3	7	40	tr	13
IDL	28	36	11	23	<1	-	63	1	4	25	-	14
LDL	7	54	9	28	tr	tr	95	tr	tr	tr	tr	<5
HDL_2	13	28	8	55	64	20	-	6	1	4	3	2
HDL_3	7	29	9	55								

The values represent the approximate % of the total lipid and protein mass (dry weight). TG=triglyceride, CE=cholesterol ester, FC=free cholesterol, PL=phospholipid, tr=trace. Adapted from references 1-7.

Table 2. Levels of apo B in normolipemic subjects measured by immunoassay

subjects		apo B (mg/dl)		cholesterol (mg/dl)			
n	♂/♀	total(SD)	LDL	total	LDL-C	method	series
349	189/160	81	72(19)		120	RIA	Albers (17)
128	60/68	87(30)		240		RIA	Bedford (16)
82	33/49	90(24)	85(18)	190	112	RIA	Bautovitch (15)
42		83(16)		213		RIA	Schonfeld (14)
42	20/22	93(25)/90(18)				RIA	Karlin (19)
256	114/142	103/101		220/234		RIA	Albers (106)
25		94(38)	86			RIA	Thompson (18)
35		71(36)		220		RIA	Ballantyne (31)
64	52/12	91(16)		184		RIA	Durrington(20)
209		89(23)		193		RID	Heuck (68)
64	32/32	83(25)			140	RID	Lees (22)
34		97(22)				RID	Havekes (67)
31			82	168	112	RID	Sniderman (49)
29		72(16)				ELISA	Holmquist (29)
200	100/100	99(17)/92(15)		210/205		EIA	Avogaro (105)
116	65/51	77(18)	74/69			EIA	Onitiri (24)
74	38/36	98(20)		208/190		EIA	Curry (25)
146		98(19)		202		EIA	Fruchart (50)
76		113(20)		223		EIA	Fagar (180)
34		105(28)				EIA	Havekes (67)
106	60/46	117(35)/121(34)		197/197		EIA	Whayne (53)

Table 3. Comparison of apo B Values Quantitated by INA

Subjects	apo B (mg/dl)	Lipids† (mg/dl)				
n	*total*	*chol*	*TG*	*LDL-C*	*method*	*series*
87	173	259	236		INA	Ballantyne (31)
	134				AIP	
	91				RIA	
35 NL	159	220	105		INA	
	112				AIP	
	71				RIA	
27 Post MI	114				INA	Ballantyne (149)
24 NL	100					
31	196				INA	Fievet-Desreumaux
	193				EIA	(30)
	211				ELISA	
477 $\frac{206♂}{271♀}$	$\frac{129}{120}$			$\frac{138*}{131}$	INA	Dedonder-Decoopman (57)
68 NL	82	197			INA	Heuck (32)
14 IIa	190	373			INA	
15 IIb	179	342			INA	
22 IV	116	246			INA	
432 NL	103	195			INA	Heuck (75)
10 NL (AC)	90	181			INA	Rosseneu (33)
	85				EIA	
10 NL (PC)	93	178			INA	
	82				EIA	
21 IIa	139	291			INA	
	150				EIA	
30 IIb	146	306			INA	
	155				EIA	
12 IV	102	219			INA	
	98				EIA	
70 Post MI	143	254			INA	DeBacker (56)
70 control	113	242			INA	
30 cord	24	65		29	INA	Van Biervliet (110)
7 days	67	108		58	INA	
30 days	70	114		59	INA	
232 cord	54				INA	Brewster (85)
	50				EIA	

NL=normolipemic, *=VLDL+LDL cholesterol, A=fasting, PC=after fatty meal
† 1 mg/dl cholesterol=0.02586 mmol/L, 1 mg/dl triglyceride=0.1665 mmol/L,
MI=myocardial infarction.

Table 4. Distribution of apo B in Human Serum

subjects		apo B					
n		*total*	*VLDL*	*LDL*	*HDL*	*method*	*series*
349	NL	81	8[α]	72[β]		RIA	Albers (17)
20	IIa		9	118		RIA	
14	IIb		16	130		RIA	
7	III		34	72		RIA	
59	IV		14	88		RIA	
17	NL	83	5	83[β]		RIA	Schonfeld (14)
18	NL	92	5	83[γ]		RIA	Durrington (179)
15	IIa		1	206		RIA	
5	IIb		14	235		RIA	
6	IV		30	160		RIA	
43	NL	90	2	85[γ]	3	RIA	Bautovitch (15)
20	NL	89	5[δ]	83[ε]		EIA	Curry (25)
		87	6	79		RIA	
		87	6	80		RID	
15	HT	128	23	100		EIA	
		113	13	92		RIA	
		113	15	103		RID	
12	NL	93	8	76[ω]	9	EIA	
30	NL	77	6♂ / 5♀	74[γ] / 69	12 / 12	EIA	Onitiri (24)
8	NL		4	88[β]		RIA	Thompson (18)
10	NL	92	3[α]	89		EIA	Franceschini (99)
12	NL-PVD	87	6	81		EIA	
10	HT		13	88		EIA	
8	HT-PVD		21	95		EIA	

α = TMU insoluble protein
β = d>1.006 gms/ml
γ = 1.006-1.063 gms/ml
δ = <1.019 gms/ml
ε = >1.019 gms/ml
ω = 1.019-1.063 gms/ml
NL=normolipemic, HT=hypertriglyceridemia, PVD=peripheral vascular disease

Ag (mg/dl)	RLS of Ag Blank 25λ	5λ
143	4.2	1.1
99	3.2	0.9
57	1.8	0.4
42	0.7	0.4
23	0.6	0.1

Table 5. Comparison of Light Scattering (RLS Units) for the Ag Blanks

Antiserum dilution	RLS
1:20	6.4
1:40	4.5
1:80	2.9
1:100	2.0

Table 6. Light Scattering (RLS Units) for the Antiserum Blanks

As	1:20		1:40		1:80		1:100	
Ag (mg/dl)	5λ	25λ	5λ	25λ	5λ	25λ	5λ	25λ
23	13	205	35	138	35	93	34	82
42	94	265	77	204	56	146	49	63
56	121	305	106	234	76	38	60	14
99	217	435	161	92	113	11	85	4
143	263	630	190	67	143	7	121	3

Table 7. Comparison of ΔRLS for the Different Reaction Mixtures. 1:20 etc., corresponds to the dilution of As used (see text for details).

Table 8. Protocol for Routine Immunoassay

1. Each serum sample is initially diluted 1:1 with PBS.
2. 0.005 (5λ) of diluted Ag is added to a clean 10 by 75 mm disposable glass tube.
3. 1.0 ml of buffer (PBS) containing 0.0125 ml of As (final dilution 1:80) is added next.
4. Each tube is gently mixed by inversion and allowed to react at room temperature for 2 hours.
5. All standards and samples are prepared in duplicate.
6. For each immunoassay, one Ag and one As blank are each prepared by dilution with 1.0 ml of PBS.
7. Proir to determination of RLS, each tube is gently mixed and wiped clean with lens paper.

n	Apo B *(mg/dl)*
1.	146
2.	149
3.	146
4.	140
5.	150
6.	147
7.	139
8.	152
9.	150
10.	146
11.	151
12.	141
13.	144
14.	145
15.	149
16.	140
mean	146

Table 9. Apo B Levels Calculated in 16 Aliquots of a Control Serum during the same Assay. The within-run Coefficient of Variation was 4%.

n	Apo B *(mg/dl)*
1.	144
2.	147
3.	140
4.	145
5.	146
6.	140
7.	141
8.	148
9.	144
10.	142
11.	146
12.	143
13.	147
14.	147
mean	144

Table 10. Apo B Levels Determined in a Control Serum in 14 Separate Assays. The Between-run Coefficient of Variation was 3%.

no.	1.006 bottom	serum INA	serum RID
1.	162	213	156
2.	165	201	141
3.	158	165	131
4.	88	103	70
5.	125	150	104
6.	125	129	95
7.	114	100	76
8.	142	146	98
9.	105	176	88
10.	118	130	86
11.	131	163	113
12.	121	198	134
mean	130	156	108

Table 11. Individual and mean values for apo B *(mg/dl)* in 12 Samples measured by INA and RID methods.

no.	1.006 bottom	1.006 top	total	total INA
1.	162	16	178	213
2.	165	17	182	201
3.	88	4	92	103
4.	125	4	129	129
5.	114	3	117	164
6.	142	2	144	142
7.	105	12	117	176
8.	125	8	133	150
9.	118	4	122	130
mean	128	8	136	156

Table 12. Individual and Mean levels for apo B *(mg/dl)* in the 1.006 bottom (INA) and 1.006 top (chemical) serum fractions estimated in a subset of 9 samples. The combined total value is significantly less than the whole serum total measured by INA.

subject	Triglyceride	Cholesterol
1.	154	215
2.	40	119
3.	117	224
4.	62	183
5.	196	250
6.	108	207
7	176	232
8.	87	210
9.	84	211
10.	143	204
11.	66	172
12.	172	212
13.	97	259
14.	71	176
15.	38	157
16.	91	123
mean	106	197
±SD	49	40

Table 13. Serum Triglyceride and Cholesterol Levels *(mg/dl)* with the Standard Deviation (±SD)

LDL apo B (mg/dl)	+LFS	-LFS	Δlight scattering
104	180	155	25
91	157	132	25
80	140	110	30
61	112	70	42
40	71	29	42
21	42	7	35

Table 14. Comparison of light scattering of the LDL standards prepared in LFS (+LFS) and serum-free salt solution (-LFS) at 2 hours.

no.	INA *SF-LDL*	INA *LFS-LDL*	RID
1.	86	67	60
2.	59	40	33
3.	87	62	66
4.	67	48	42
5.	107	92	97
6.	92	73	76
7.	80	61	56
8.	65	45	43
9.	86	67	66
10.	71	51	51
11.	83	64	66
12.	94	76	72
13.	75	56	50
mean	80	62	60

Table 15. Individual and mean levels of apo B *(mg/dl)* in the 1.020 bottom fraction in 13 subjects measured by RID and INA using serum-free (SF) and LFS-LDL standards.

no.	INA	RID
1.	131	117
2.	84	69
3.	124	132
4.	91	80
5.	124	131
6.	146	152
7.	122	112
8.	93	88
9.	121	119
10.	110	110
11.	131	135
12.	133	126
13.	90	80
mean	115	112

Table 16. Comparison of Individual and mean apo B levels *(mg/dl)* in the 1.02 bottom serum fraction of 13 subjects measured by INA and RID.

no.	INA	RID
1.	63	62
2.	45	33
3.	73	67
4.	53	50
5.	87	90
6.	77	69
7.	68	55
8.	53	45
9.	67	64
10.	53	48
11.	67	64
12.	77	69
13.	57	44
mean	63	57

Table 17. Comparison of Individual and mean apo B levels *(mg/dl)* in the 1.006 bottom fraction of 13 subjects measured by INA and RID.

no.	INA	RID
1.	135	136
2.	94	71
3.	151	141
4.	88	85
5.	127	135
6.	160	147
7.	138	113
8.	91	79
9.	121	118
10.	117	108
11.	138	134
12.	152	138
13.	112	88
mean	125	115

Table 18. Comparison of individual and mean apo B levels *(mg/dl)* in the 1.006 bottom serum fraction of 13 subjects measured by INA and RID.

no.	INA	RID
1.	153	115
2.	97	72
3.	180	131
4.	110	80
5.	185	145
6.	187	131
7.	142	109
8.	100	79
9.	155	122
10.	134	100
11.	154	133
12.	150	127
13.	121	85
mean	143	110

Table 19. Comparison of serum apo B levels (mg/dl) measured by INA and RID

no.	serum	plasma
1.	153	152
2.	97	93
3.	180	171
4.	110	110
5.	185	174
6.	187	178
7.	142	140
8.	100	101
9.	155	148
10.	134	114
11.	154	151
12.	150	152
13.	121	122
14.	126	128
15.	94	90
mean	138	135

Table 20. Comparison of Total apo B Levels *(mg/dl)* in Serum and Plasma by INA.

1.02 top	1.006 top	1.006-1.02
16	10	6
9	4	5
17	12	5
8	6	2
26	18	8
22	14	8
12	6	6
6	3	3
12	8	4
8	3	5
14	10	4
12	5	7
13	8	5
mean 13.5	8.3	5.2

Table 21. Apoprotein B (mg/dl) in the d 1.02, d 1.006 and d 1.006-1.02 serum fractions determined chemically.

no.	1.020 bottom	1.006 bottom	serum
1.	117	136	115
2.	69	71	72
3.	132	141	131
4.	80	85	80
5.	131	135	145
6.	152	147	131
7.	112	113	109
8.	78	88	79
9.	119	118	122
10.	110	108	100
11.	135	134	133
12.	126	138	127
13.	80	88	85
mean	112	115	110

Table 22. Values for apo B (mg/dl) measured by RID assay in fractionated and unfractionated serum.

no.	RID	IDL	total	INA
1.	136	6	142	135
2.	71	5	76	94
3.	141	5	147	151
4.	85	2	87	88
5.	135	8	143	127
6.	147	8	155	160
7.	113	6	119	138
8.	79	3	82	91
9.	118	4	122	121
10.	108	5	113	117
11.	134	4	138	138
12.	138	7	145	152
13.	88	5	93	112
mean	115	+ 5.3 =	120.3	vs 125

Table 23. Individual and mean values for apo B *(mg/dl)* in the 1.006 bottom serum fraction (RID) and IDL (chemical) fraction. The combined value is lower than the estimate by INA of the 1.006 bottom serum fraction but the difference is not significant.

no.	1.02 bottom	IDL	total	1.006 bottom
1.	131	6	137	135
2.	84	5	89	94
3.	124	5	129	151
4.	91	2	93	88
5.	124	8	132	127
6.	146	8	152	160
7.	122	6	128	138
8.	93	3	96	91
9.	121	4	125	121
10.	110	5	115	117
11.	131	4	135	135
12.	133	7	140	152
13.	90	5	95	112
mean	115	+ 5.3 =	120.3	vs 125

Table 24. Individual and mean values for apo B *(mg/dl)* in the 1.02 bottom serum fraction and IDL. The combined value is not significantly different from the 1.006 bottom serum estimate.

no.	1.020 bottom		1.020 top		total		serum INA
1.	131		16		147		153
2.	84		9		93		97
3.	124		17		141		180
4.	91		8		99		110
5.	124		26		150		185
6.	146		22		168		187
7.	122		12		134		142
8.	93		6		99		100
9.	121		12		133		155
10.	110		8		118		134
11.	131		14		145		154
12.	133		12		145		150
13.	90		13		103		121
mean	115	+	13	=	128	vs	143

Table 25. °Comparison of individual and mean values for total serum apo B *(mg/dl)* by combined and whole serum estimates.

1.006 botton		1.006 top		total		INA total
139		10		149		153
97		4		101		97
153		12		165		180
90		6		96		110
131		18		149		185
164		14		178		187
139		6		145		142
93		3		96		100
124		8		132		155
119		3		122		134
141		10		151		154
154		5		159		150
117		8		125		121
mean 125	+	8.3	=	133.3	vs	143

Table 26. Comparison of individual and mean values for total serum apo B (*mg/dl*) by combined 1.006 bottom and 1.006 top and whole serum estimates.

no.	VLDL apo B	Triglyceride
1.	10	154
2.	4	40
3.	13	117
4.	6	62
5.	18	196
6.	14	176
7.	6	87
8.	3	84
9.	8	143
10.	3	66
11.	10	172
12.	5	124
13.	8	71
mean	8.3	116

Table 27. Comparison of individual and mean VLDL apo B and Serum triglyceride levels.

	apo B (mg/dl)	RLS
VLDL	11.0	10.0
	5.5	6.0
LDL	11.5	1.5
	23.0	2.0

Table 28. Light scattering of VLDL (25λ) and LDL (25λ) blanks.

no.	1.02 bottom	1.02 bottom +VLDL *expected*	1.02 bottom +VLDL *observed*
1.	68	79	86
2.	44	55	62
3.	60	71	75
4.	45	56	57
5.	90	101	113
6.	75	86	91
7.	63	74	83
8.	41	52	62
9.	67	78	87
10.	53	64	78
11.	66	77	76
12.	80	91	95
13.	50	61	65
mean	62	73	79

Table 29. Comparison of expected and observed apo B values *(mg/dl)* with the addition of VLDL (11 mg/dl) to the 1.02 bottom serum samples

no.	1.02 bottom	1.02 bottom + LDL *expected*	1.02 bottom + LDL *observed*
1.	68	79.5	80
2.	44	55.5	51
3.	60	71.5	71
4.	45	56.5	57
5.	90	101.5	104
6.	75	86.5	79
7.	63	74.5	70
8.	41	52.5	51
9.	67	78.5	79
10.	53	64.5	65
11.	66	77.5	69
12.	80	91.5	88
13.	50	61.5	59
mean	62	73.5	71

Table 30. Comparison of expected and observed apo B values *(mg/dl)* with the addition of LDL (11.5 mg/dl) to the 1.02 bottom serum samples.

	conc.	tween 20	triton X-100	ODA-POE
VLDL	0.001%	102	100	55
	0.01%	63	47	30
	0.1%	30	25	35
LDL	0.001%	101	104	110
	0.01%	95	100	90
	0.1%	85	65	70

Table 31. Effects of the three detergents on the light scattering of VLDL and LDL. The values shown are the % of the ΔRLS produced by the corresponding reactions in the absence of detergent.

APPENDIX II - FIGURES

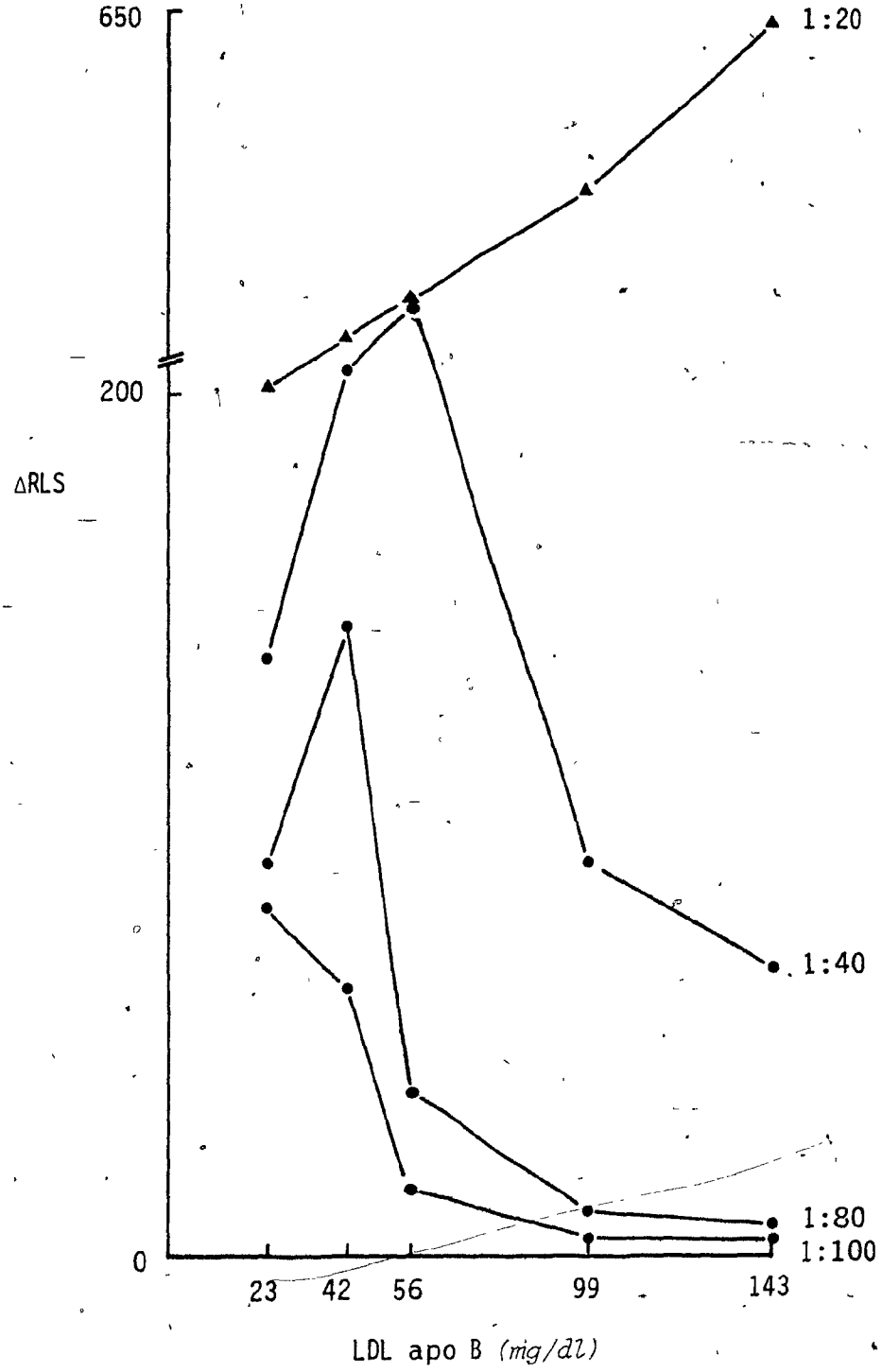


Figure 1. Light Scattering of Reaction Mixtures Containing 25λ of Antigen (2 hours).

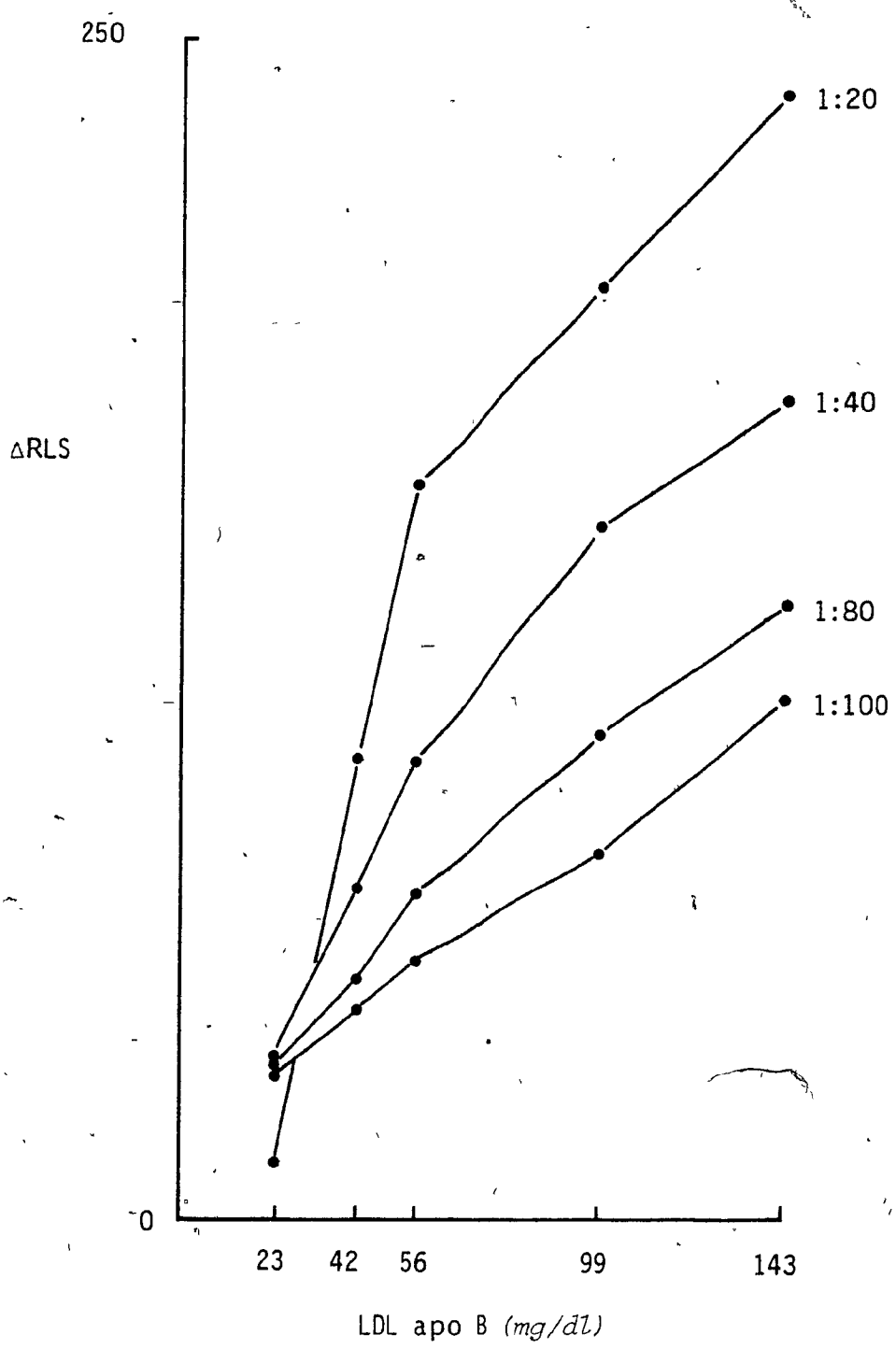


Figure 2. Light Scattering of Reaction Mixtures Containing 5λ of Antigen *(2 hours)*.

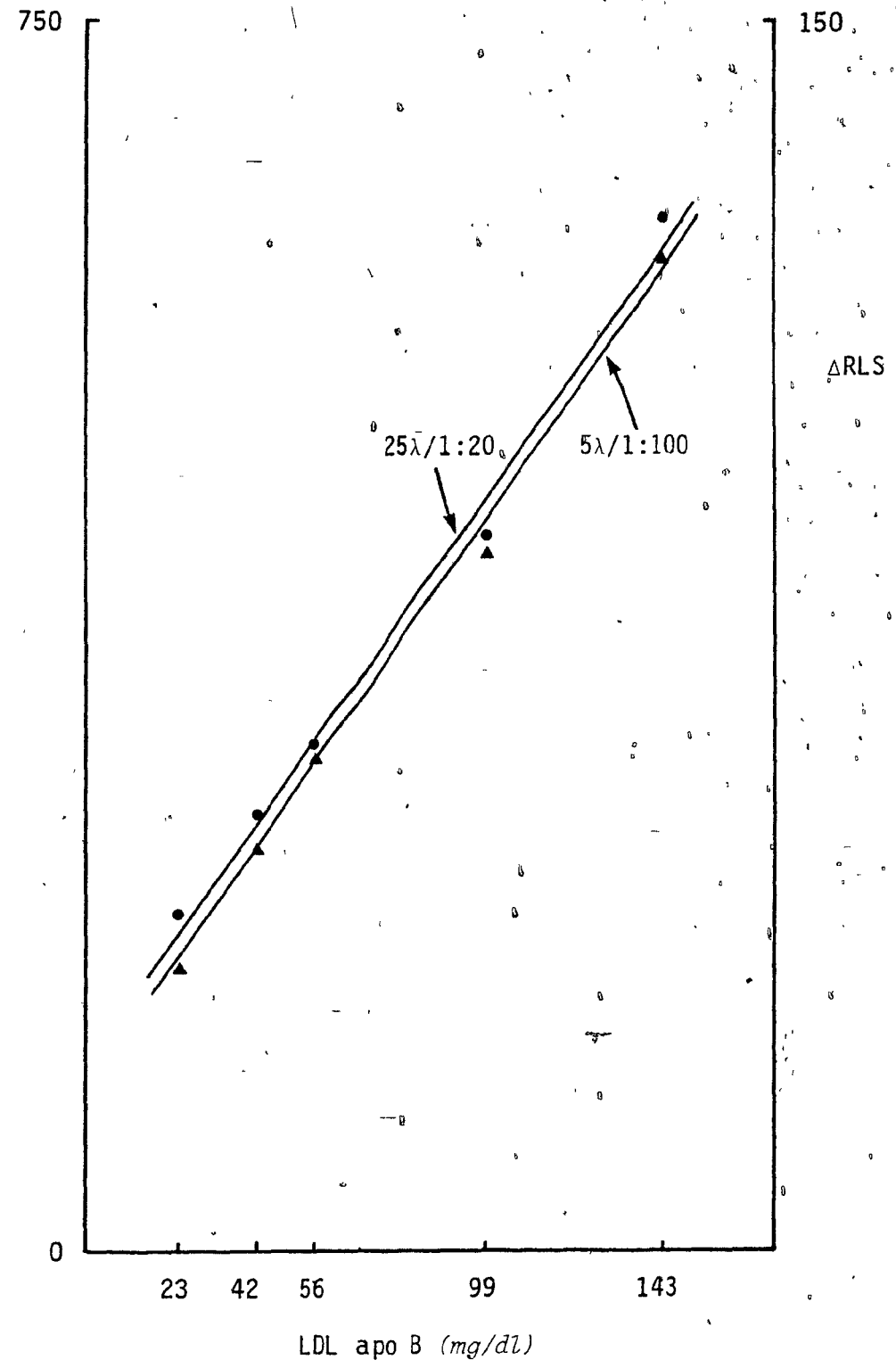


Figure 3. Comparison of Light Scattering of the Reaction Mixtures Containing 25λ Ag 1:20 Dilution of Antiserum and 5λ Ag 1:100 Dilution of Antiserum.

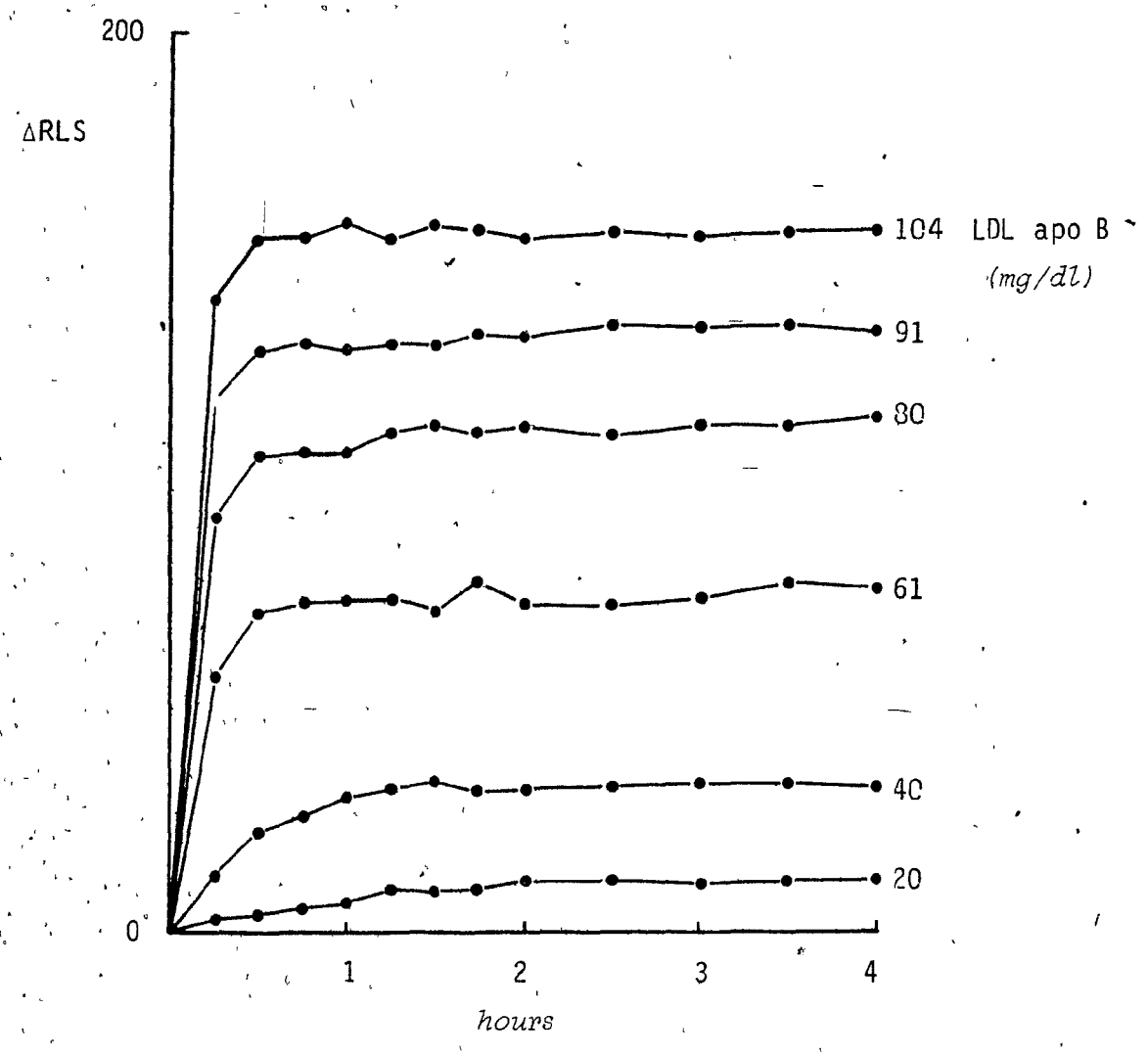


Figure 4. Time Course of LDL apo B-Anti-LDL apo B Immunoreactivity.

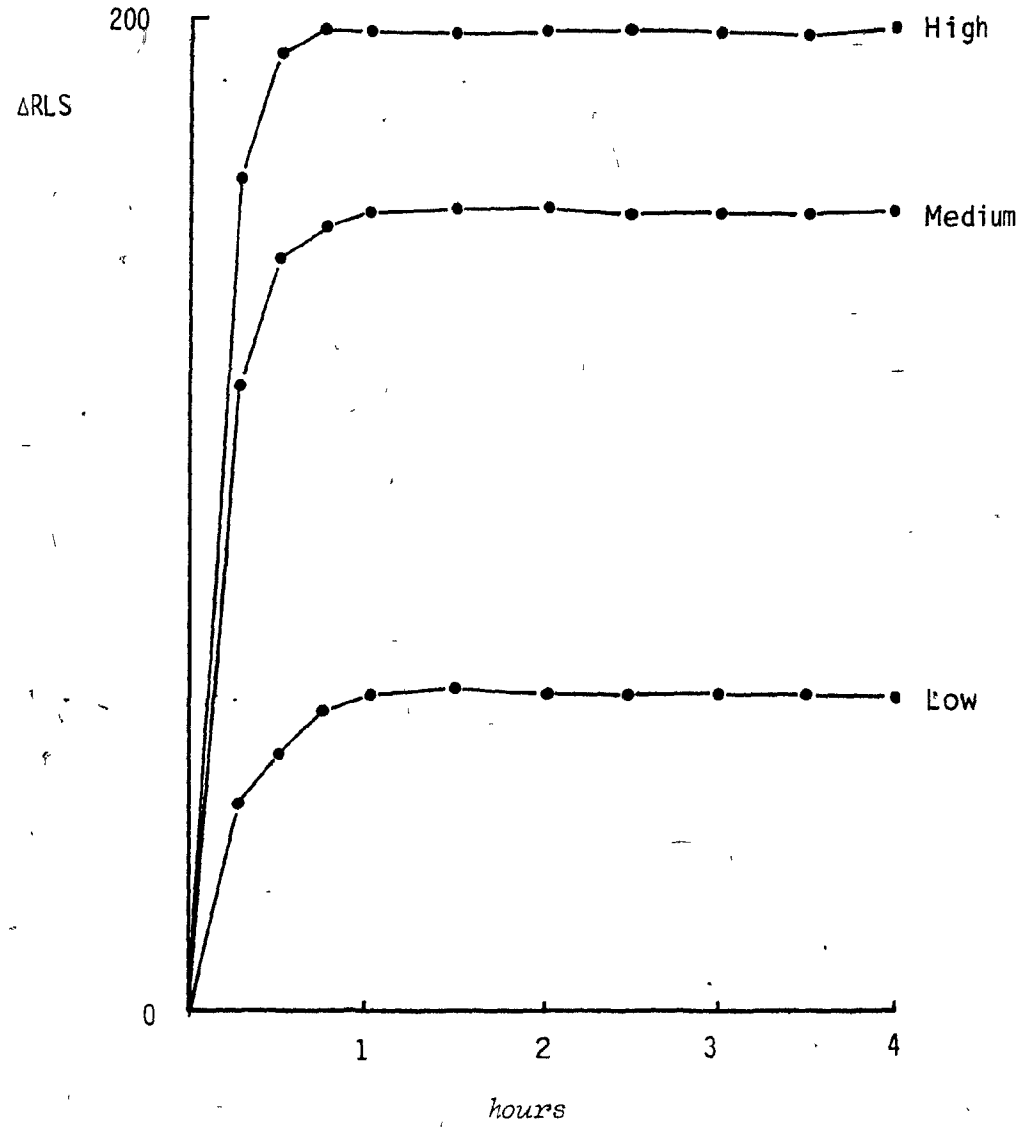


Figure 5. Time Course of the Immunoreactivity of the three Serum Samples.

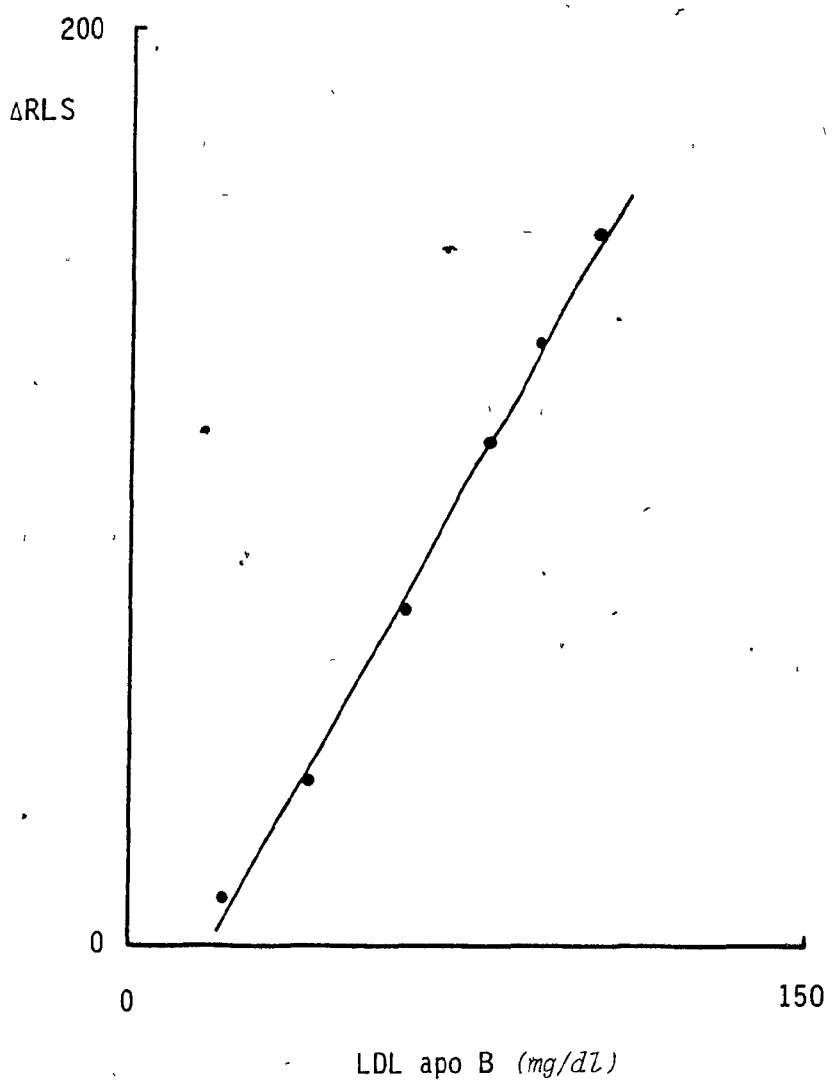


Figure 6. Precipitan Curve for LDL apo B-Anti-LDL apo B *(2 hours)*.

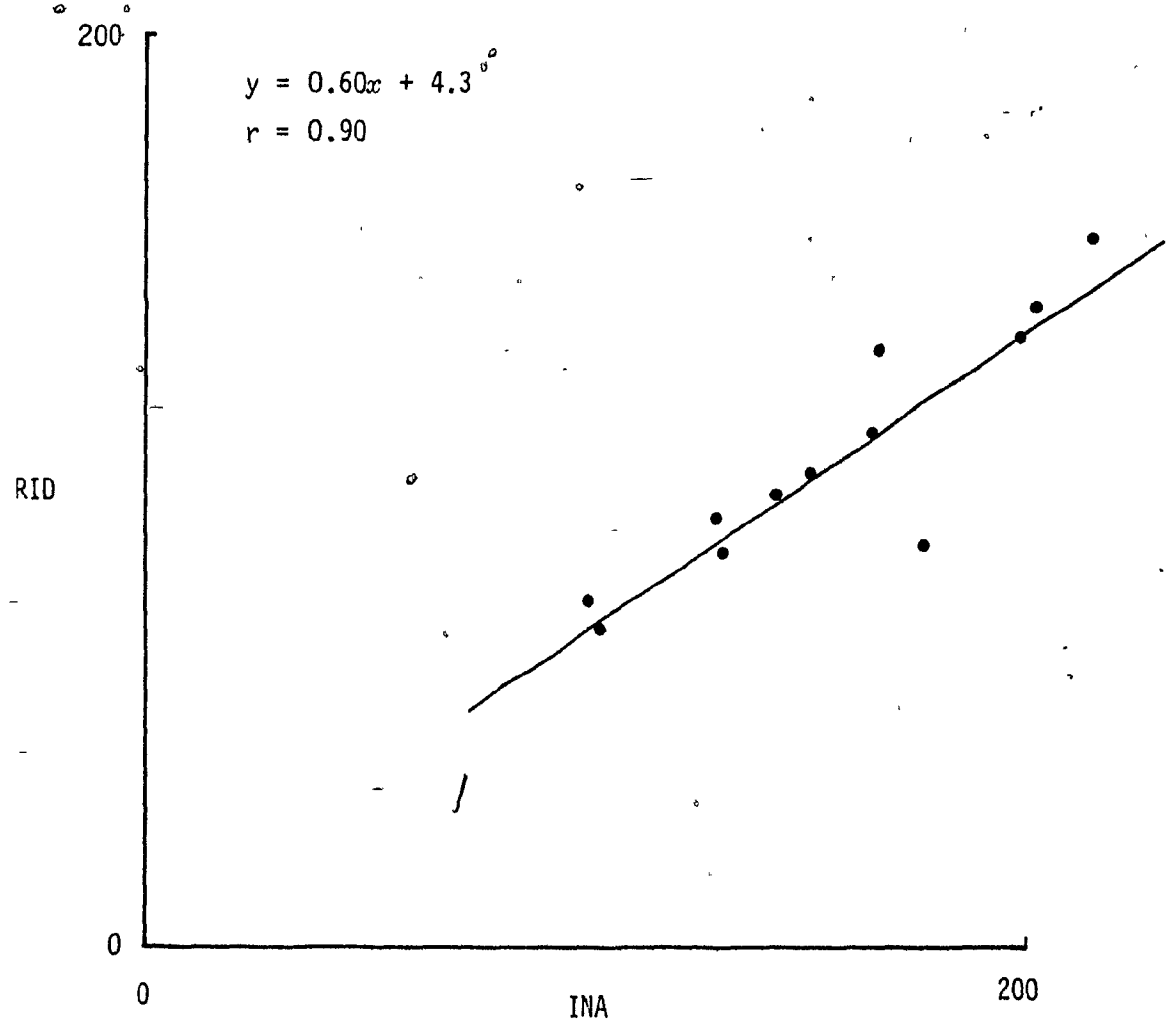


Figure 7. Comparison of Apo B Levels *(mg/dl)* Estimated in Whole Serum by INA and RID.

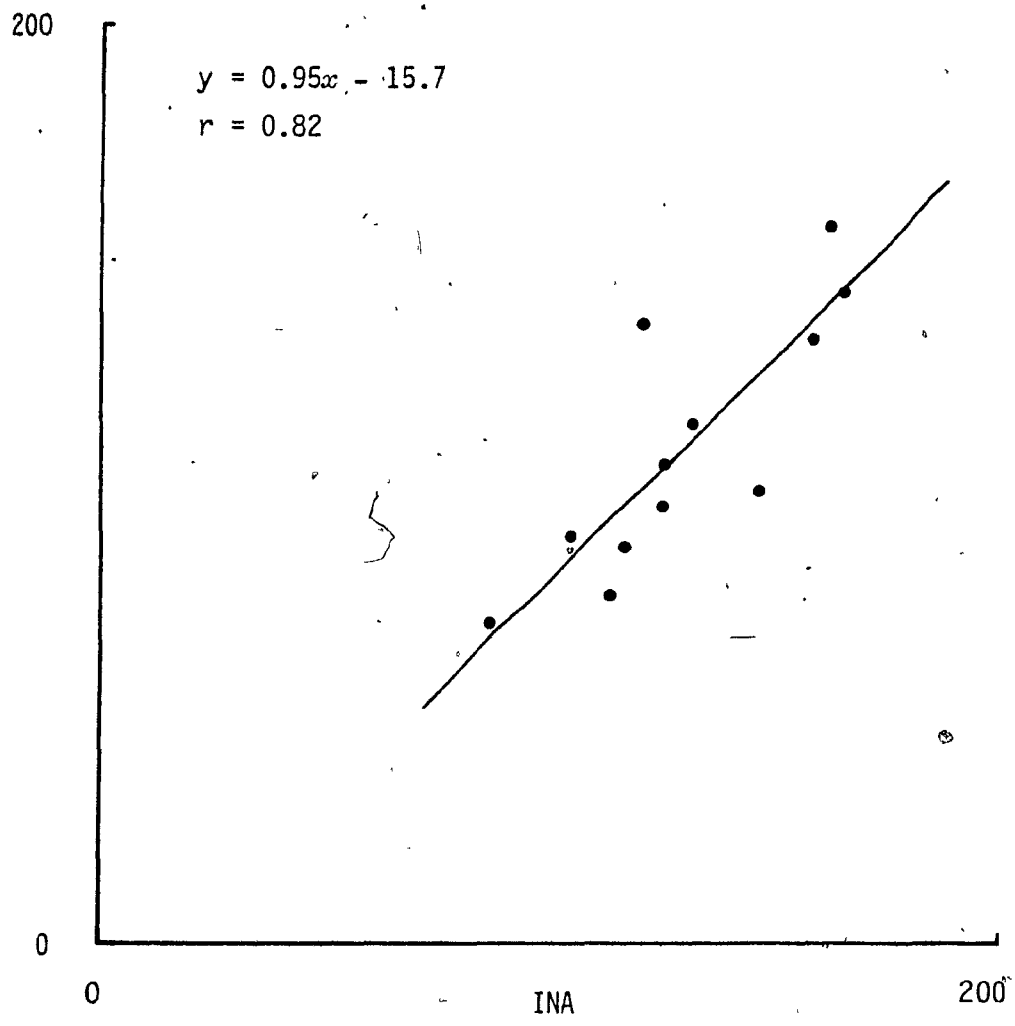


Figure 8. Comparison of apo B *(mg/dl)* in the 1.006 Bottom Serum Fraction Measured by INA and RID.

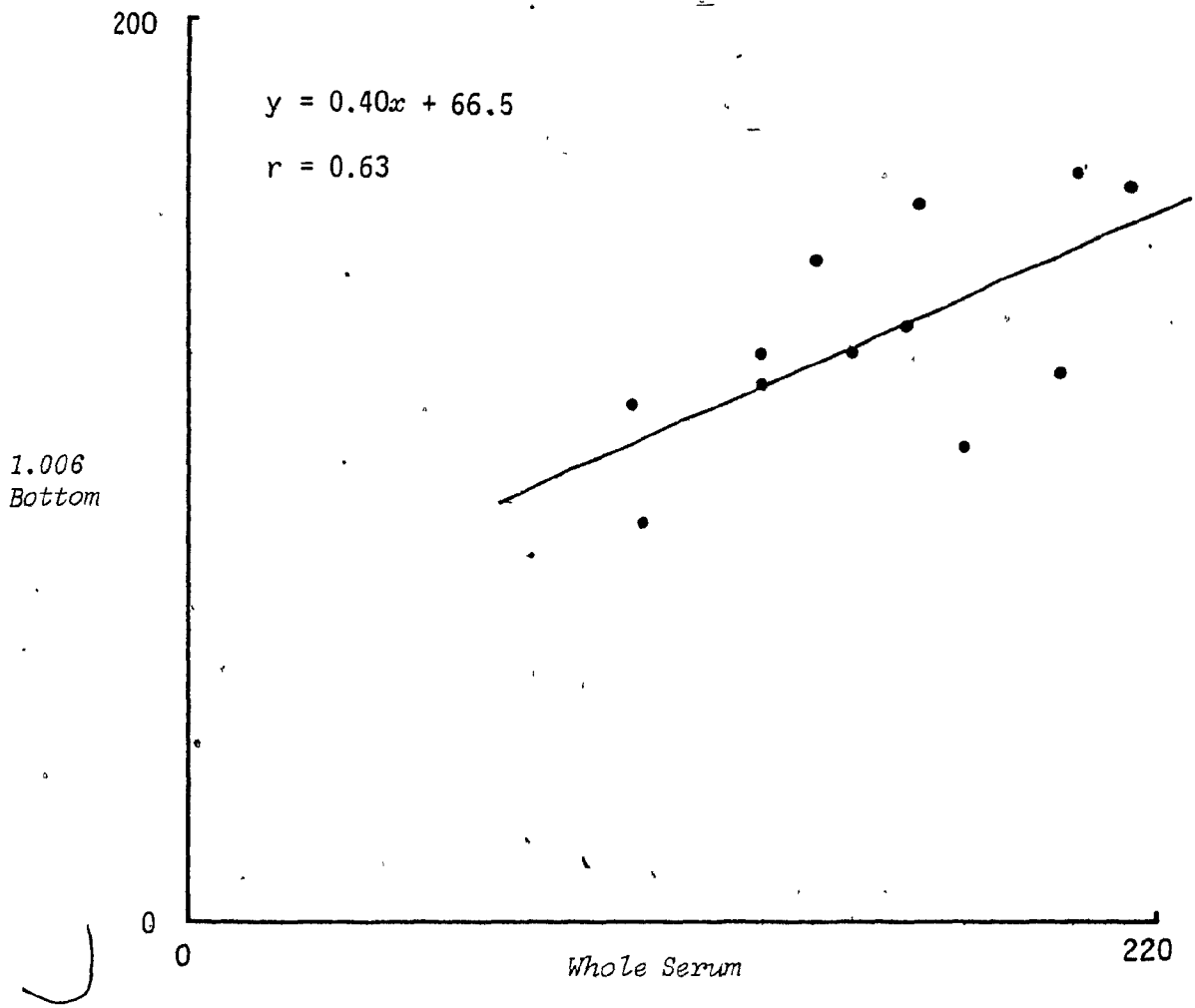


Figure 9. Comparison of apo B Levels *(mg/dl)* in Whole Serum and the 1.006 Bottom Serum Fraction Measured by INA.

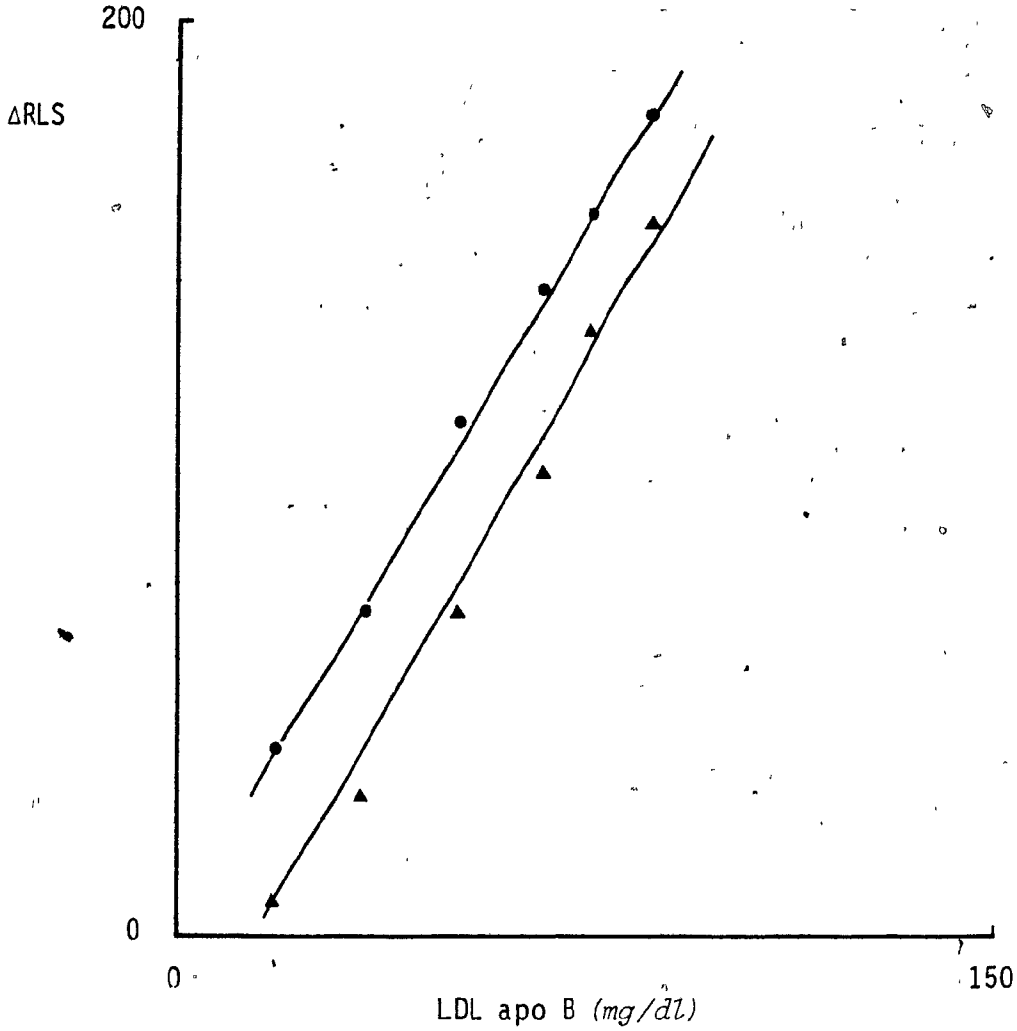


Figure 10. Comparison of the Precipitan Curves using Serum-Free ▲——▲ and LFS ●——● LDS Standards.

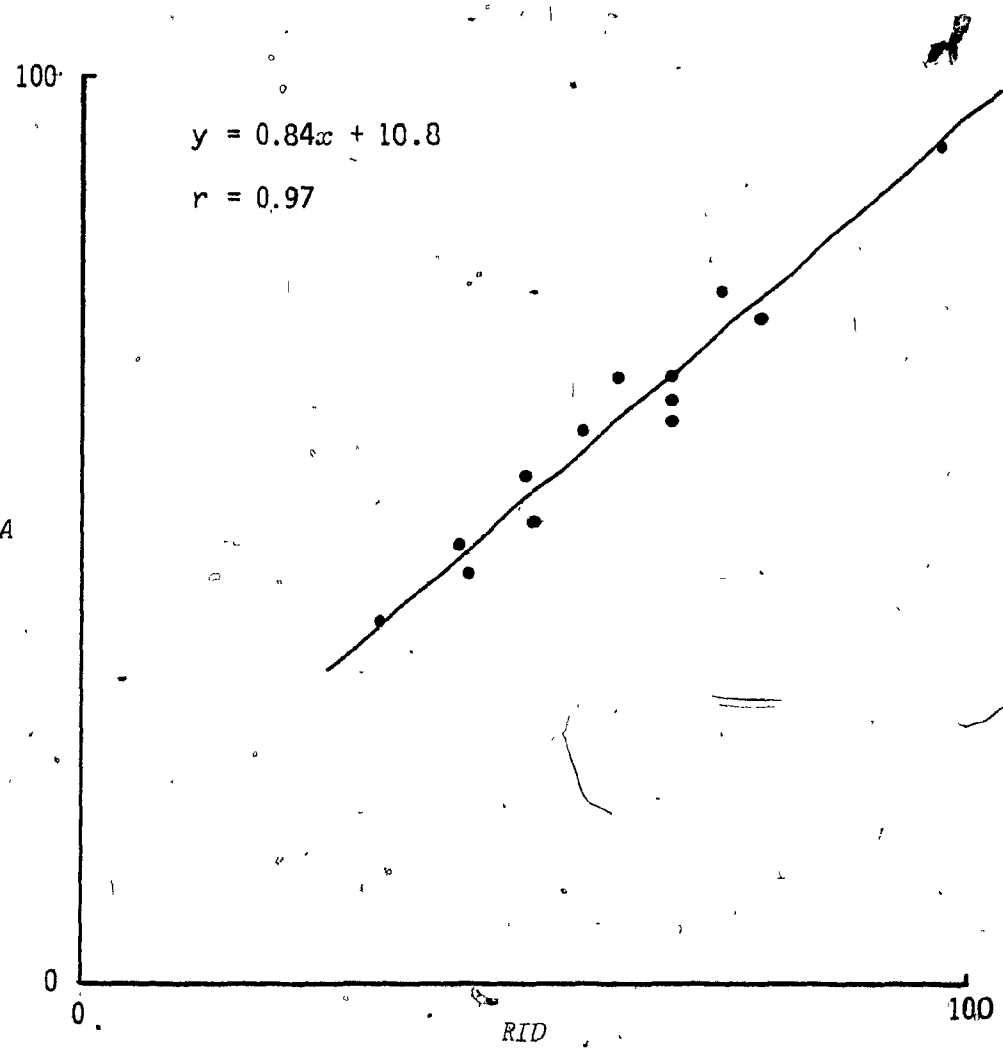


Figure 11. Comparison of Apo B Levels *(mg/dl)* estimated in the 1.02 Bottom Fraction by INA and RID.

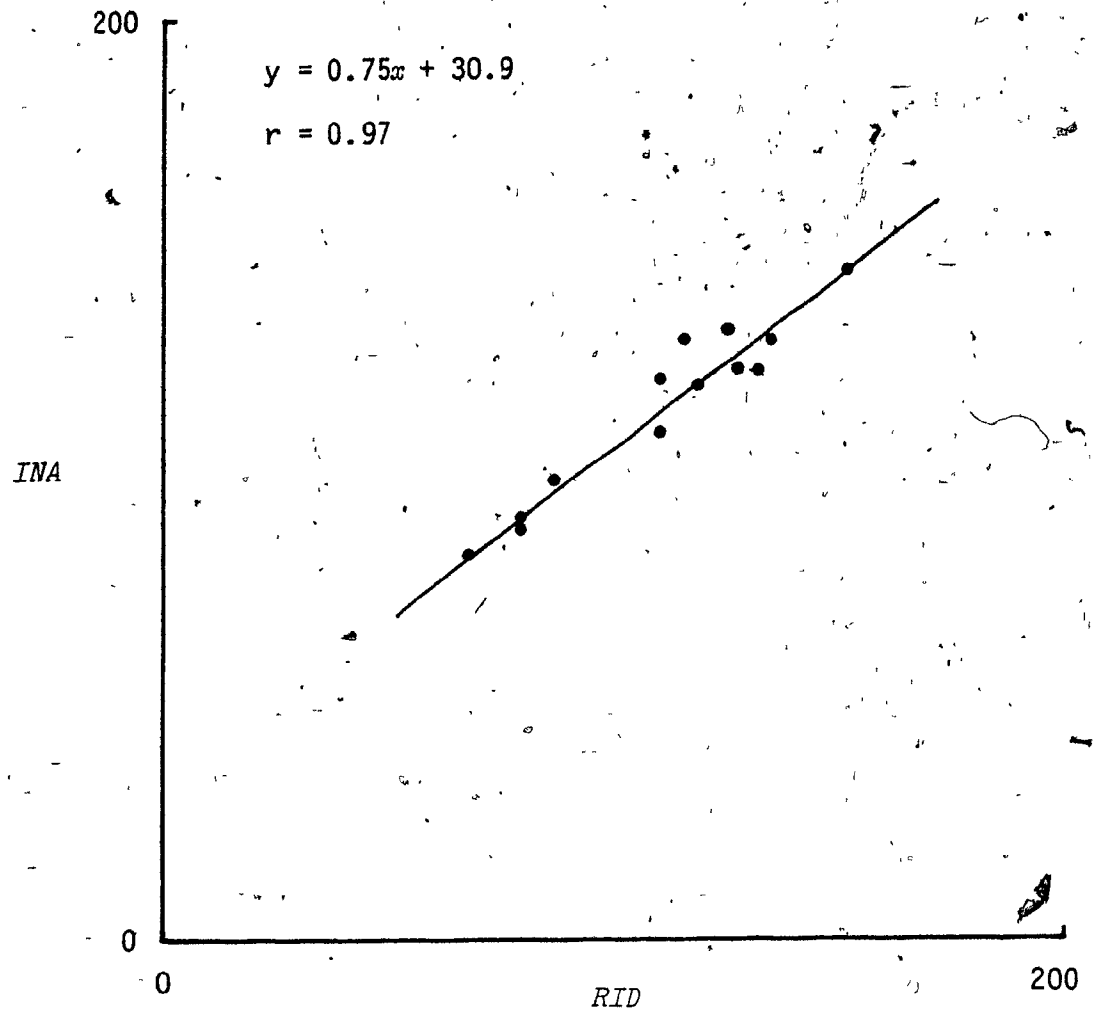


Figure 12. Comparison of Apo B Levels *(mg/dl)* Estimated in the 1.02 Bottom Serum Fraction by INA and RID.

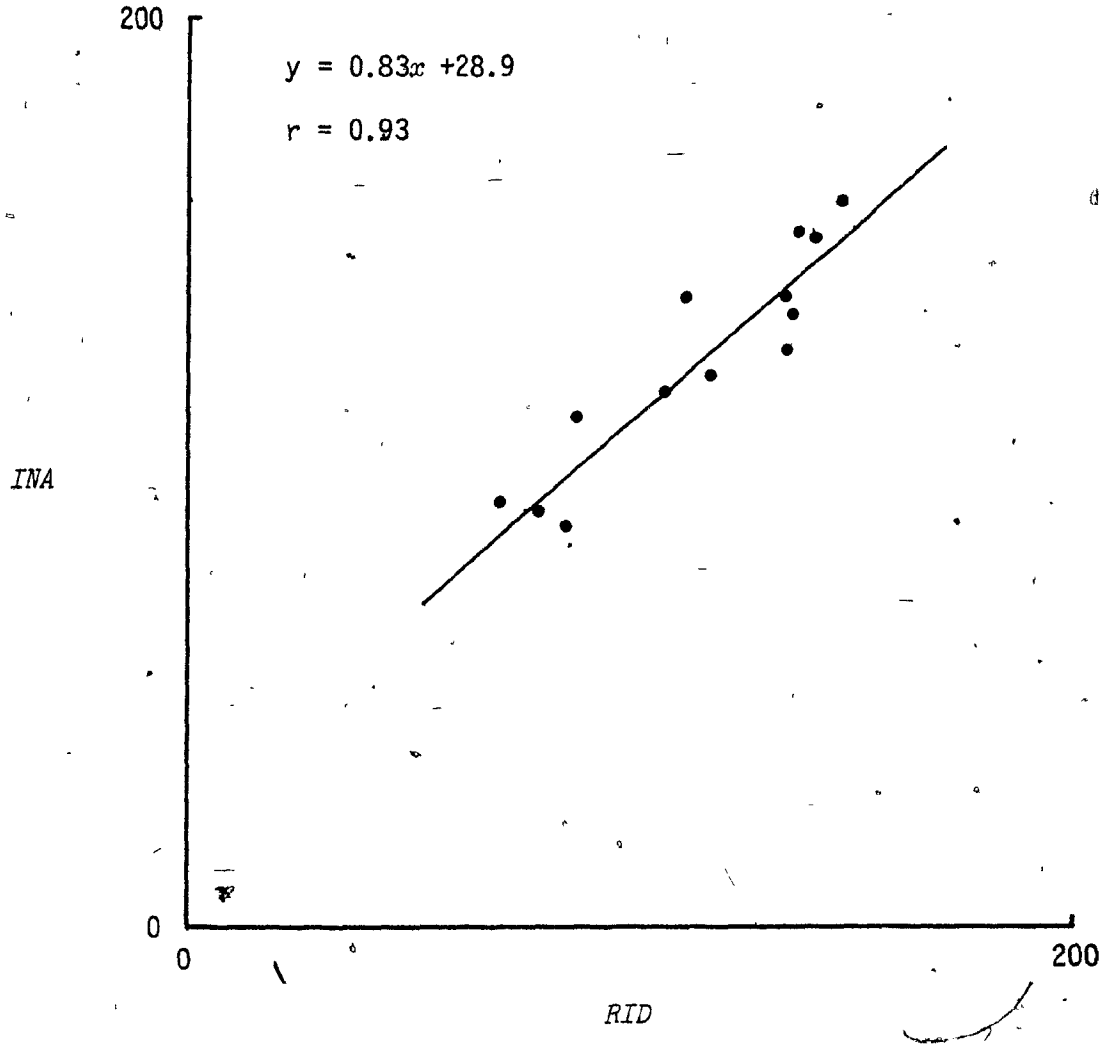


Figure 13. Comparison of Apo B levels *(mg/dl)* Estimated in the 1.006 Bottom Serum Fraction by INA and RID

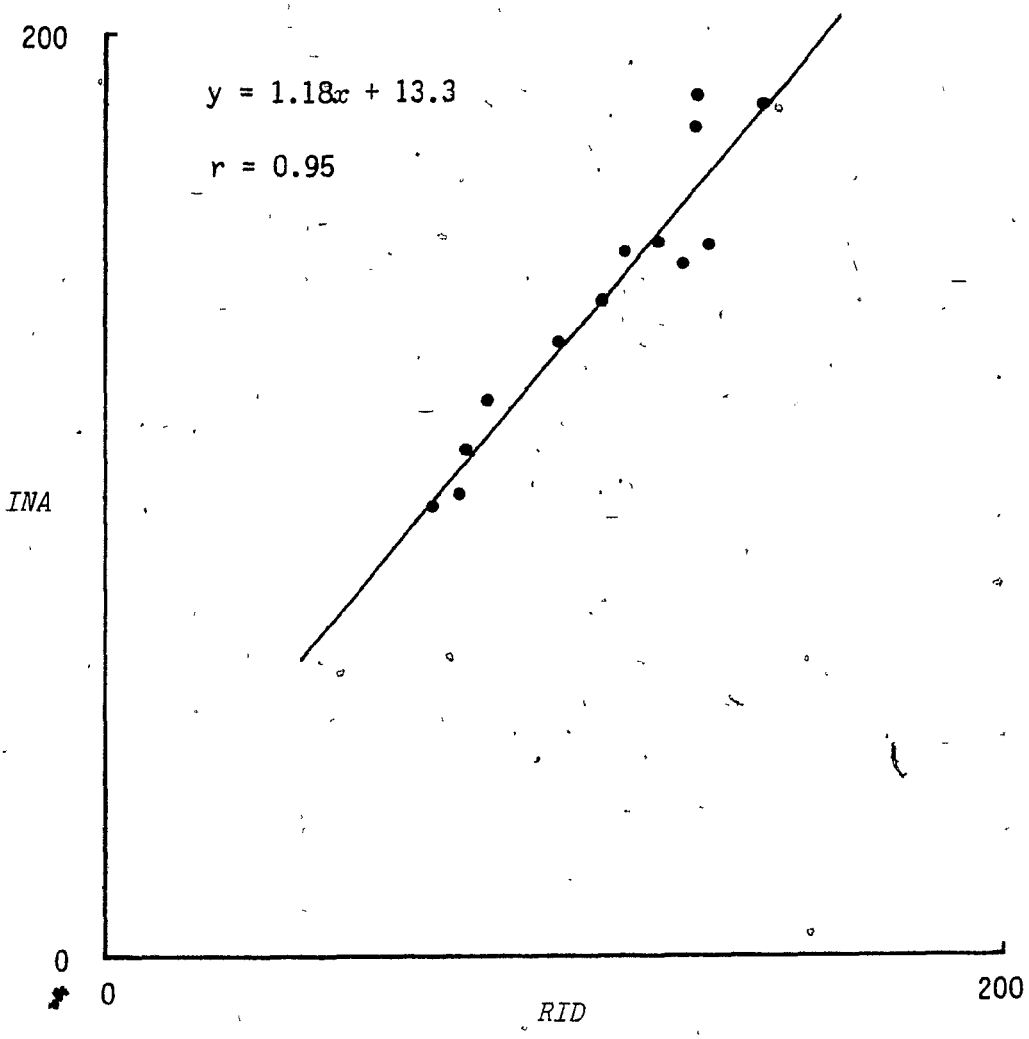


Figure 14. Comparison of Apo B Levels *(mg/dl)* Estimated in Whole Serum by INA and RID.

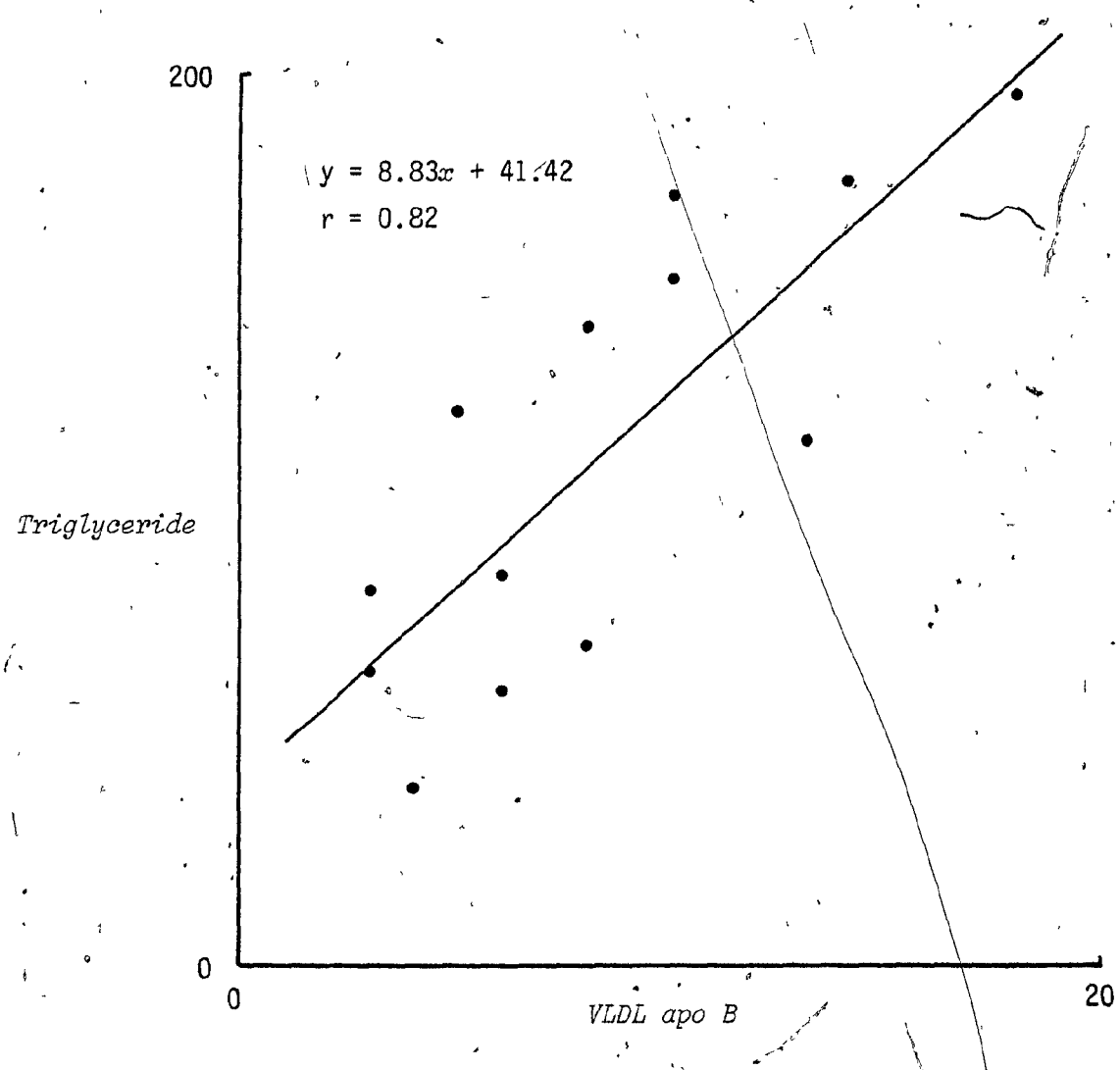


Figure 15. Comparison of Serum Triglyceride *(mg/dl)* and VLDL Apo B *mg/dl)* Levels.

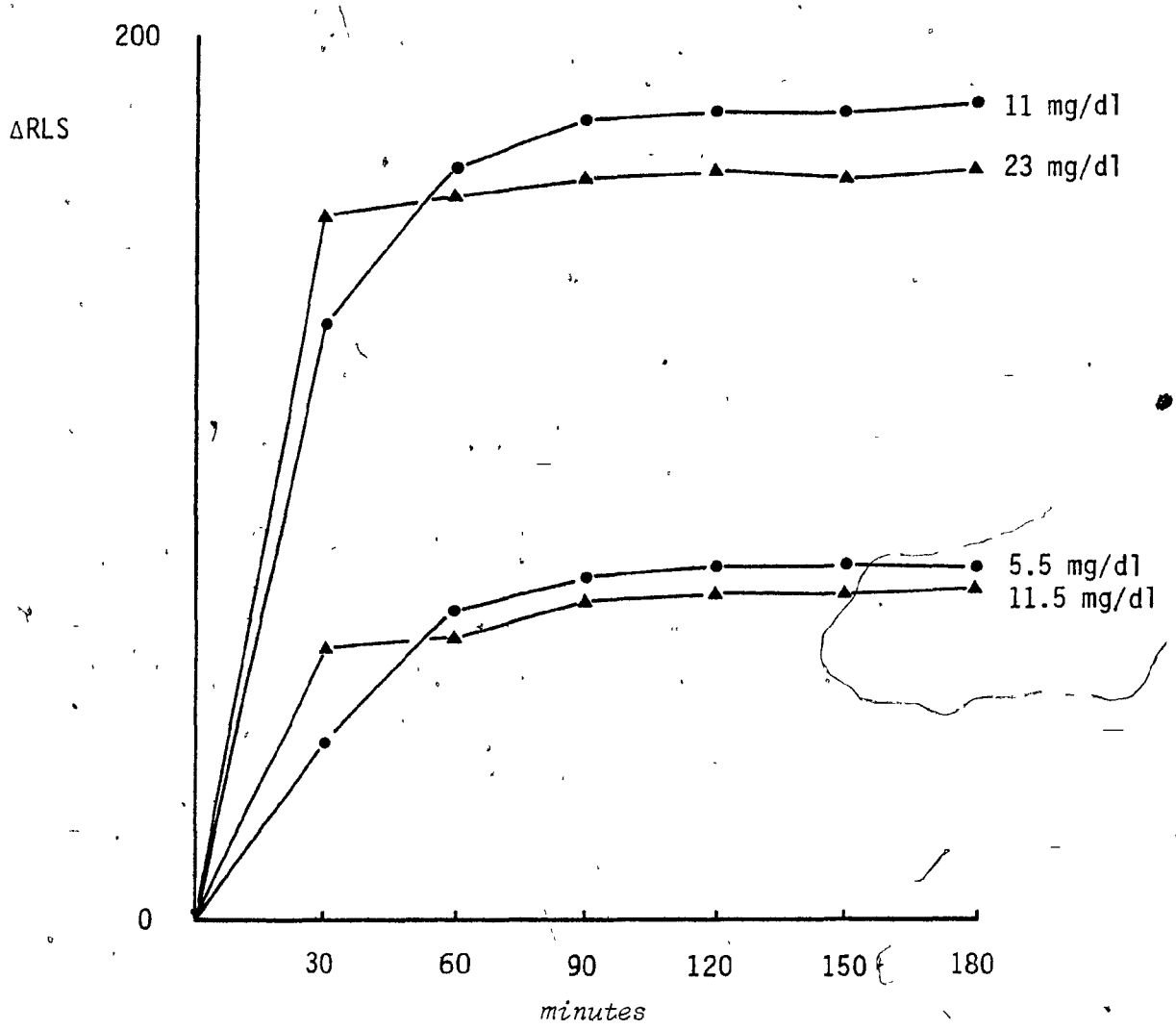


Figure 16. Comparison of the Immunoreactivity Over Time of VLDL●——● and LDL ▲——▲ .

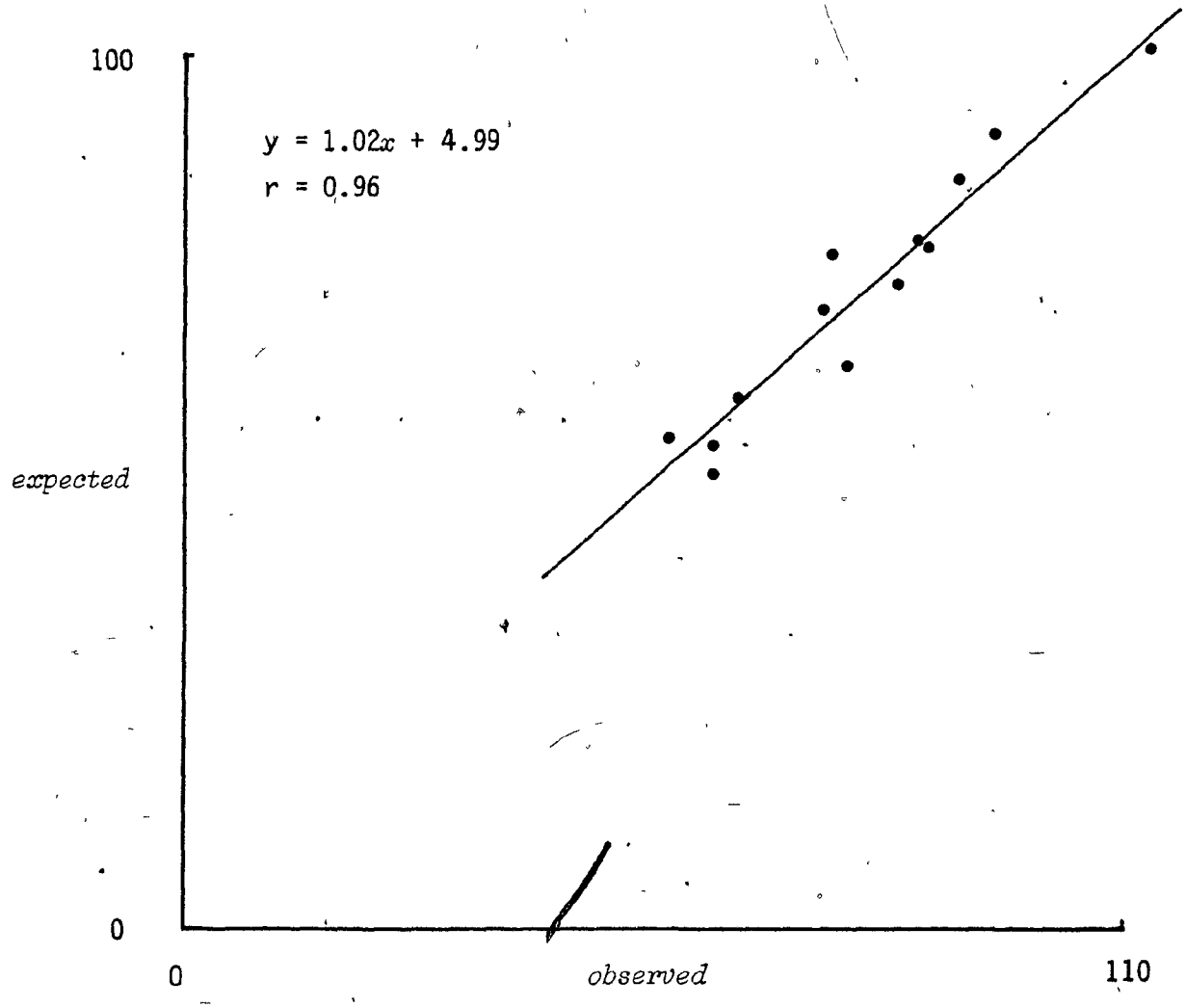


Figure 17. Comparison of Expected and Observed Apo B Levels *(mg/dl)* With the Addition of VLDL (11 mg/dl) to the 1.02 Bottom Serum Samples.

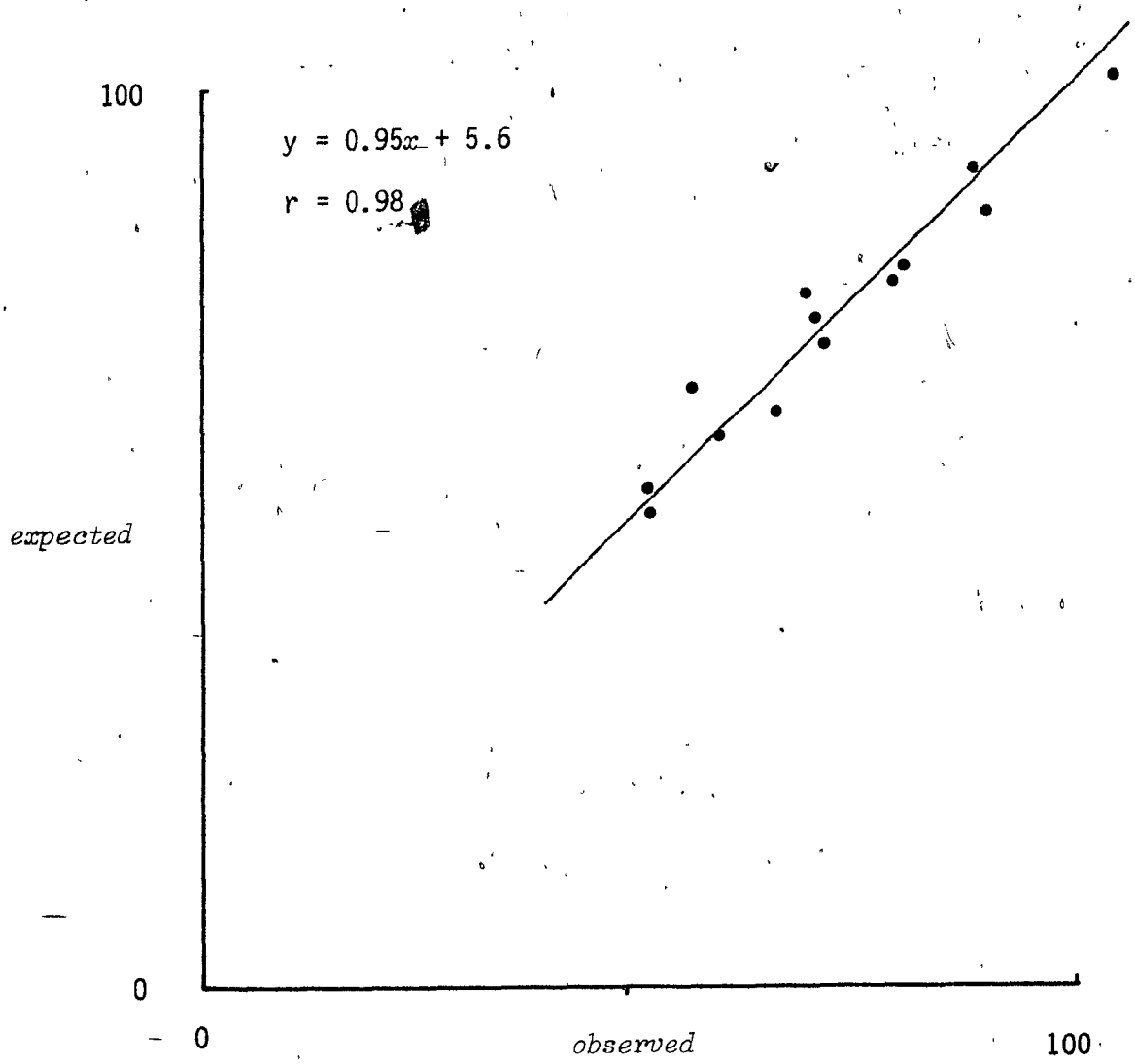


Figure 18. Comparison of Expected and Observed Apo B Levels *(mg/dl)* With the Addition of LDL (11.5 mg/dl) to the 1.02 Bottom Serum Samples.

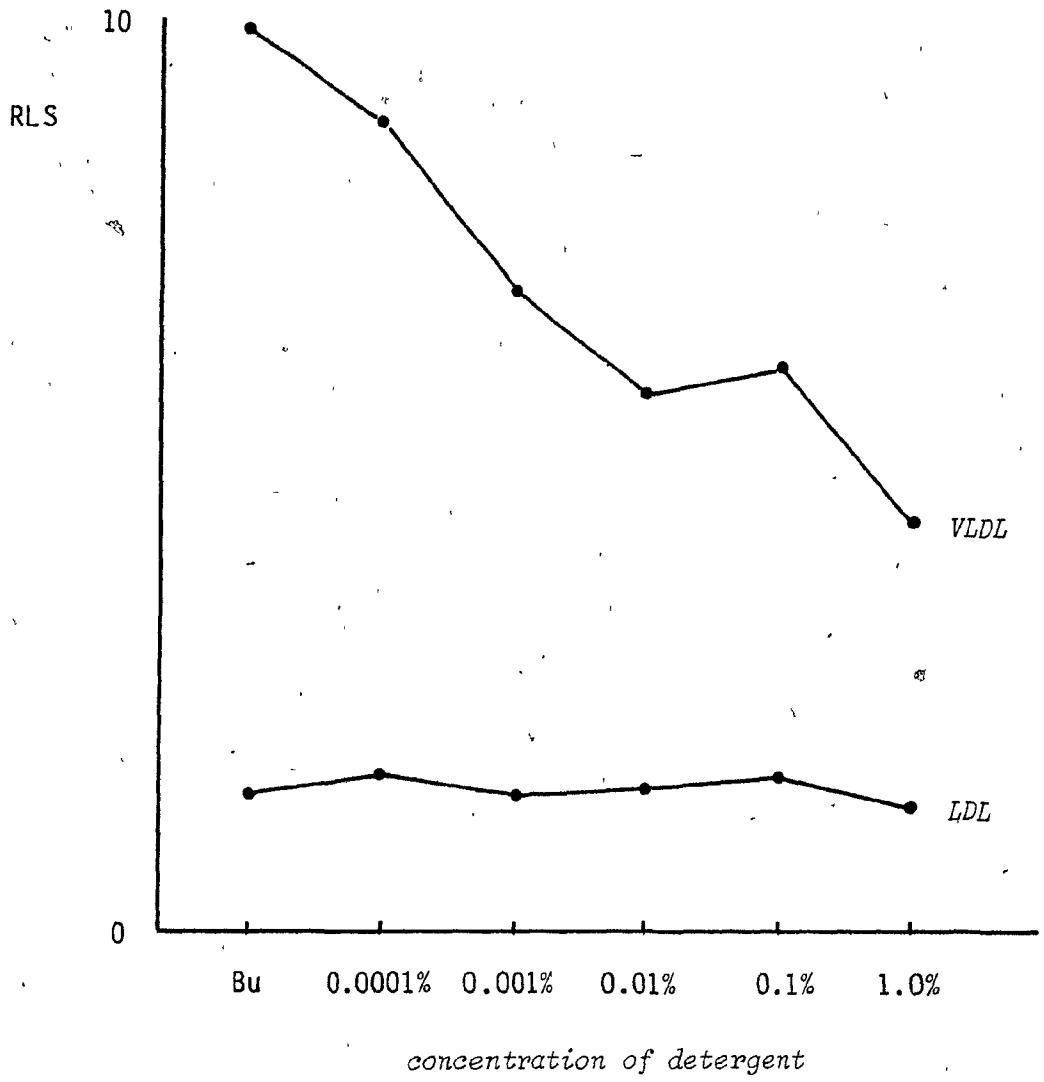


Figure 19. Effect of ODA-POE on the Light Scattering of the VLDL and LDL Blanks.

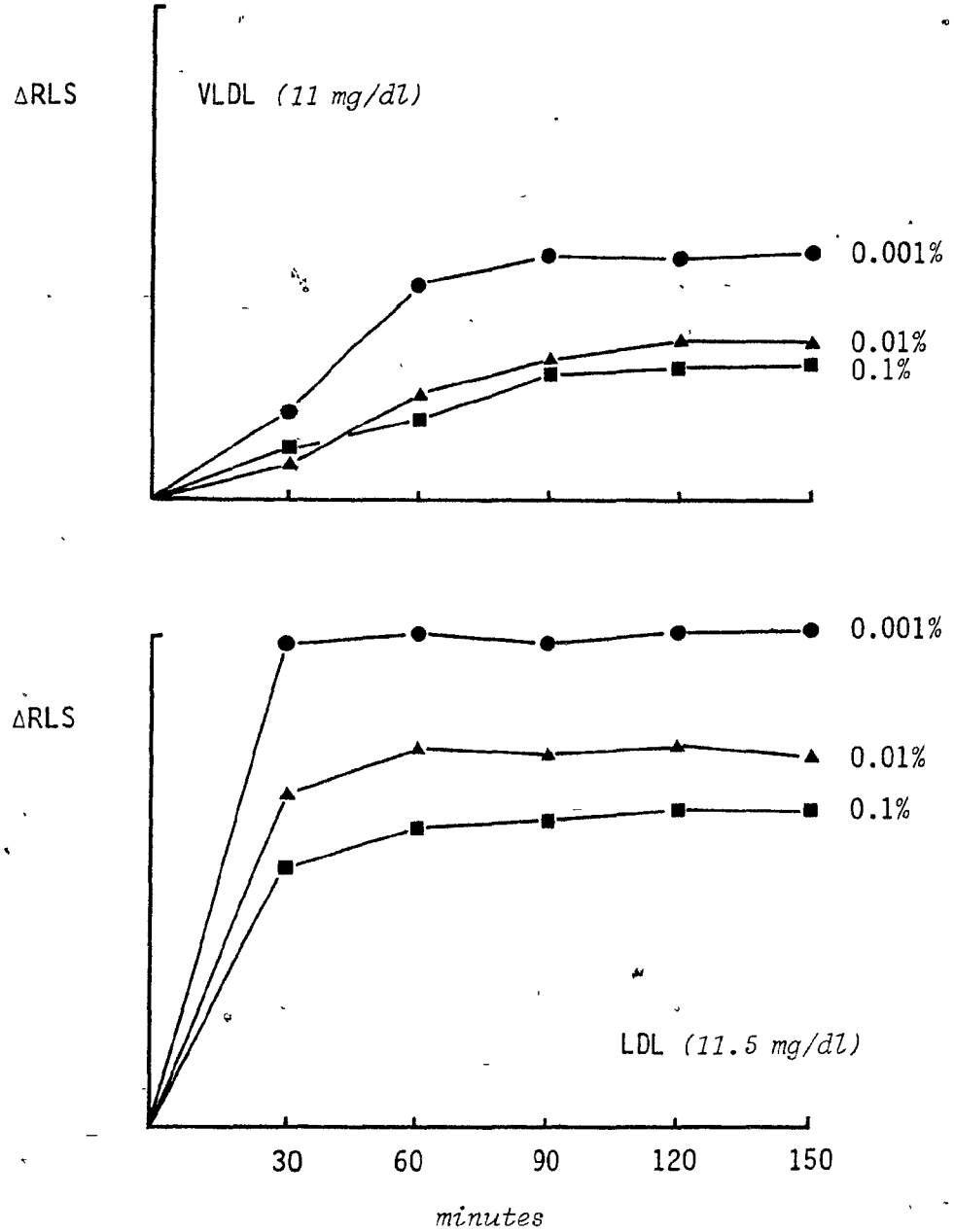


Figure 20. Comparison of the Immunoreactivity Over Time of VLDL (top) and LDL (bottom) in the Presence of Tween 20. The Effect Shown for Tween is Representative of the Effect of the Other Detergents Tested as well.

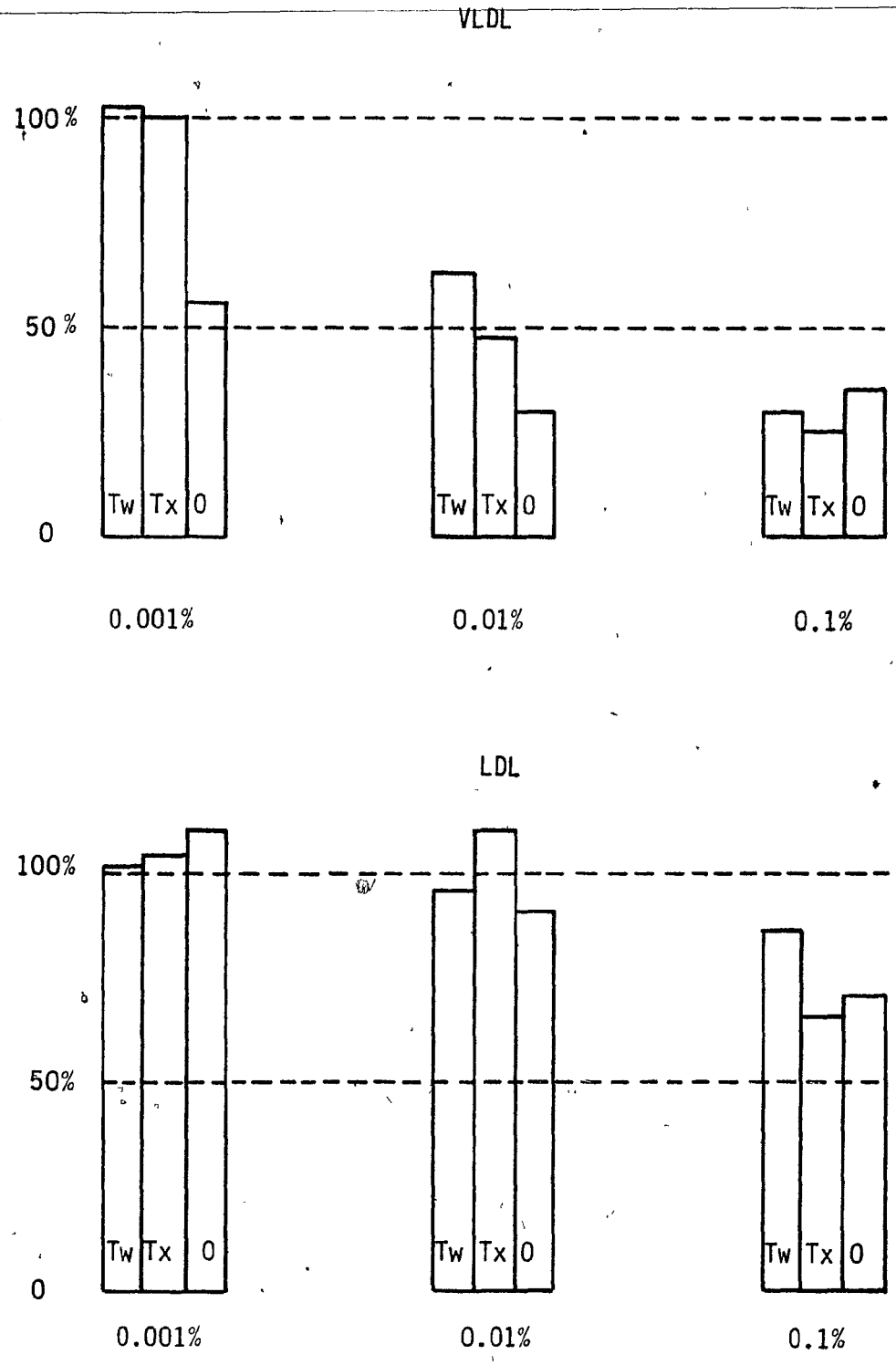


Figure 21. The Graphs Illustrate the Effects of the Three Detergents on the Light Scattering of VLDL (top) and LDL (bottom). The Height of the Bar Represents the % of the Light Scattering of the Corresponding Reaction in the Absence of Detergent. The non-detergent reaction = 100%. Tw = Tween 20, Tx = Triton X-100 and O = ODA-POE.

APPENDIX III - ENDNOTES

1. A primary characteristic of a particle with regard to its light scattering properties, is the ratio of its size to its wavelength of illumination, λ. For small particles with a diameter (d) < 0.1, the angular distribution of scattered light is symmetrical about the 90° axis, and forward scatter is equal to backward scatter. The scattering exhibited by particles of this size is known as Rayleigh scattering. Albumin (d/λ=0.02), HDL (d/λ=0.03), LDL (d/λ=0.06), and IgG (d/λ=0.07), are examples of this pattern of scattering. As the particle size increases relative to the wavelength of illumination, the intensity of light in the forward direction increases over that scattered in the backward direction, making the dissymmetry ratio (intensity of forward light scatter/intensity of backward light scatter) greater than unity. This form of scattering where $d \leq \lambda$ is known as Rayleigh-Debye. Many of the small aggregates formed as a result of immunological reactions are typical examples. Mie scattering occurs when $d > \lambda$; for such large particles, most of the scattered light is concentrated within a narrow angular region in the forward direction. Bacteria, blood cells, dust particles and immunocomplexes formed with lipoprotein particles are representative of particles whose $d > \lambda$. In mie scattering, the potential interfering effects of small particles such as Rayleigh scatterers is increased because the particles may reach a point of observation in phase and add, producing an intensity maximum, or out of phase and cancel (either partially or completely), producing an intensity minimum. (For a more complete discussion, please refer to reference 135).

2. The instrument is equipped with a sensitivity setting which is used to increase or decrease the electronic signal of the photomultiplier tube. For any given sensitivity settimg, the maximum number of RLS units displayed is 200 (see reference 137).

3. Henceforth, the LFS-LDL standards were used for all apo B determinations made by INA.

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