papers and notes on methodology

Evaluation of the measurement of B protein of plasma low density lipoprotein by radial immunodiffusion

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Abstract Radial immunodiffusion (RID) has been used for determination of low density lipoprotein (LDL) B protein in plasma. During measurement of B protein in plasma and the d < and d > 1.019 g/ml plasma fractions by RID in 1.0%, 1.5%, 2.0%, and 2.5% agarose, the d < 1.019 g/ml lipoproteins diffuse in the agarose and produce precipitin rings. Among normotriglyceridemic subjects, the B protein values in whole plasma obtained by RID using 1.5 to 2.5% agarose were only slightly higher than the values in the d > 1.019 g/ml fraction obtained by RID and closely approximated the values obtained in the d > 1.019 g/ml fraction by radioimmunoassay. However, among the hypertriglyceridemic subjects, the RID measurement of B protein in plasma using 1.0 to 2.5% agarose overestimated the LDL B protein levels in plasma. The RID procedure at agarose concentrations of 1.5% to 2.5% can be used to estimate plasma LDL B protein levels in normotriglyceridemic subjects. However, measurement of LDL B protein by RID in plasma of hypertriglyceridemic subjects must be interpreted with caution; the LDL B protein is overestimated by this procedure because of the contribution by the d < 1.019 g/ml lipoproteins to the B protein value. — **Lutalo-Bosa, A. J., J. L. Adolphson, and J. J. Albers.** Evaluation of the measurement of B protein of plasma low density lipoprotein by radial immunodiffusion. *J. Lipid Res.* 1985. **26:** 995–1001.

Supplementary key words hypertriglyceridemia • normotriglyceridemia • VLDL • radioimmunoassay

The association of low density lipoproteins (LDL) with increased risk of coronary artery disease (1, 2) has stimulated extensive studies of the metabolism of LDL and provided a rationale for the measurement of LDL in the clinical laboratory. The radial immunodiffusion (RID) technique has been used for quantification of immunoglobulins and various plasma proteins (3–5). Sniderman, Teng, and Jerry (6) reported the use of RID to measure LDL B protein in whole plasma, and this technique has been used subsequently for the identification of subjects with elevations of LDL B protein but normal LDL cholesterol levels whose risk of premature coronary artery disease is increased (7). Sniderman et al. (6) provided evidence that under non-equilibrium conditions in 1.5% agarose the very low density lipoproteins (VLDL) of d < 1.006 g/ml do not contribute to the ring size of the precipitate, and therefore suggested that this RID method provides an approach to measure LDL B protein in plasma without ultracentrifugation. The purpose of the present study was to further evaluate the RID technique for the measurement of LDL B protein in plasma.

MATERIALS AND METHODS

Low density lipoprotein of d 1.019–1.063 g/ml was isolated from fresh plasma by sequential ultracentrifugation as described (8). Rabbit anti-human LDL specific to B protein was identical to that used previously (8). In order to separate plasma into the d > 1.019 and d < 1.019 g/ml plasma fractions, 5 ml of plasma was pipetted into a 6.5-ml tube (Beckman Instruments, Inc., Palo Alto, CA) and the nonprotein solvent density was brought to 1.019 g/ml by the addition of 0.092 g of KBr and centrifuged at

Abbreviations: RID, radial immunodiffusion; LDL, low density lipoproteins; VLDL, very low density lipoproteins.

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40,000 rpm for 18 hr. The top 12 mm of the tube, which was sliced off with a tube slicer, contained the d < 1.019 g/ml plasma fraction. The remaining fraction consisted of the d > 1.019 g/ml plasma fraction. Recovery of LDL B protein in the d > 1.019 plasma fraction was determined by the addition of 10 μl of ^{125}I-labeled LDL to the plasma before the centrifugation. B protein was measured in the plasma fractions by double antibody radioimmunoassay (8). All samples are analyzed in triplicate with the coefficient of variation of 5% to 6%. The absolute value of B protein is reported in terms of Lowry protein using bovine serum albumin (Armour Pharmaceutical Co., Phoenix, AZ) as a reference. Normal levels of plasma B protein obtained by this technique are age- and sex-dependent but average approximately 95 ± 21 mg/dl (mean ± SD) for healthy adults. For analysis of plasma B protein, fresh plasma was obtained from two normolipidemic subjects (subjects 2 and 3), one subject with hypercholesterolemia (subject 1), two with hypertriglyceridemia (subjects 5 and 7), and two subjects (numbers 4 and 6) with dysbetalipoproteinemia and deficiency of E_3 (9). Lp(a) lipoprotein was measured in plasma by RID (10). For comparison of equilibrium and non-equilibrium conditions for radial immunodiffusion, fresh plasma was obtained from six additional subjects: three normolipidemic subjects (subjects 11, 12, and 13), subject 8 with familial combined hyperlipidemia, subject 9 with hypercholesterolemia, and subject 10 with hypertriglyceridemia.

Radial immunodiffusion technique

Appropriate quantities of agarose type II: medium EEO, (A-6877, Sigma Pharmaceutical Company, Uppsala, Sweden) were suspended in 10–20 ml of 0.02 M Tris-EDTA buffer, pH 8.0, containing 0.02% (w/v) NaN_3 in a conical flask. The suspended agarose was melted by heating it in a bath of boiling water for approximately 10–15 min. To another conical flask was added rabbit anti-human LDL serum diluted 1:40 to 1:60 with 2% (w/v) bovine serum albumin in 0.02 M Tris-EDTA buffer, pH 8.0. The melted agarose and the antibody solution were brought to a temperature of 55–56°C in a constant temperature water bath and the agarose solution was added to an equal volume of the antibody solution to give a final gel concentration of 1.0 to 2.5%. The antisera-agarose solutions were mixed, and added to preheated (55°C) plastic dishes as described (10). Wells (36/dish) of 1.8 mm diameter were made at a distance of 12 mm apart in the agarose gel of 1.5 mm thickness. Samples and standards were introduced undiluted into the wells in duplicate as described (10), except that the wells were filled flush to the gel surface rather than to a premeasured volume. The within-plate coefficient of variation of ring diameters when the samples are filled flush to the gel surface is 4%, which is comparable to that obtained by adding a premeasured volumn of 4 μl (4%). Six standards were added to each dish with the following LDL B protein concentrations: 40, 80, 120, 160, 200, and 240 mg/dl, respectively. Precipitin ring diameters were measured using a Transidine calibrating viewer (Transidyne General Corp., Chaska, MN).

RESULTS

Five to six days were required for the precipitin rings to attain maximal ring diameters for LDL sample concentrations of 200 mg/dl (**Fig. 1**). At lower LDL concentration, less incubation time was required to obtain maximal ring diameters. At incubation times under 5 days, the square of the ring diameter was not linear with the LDL B protein concentration up to 200 mg/dl, regardless of gel concentration of 1.0% to 2.5%. Thus, approximately 5–6 days were required to obtain linearity of precipitin ring area and LDL concentration. However, the final ring diameter decreased slightly as the agarose gel concentration increased (Fig. 1). The ring diameters obtained with 2.5% agarose was significantly less than that obtained with 1% agarose (Fig. 1). Ring diameters intermediate to those obtained with 1% and 2.5% agarose were obtained with 1.5 and 2% agarose, respectively (not shown). Results consistent with those shown in Fig. 1 were obtained in four separate experiments.

The diffusion rate of the antigen decreases as the agarose gel concentration increases and, therefore, the time needed to reach equilibrium will be increased. Therefore, it would be expected that the ring diameter would vary inversely with gel concentration if measured at a single time before equilibrium is reached. However, it should be noted that the ring diameter varied inversely with gel concentration even at equilibrium conditions (**Fig. 2**). Also, the final ring diameter decreased as the gel concentration increased from 1.0% to 2.5%, regardless of incubation temperature, i.e., 20°C or 37°C (Fig. 2). Standard curves obtained at 20°C were not identical to those obtained at 37°C (Fig. 2). At 37°C the final ring diameter was consistently slightly larger than that obtained at 20°C incubation (four out of four experiments).

B protein was determined by radial immunodiffusion by the endpoint technique and by radioimmunoassay in plasma and in the d < and d > 1.019 g/ml plasma fractions from subjects having a wide range of triglyceride levels (**Table 1**). The B protein associated with the Lp(a) lipoprotein represented a small percentage (<0.3 to 4%) of the B protein in plasma. The value for plasma B protein obtained by RID generally decreased as the agarose gel concentration increased from 1% to 2.5%. The B protein values in the d > 1.019 g/ml fraction obtained by RIA were very similar to but tended to be slightly higher than the values obtained by RID for five out of seven samples. All subjects had B protein in the d < 1.019 g/ml

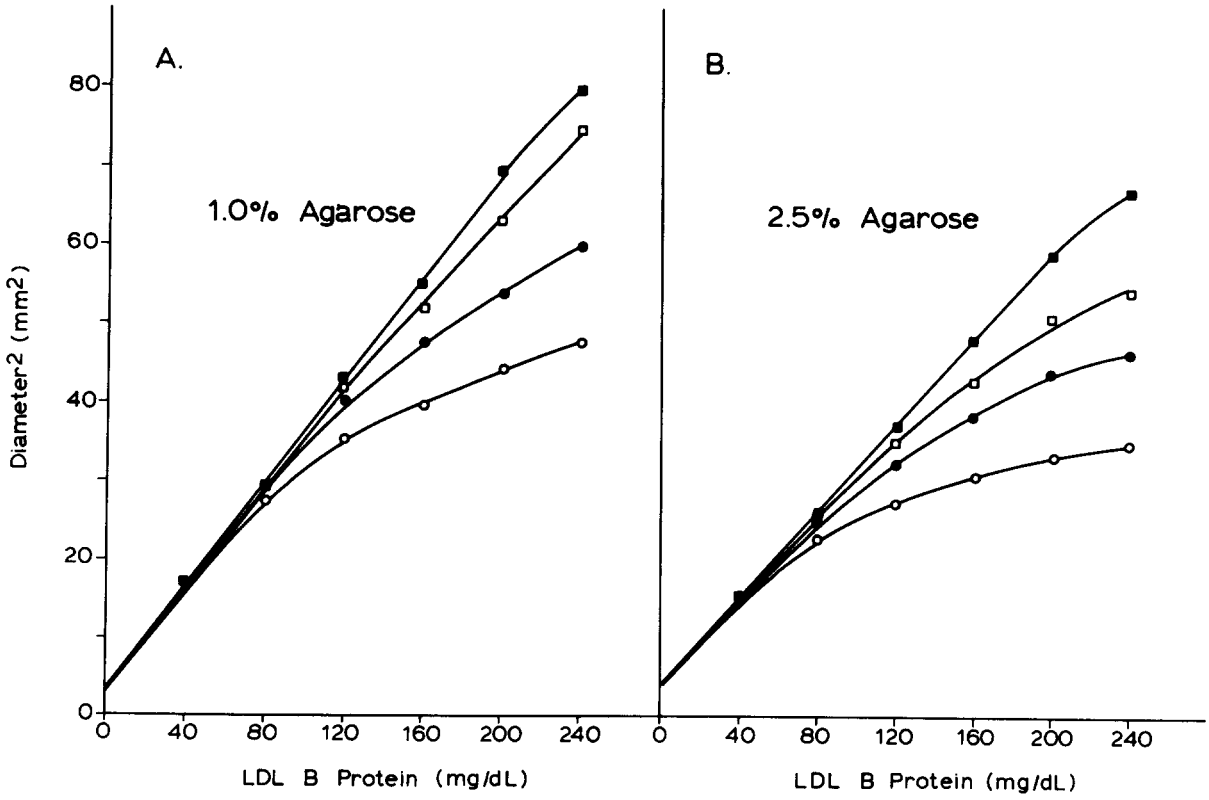

Fig. 1. Effect of incubation time on precipitin ring diameter of LDL standards. Incubation time 20 hr, ○; 43 hr, ●; 68 hr, □; 139 hr, ■. Panel A, performed in 1% agarose; panel B, 2.5% agarose.

fraction detectable by RID at all agarose concentrations examined (Table 1). However, among the hypertriglyceridemic subjects, B protein in the d < 1.019 g/ml fraction measured by RID tended to decrease as the agarose gel concentration increased. Among the normotriglyceridemic subjects, the B protein values obtained in whole plasma by RID were only slightly higher than the values obtained in the d > 1.019 g/ml fraction by RID (approximately 10% higher when determinations were performed in 1% agarose and approximately 4% higher if determinations were made in 2.5% agarose).

To quantitatively assess the "accuracy" of the radial immunodiffusion method for measurement of LDL B protein in plasma, the ratio of B protein in plasma obtained by RID to B protein in the d > 1.019 g/ml fraction obtained by RIA or RID was computed (**Table 2**). The LDL B protein is operationally defined as the B protein found in the d > 1.019 g/ml plasma fraction. For subjects 1-3, the B protein values obtained by RID of plasma using 2.0 or 2.5% agarose approximated the B protein values of the d > 1.019 g/ml fraction obtained by RIA (Table 2). Comparison of RID values of plasma versus the d > 1.019 g/ml plasma fraction from subjects 1-3 suggested that the RID method slightly overestimated (1% to 7%) the plasma LDL B protein level. For subject 4 with mild hypertriglyceridemia, the plasma B protein value by RID was approximately 10% higher than the B protein in the d > 1.019 g/ml fraction by RID. Furthermore, in hypertriglyceridemic subjects 5 and 7, the RID procedure significantly overestimated the LDL B protein levels even with 2.5% agarose. These differences could not be ascribed to losses of LDL B protein during centrifugation because the recovery of ^{125}I-labeled LDL during the centrifugation and sample handling was 98 ± 1%. Furthermore, all hypertriglyceridemic samples contained a significant amount of B protein in the d > 1.019 g/ml fraction by the RID procedure. Thus, the RID procedure done with 2.0 or 2.5% agarose appears to be a reasonable estimate of LDL B protein in normolipidemic subjects. Measurement of B protein in plasma of hypertriglyceridemic subjects by the RID procedure using endpoint or equilibrium conditions will overestimate the LDL B protein level because of the inclusion of B protein containing lipoproteins of d < 1.019 in the measurement.

B protein was then determined by RID under both equilibrium and non-equilibrium conditions in plasma and in the d < and > 1.019 g/ml plasma fractions in six subjects (**Table 3**). B protein values obtained by the endpoint technique in plasma or plasma fractions were not systematically different from B protein obtained by non-

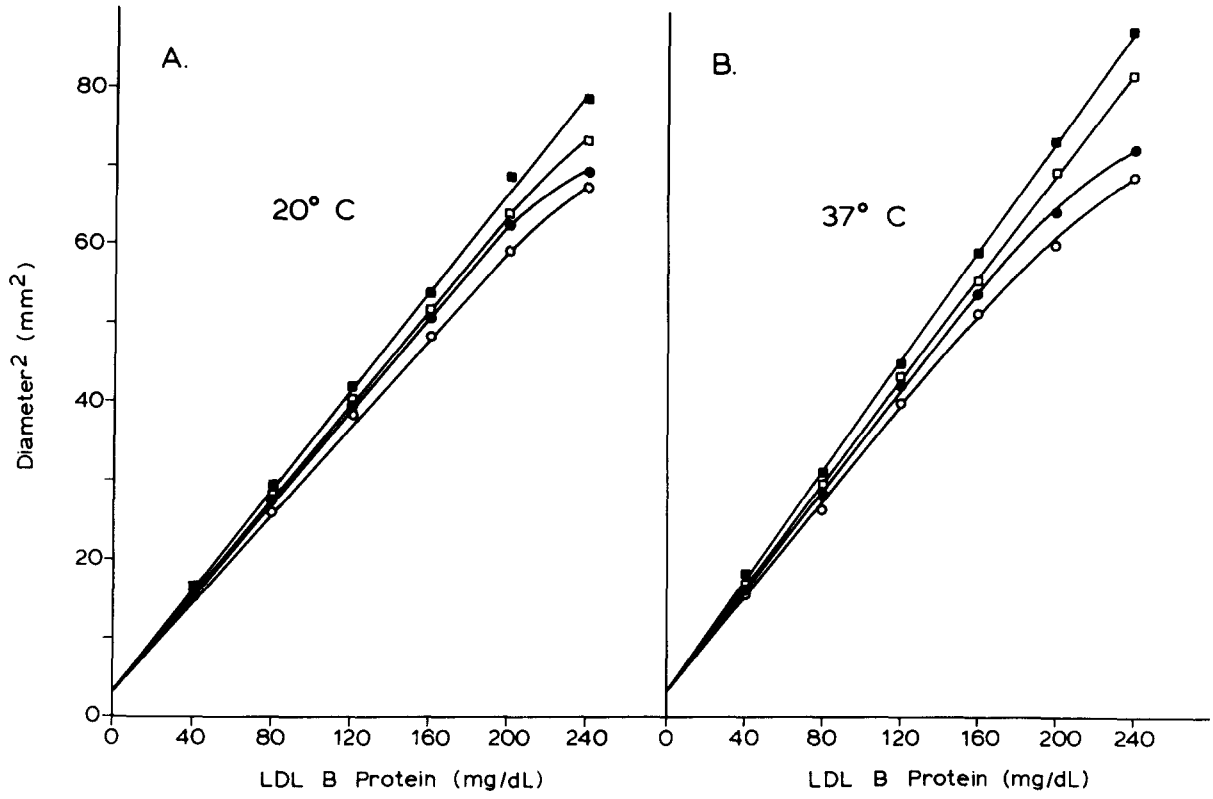

Fig. 2. Effect of incubation temperature and agarose gel concentration on precipitin ring diameter of LDL standards. 1% agarose; ■; 1.5% agarose, □; 2% agarose, ●; 2.5% agarose, ○. Panel A, incubation performed for 139 hr at 20°C; panel B, incubation performed for 139 hr at 37°C.

equilibrium conditions in both 1.5% or 2.5% agarose. All subjects had B protein in the d < 1.109 g/ml fraction detectable by RID under both equilibrium and non-equilibrium conditions. Among the four normolipidemic subjects (subjects 9, 11, 12, 13), the B protein values obtained in whole plasma by RID at non-equilibrium conditions were consistently higher (4% to 18%, mean 12% in 1.5% agarose; 6% to 25%, mean 12% in 2.5% agarose) than the values obtained in the d > 1.019 g/ml fraction by RID under identical conditions. Using equilibrium conditions, similar results were obtained with the plasma B protein values slightly higher (1% to 16%, mean 9% in 1.5% agarose; 2% to 7%, mean 4% in 2.5% agarose) than B protein values obtained in the d > 1.019 fraction.

For subjects 1 and 3 with mild hypertriglyceridemia the plasma B protein value by RID was significantly higher than the B protein in the d > 1.109 g/ml fraction by RID regardless of whether or not the analysis was performed at equilibrium or non-equilibrium conditions (Table 3). At non-equilibrium conditions, the plasma B protein values were 35% to 45% higher than B protein in the d > 1.019 g/ml fraction when using 1.5% agarose and 23% and 21% higher when using 2.5% agarose. Similar results were obtained using equilibrium conditions. Furthermore, the d < 1.019 g/ml fractions from the two hypertriglyceridemic subjects contained a significant amount of B protein when measured at either non-equilibrium or equilibrium conditions in 1.5% or 2.5% agarose. Therefore, measurement of B protein in plasma of hypertriglyceridemic subjects by RID at either non-equilibrium on equilibrium conditions will overestimate the LDL B protein level.

DISCUSSION

Mancini, Carbonara, and Heremans (4) investigated the applicability of the single radial immunodiffusion method for quantification of proteins in mixtures such as serum, and stressed the importance, if optimal quantitative results are desired, of taking readings of diameters of the precipitin rings at equilibrium, i.e., when there is no further growth of the precipitin rings. If the square of the diameter of the precipitin ring is plotted against antigen concentration on a linear scale, a linear relationship is obtained. In the present study, a linear relationship was obtained between the square of the diameter and LDL B protein concentration between 40 and 200 mg/dl, but it required at least 5 days of diffusion. At very high antigen

TABLE 1. B protein concentration in plasma and plasma fractions determined by RIA and RID[a]

Subject	Fraction	Chol	TG	B Protein in Lp(a) Lipoprotein[b]	B Protein RIA	B Protein RID (% Agarose) 1.0%	1.5%	2.0%	2.5%
1	Plasma	294	64	1	161	170	185	164	144
	d > 1.019				148	151	163	152	134
	d < 1.019				10	8	13	8	7
2	Plasma	222	108	4	108	107	110	102	93
	d > 1.019				102	100	103	90	89
	d < 1.019				12	8	8	7	8
3	Plasma	204	209	1	192	170	164	161	156
	d > 1.019				166	154	143	148	154
	d < 1.019				22	10	11	11	10
4	Plasma	338	376	6	204	181	174	178	174
	d > 1.019				175	158	159	160	160
	d < 1.019				45	27	29	26	26
5	Plasma	234	481	2	106	86	86	84	85
	d > 1.019				74	62	61	64	66
	d < 1.019				41	23	22	21	19
6	Plasma	660	1080	<0.3	169	114	113	107	106
	d > 1.019				62	52	52	60	52
	d < 1.019				109	56	54	42	34
7	Plasma	415	1152	0.3	157	128	127	124	116
	d > 1.019				91	87	87	88	88
	d < 1.019				72	33	29	23	20

[a]Results expressed in mg/dl.
[b]Lp(a) lipoprotein determined in plasma by RID (10). B protein in the Lp(a) lipoprotein was estimated by assuming that B protein in the Lp(a) lipoprotein represents 30 ± 4% of the total protein of the Lp(a) lipoprotein as determined by RIA of B protein in five Lp(a) lipoprotein preparations.

concentration, above 200 mg/dl, a slight deviation from linearity was observed, suggesting that at this high antigen concentration equilibrium had not been reached during 5 days of incubation. The present study has also confirmed the observation of others (4, 11), that lower antigen concentrations, that give rise to smaller precipitin rings, reach equilibrium earlier than do higher antigen concentrations. For example, at LDL B protein levels of 40 and 80 mg/dl, equilibrium was obtained by 20 hr of incubation.

Increased gel concentration resulted in a precipitin ring of smaller diameter and equilibrium appeared to be attained somewhat later at the higher gel concentrations (Fig. 2). Also, at higher gel concentrations, e.g., 2.0 and 2.5%, respectively, the edge of the precipitin ring appeared to be sharper than did precipitin rings obtained at lower gel concentrations. However, the higher gel concentrations, particularly 2.5%, were more difficult to prepare because the agarose solidified more rapidly than at the lower gel concentrations. Sniderman and colleagues

TABLE 2. Ratio of B protein in plasma obtained by RID to B protein in the d > 1.019 g/ml fraction obtained by RIA or RID

Subject	TG	RID (% Agarose) 1.0%		1.5%		2.0%		2.5%	
	mg/dl								
1	64	1.15[a]	1.12[b]	1.25[a]	1.13[b]	1.11[a]	1.08[b]	0.97[a]	1.07
2	108	1.05	1.07	1.08	1.07	1.00	1.13	0.91	1.04
3	209	1.02	1.10	0.99	1.15	0.98	1.09	0.94	1.01
4	376	1.03	1.14	0.99	1.09	1.02	1.11	0.99	1.09
5	481	1.16	1.39	1.16	1.41	1.14	1.31	1.15	1.29
6	1080	1.84	2.19	1.82	2.17	1.73	1.78	1.71	2.04
7	1152	1.41	1.47	1.40	1.46	1.36	1.41	1.27	1.32

[a]Ratio plasma B protein by RID/B protein in the d > 1.019 g/ml fraction by RIA.
[b]Ratio plasma B protein by RID/B protein in the d > 1.019 g/ml fraction by RID.

TABLE 3. B protein concentration in plasma and plasma fractions determined by RIA and RID. Comparison of equilibrium and non-equilibrium conditions for RID

				B Protein[a]				
				RIA	RID (% Agarose)			
					1.5%	2.5%	1.5%	2.5%
Subject	Fraction	Chol	TG		18 hr[b]		160 hr[b]	
8	Plasma	271	357	120	112	118	113	114
	d > 1.019			80	82	96	74	82
	d < 1.019			40	28	28	36	28
9	Plasma	242	35	115	109	114	102	105
	d > 1.019			110	105	108	101	99
	d < 1.019			4	4	3	3	5
10	Plasma	155	355	53	45	39	45	53
	d > 1.019			35	31	32	32	42
	d < 1.019			17	11	9	12	15
11	Plasma	186	96	100	95	100	90	90
	d > 1.019			91	84	91	83	87
	d < 1.019			6	6	5	5	3
12	Plasma	153	42	61	58	57	57	64
	d > 1.019			54	51	53	51	60
	d < 1.019			5	5	5	4	8
13	Plasma	196	68	89	97	99	87	84
	d > 1.019			83	82	79	75	82
	d < 1.019			5	5	5	6	8

[a]Results expressed in mg/dl.
[b]Incubation time at 20°C.

(6, 7) reported measurement of B protein in plasma by the RID technique after 18 hr of incubation in 1.5% agarose. Under these non-equilibrium incubation times, the diameter of the precipitin ring is linear if plotted against the log of the antigen concentration. These workers also reported that "the precipitin interface was clearest with this timing" (18 hr). However, it was our experience that the edge of the precipitin ring was not as well defined when measured at pre-equilibrium conditions or measured under conditions when the precipitin rings had not yet reached the endpoint. Measurement errors are likely to be greater if the precipitin rings are read at pre-equilibrium conditions.

Sniderman et al. (6) reported that RID assay of B protein using the non-equilibrium conditions of 18 hr "is insensitive to large increments of added VLDL protein." Havekes, Hemmink, and de Wit (12) reported that in the presence of plasma proteins VLDL is not completely excluded from the 1.5% agarose gel, and therefore recommended that plasma be frozen prior to RID analysis of LDL B protein to minimize the contribution of VLDL B protein to the estimation of LDL B protein. In the present study, we have evaluated the measurement of B protein in the d < 1.019 g/ml fraction rather than in the d < 1.006 g/ml fraction because of the possible contribution of intermediate density lipoproteins to the measurement of LDL B protein. In the present study, the d < 1.019 g/ml lipoproteins were not excluded from the gel even when the agarose gel concentration was increased to 2.5%. Furthermore, the d < 1.019 g/ml lipoproteins contributed to the measurement of B protein in plasma when measured at 18 hr at non-equilibrium conditions or at equilibrium conditions of 5 to 6 days.

We demonstrated that B protein measured in whole plasma was always greater than the B protein measured in the d > 1.019 g/ml fraction and that this difference closely approximated the B protein measured in the d < 1.019 g/ml fraction. It is possible, however, that under different conditions, e.g., use of a different kind of agarose or use of an antisera with a different specificity for the epitopes of the B protein, the contribution of the d < 1.019 g/ml lipoproteins to the precipitin ring size could be negligible even in hypertriglyceridemic subjects. Precipitin rings obtained with the d < 1.019 g/ml fraction are not entirely explained by the presence of intermediate density lipoproteins d 1.006–1.019 g/ml because precipitin rings were obtained at all gel concentrations if the lipoproteins of d < 1.006 g/ml were added to gel (A. J. Lotalo-Bosa, J. L. Adolphson, and J. J. Albers, unpublished observations).

Among all seven normotriglyceridemic subjects examined, the amount of measurable B protein by RID in the d < 1.019 g/ml fraction was quite small in relationship to the plasma LDL B protein level. For example, at equilibrium conditions in 1.5% agarose, the B protein in the d < 1.019 g/ml fraction represented 6 ± 2% of the

protein measured in plasma. Therefore, the RID procedure performed in 1.5 to 2.5% agarose may be appropriate for estimation of LDL B protein in plasma in normotriglyceridemic subjects. On the other hand, for the four subjects with mild hypertriglyceridemia (triglyceride between 300 and 500 mg/dl), the B protein measured in the d < 1.019 g/ml fraction at equilibrium conditions was a significant portion of the B protein measured in plasma (25 ± 6% in 1.5% agarose and 23 ± 6% in 2.5% agarose). For the two subjects with triglyceride over 1000 mg/dl, the B protein measured in the d < 1.019 g/ml fraction also represented a significant portion of the B protein measured in plasma. Also, at non-equilibrium conditions, the plasma B protein values in the hypertriglyceridemic samples were significantly higher than B protein in the d > 1.019 g/ml fraction. Therefore, application of the RID procedure to whole plasma for estimation of LDL B protein in hypertriglyceridemic subjects must be interpreted with caution considering that a significant proportion of the B protein measured by this technique may reflect the presence of lipoproteins of d < 1.019 g/ml or non-LDL B protein.

The Lp(a) lipoprotein fraction (primarily of d 1.050–1.090 g/ml) also contains B protein (13, 14). However, the Lp(a) lipoprotein appears to be under separate metabolic and genetic control (14, 15). Therefore, any measurement of B protein in plasma would also contain a contribution of B protein from the Lp(a) lipoprotein. The B protein of Lp(a), however, usually represents a small proportion of the total plasma B protein. Thus, it would be relatively rare that "LDL B protein levels" in plasma as measured by the RID procedure would appear high as the result of an elevation of the Lp(a) lipoprotein. However, the contribution of the Lp(a) lipoprotein to the measurement of the plasma B protein should not be overlooked.

Subjects with hyperapobetalipoproteinemia (15) and those with familial combined hyperlipidemia (16) have LDL particles that are smaller than average size LDL and relatively enriched in apoprotein B. These two subject groups represent similar but non-identical populations (17). In RID the smaller particles will diffuse more rapidly into the gel than larger particles. Thus, the RID technique may tend to overestimate the B protein in "small" LDL particles, particularly if measured at non-equilibrium conditions, when the smaller apoprotein B-containing particles form the leading edge of the precipitate.

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