notes on methodology

Determination of B protein of low density lipoprotein directly in plasma

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Summary Quantitation of the apoprotein constituents of lipoproteins has extended our knowledge of plasma lipid transport. Previously, B protein content of low density lipoprotein could be measured by radial immunodiffusion only after ultracentrifugation. However, if performed in 1.5% agarose gel with standards and measured at 18 hr rather than at equilibrium, low density lipoprotein B protein can be measured directly in plasma, eliminating the need to separate very low density lipoprotein.

Supplementary key words hyperlipidemia · radial immunodiffusion

Understanding lipoprotein physiology is contingent upon the ability to quantify. Usually, lipoprotein concentration is expressed in terms of lipids; recent studies, however, focus on the protein moiety (1, 2). In low density lipoprotein (LDL), this is composed almost exclusively of B protein (3, 4), whereas in very low density lipoprotein (VLDL), several additional apoproteins are present (5). Previously, before measuring LDL B protein by immunological techniques, VLDL has been separated from plasma by ultracentrifugation. The present study reports a modified radial immunodiffusion assay that allows LDL B protein to be measured directly in plasma.

Isolation of LDL. The blood of fasting subjects was collected in tubes containing 1 mg/ml disodium EDTA. After centrifugation for 20 min at 2500 *g*, 4°C, the density of the separated plasma was adjusted to 1.019 g/ml by addition of NaCl-KBr. The sample was then ultracentrifuged for 18 hr at 105,000 *g* (Beckman model L2-65B with a Ti-50 rotor). The supernate was removed and its density was adjusted to 1.050 g/ml; the ultracentrifugation was then repeated. The supernate was "washed" by an additional centrifugation at 1.050 g/ml. The final supernate, LDL, served as the immunizing antigen and the assay standard. Similarly, VLDL, d 1.006 g/ml, and high density lipoprotein (HDL), d 1.063–1.21 g/ml, were isolated by preparative ultracentrifugation.

The reference method for measuring protein concentration was that of Lowry et al. (6).

Immunoassay for LDL. Antisera were prepared in rabbits. A total of 1 mg of LDL emulsified with complete Freund adjuvant 1:1 (v/v) was given subcutaneously to each of the four animals in five divided doses at weekly intervals. The animals were bled in 6 wk and the separated sera were stored at −20°C with 0.02% sodium azide added as preservative. The specificity of the antisera was examined by the Ouchterlony technique (7) and immunoelectrophoresis (8).

Radial immunodiffusion was carried out by the method of Mancini, Carbonara, and Heremans (9). Unabsorbed antiserum (usually in a dilution of 1/10) was mixed with veronal buffer, pH 8.6 and ionic strength 0.05, in agarose of final concentration 1.5%. The sample wells held a volume of 5 μl. All assays were done in triplicate at room temperature in a humidity chamber. Rather than being continued to equilibrium, the assay was terminated at 18 hr. A set of five serially diluted standards was included with each assay. Isolated LDL with 0.02% sodium azide was stable at 4°C for at least 4 months for use as standard.

Studies of patients. Plasma was obtained from 48 fasting patients. From each patient, a d < 1.006 g/ml infranate and a d 1.006–1.063 g/ml supernate were prepared, and then the B protein concentration was determined by radial immunodiffusion in each sample. The patients were selected from those on a general medical hospital service. None had obstructive jaundice. Phenotyping was done by lipoprotein electrophoresis and quantitation of LDL cholesterol in the 1.006–1.063 supernate (10).

Characteristics of antiserum and antigen (B protein) in LDL and VLDL. When examined by Ouchterlony's technique (7), there was no reaction of the antisera against HDL, albumin, or the 1.21 infranate. Only a single precipitin arc was seen by immunoelectrophoresis against whole plasma.

Because B protein is common to LDL and VLDL, a line of identity is produced in Ouchterlony plates between these two lipoproteins (**Fig. 1**). Note that the LDL band is thicker and penetrates the 1.5% agarose gel more readily; this difference in mobility may be due at least in part to the lower molecular weight of LDL. Moreover, if both VLDL and LDL are in the same well in equal concentration, only a single precipitin line with the faster-moving LDL is seen, even at long incubation times up to 72 hr. This occurred with each of four consecutive antisera to the LDL antigen.

Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein, HDL, high density lipoprotein.

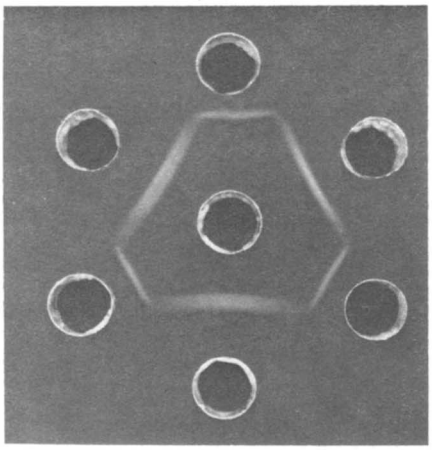

Fig. 1. With antiserum in central well and LDL and VLDL in alternate peripheral wells, two different precipitin arcs form a line of identity. The thicker, longer, more advanced arc is LDL, and the other is against VLDL.

To confirm the assay as a measure of protein, we compared the B protein concentration (measured by radial immunodiffusion) and the protein concentration (measured chemically by the technique of Lowry et al. [6]). For this, 30 samples of LDL (d 1.006–1.063) were used. The correlation coefficient was 0.96, and the regression equation was $y = 0.95x - 3.4$ (SE 3.4). The interassay coefficient of variation of the immunodiffusion measurement was less than 7%. A representative standard curve is shown in **Fig. 2.**

Selective determination of LDL B protein. The B protein concentration in 48 samples of fasting plasma was compared with the B protein concentration of the 1.006 infranate. The regression line (**Fig. 3**) was $y = 1.03x - 2.42$ (r^2 0.99). Similarly, when the B protein concentration of isolated LDL was compared with that of plasma, the regression line was $y = 0.99x + 5.05$ (r^2 0.99). Both relationships were linear over a range of 30–220 mg/100 ml.

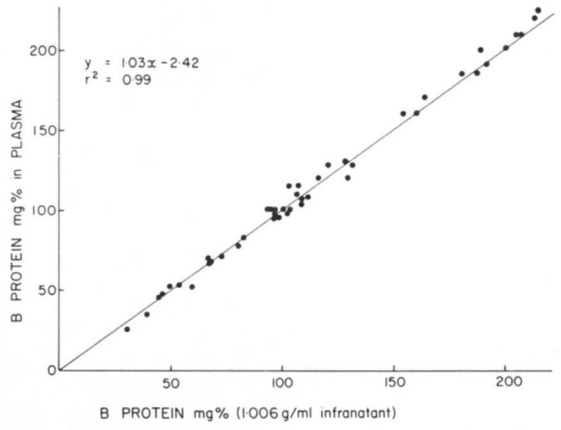

Fig. 3. Correlation of B protein in plasma with B protein in d < 1.006.

Two further techniques were employed to test the selectivity of the assay. Known amounts of LDL B protein (from 10 to 63 mg/100 ml) were added to plasma samples with different initial B protein concentrations (62 to 100 mg/100 ml). The plasma samples were reassayed, and the measured increments in B protein concentration were compared with those calculated. The results, shown in **Fig. 4,** gave a regression line of $y = 0.98x + 0.77$ (r^2 0.99).

To show that only B protein in LDL was measured, increasing amounts of VLDL were added to a constant but low amount of LDL. A representative study is shown in **Fig. 5.** Analysis of the composition of isolated VLDL revealed a mean particle size of approximately 300 Å (11). When this was added in increasing amounts up to 160 mg/100 ml (Lowry protein) to LDL (34 mg/100 ml B protein), the measured B protein was essentially unchanged. Thus, in vitro, the assay is insensitive to large increments of added VLDL protein (even at low LDL concentration) but sensitive to small amounts of added LDL. Further, as shown in **Table 1,** there is a close correspondence between B protein measured in plasma and in LDL

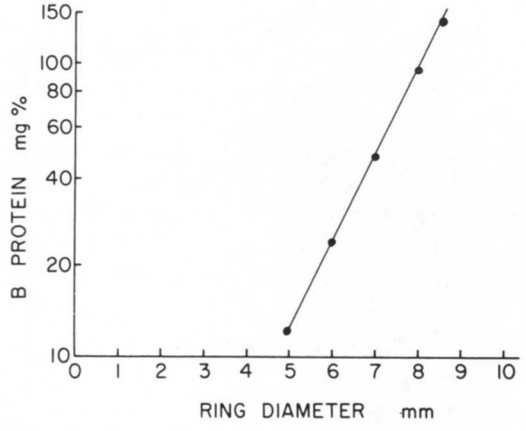

Fig. 2. Standard curve with B protein plotted against ring diameter.

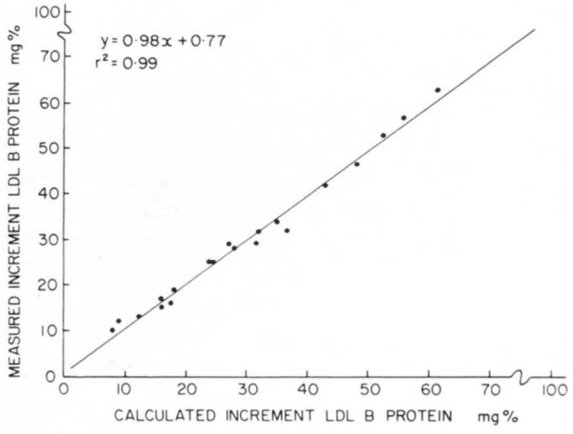

Fig. 4. Measured increase in B protein plotted against the predicted rise after addition of LDL to plasma.

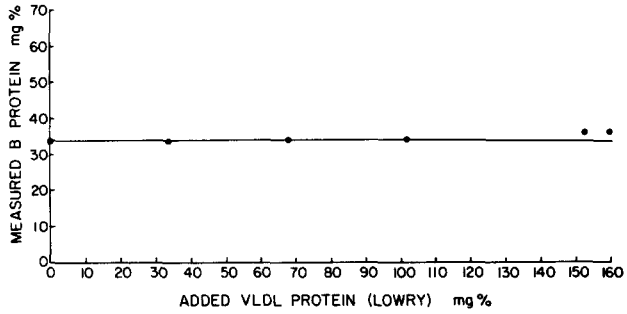

Fig. 5. Specificity of the LDL B protein analysis. Increasing amounts of VLDL were added to a known quantity of LDL B protein, and total B protein was then remeasured.

in nine patients with type IV hyperlipidemia. Therefore, the assay remains valid in patients with high levels of VLDL and low levels of LDL.

Lees (1) successfully applied the Mancini technique to measure the B protein content of the 1.006 infranate and, with his colleagues, developed a radioimmunoassay method to measure total B protein in plasma (2). We have shown in the present study that LDL B protein may be measured directly in plasma. By avoiding any separation, the technique is simpler, less expensive, and less subject to error. The principal modifications introduced are a 1.5% agarose gel and a fixed incubation time of 18 hr rather than the longer period of 72–100 hr necessary for equilibrium. The 18-hr duration was selected partly for convenience but also because the precipitin interface was clearest with this timing. The assay remains valid for at least 30 hr. The validity of the present assay to measure LDL B protein directly in plasma has been established by the following observations. First, plasma B protein is virtually the same as the B protein of either the d < 1.006 infranate or the d 1.006–1.063 isolate; second, increments of LDL B protein added to plasma can be accurately measured; and third, the measurement of small amounts of LDL B protein is undisturbed in both in vivo and in vitro in the presence of large amounts of VLDL.

TABLE 1. Results in patients with type IV hyperlipidemia

	Patient								
	1	2	3	4	5	6	7	8	9
	mg/100 ml of plasma								
Plasma B protein	52	25	63	45	35	52	68	48	53
LDL B protein (d 1.006–1.063 g/ml)	49	30	60	44	39	59	68	46	53

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