Comparison of commercial kits for apoprotein A-I and apoprotein B with standardized apoprotein A-I and B radioimmunoassays performed at the Northwest Lipid Research Center

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Abstract There has recently been a proliferation of commercial kits available for apoproteins A-I and B. Since reference procedures for apoproteins have not yet been established we have elected to compare apoprotein kit methods with highly standardized apoprotein B and A-I radioimmunoassays developed at the Northwest Lipid Research Center. Commercial radial immunodiffusion kits for apoproteins A-I and B were obtained from three separate companies, Calbiochem, Daiichi Pure Chemicals, and Tago, and a commercial radioimmunoassay kit for apoprotein A-I was obtained from Ventrex Laboratories. Considerable differences were observed between the commercial kit methods and the Northwest Lipid Research radioimmunoassay methods. Some of the differences between methods were related to the assigned value of the reference materials. Other differences between methods were clearly method-dependent. — **Albers, J. J., and J. L. Adolphson.** Comparison of commercial kits for apoprotein A-I and apoprotein B with standardized apoprotein A-I and B radioimmunoassays performed at the Northwest Lipid Research Center. *J. Lipid Res.* 1988. **29:** 102–108.

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Apoprotein measurements are of growing clinical interest as predictors of the risk of developing coronary heart disease and peripheral vascular disease, for the monitoring of the progress of therapeutic intervention, and for the differential diagnosis of the dyslipoproteinemic states. Because of this increased demand of apoprotein measurements in the clinical laboratory, there has recently been a proliferation of commercial kits available for apoprotein measurements, particularly for apoprotein A-I (apoA-I) and apoprotein B (apoB). Normal values for each of the apoproteins vary from laboratory to laboratory. The lack of comparability of results indicates a need for standardization of reagents and methods (1). If the commercial kit apoprotein methods are to be used for epidemiological studies or as indicators of coronary disease risk, it is important that the results of these methods be compared to apoprotein reference procedures. The Apoprotein and Antibody Standardization Program (AASP), sponsored by the National Heart, Lung, and Blood Institute, is currently developing reference methods for apoproteins A-I and B. Furthermore, the central laboratory of the AASP has available common reference materials for apoprotein measurements (contact Janet Adolphson, Harborview Medical Center, 326 Ninth Avenue, Seattle, WA 98104). Since reference procedures for apoproteins A-I and B have not as yet been established, we have elected to compare the commercial apoprotein kit methods with highly standardized apoB and apoA-I radioimmunoassays (RIA) developed and currently used at the Northwest Lipid Research Center (NWLRC), Department of Medicine, University of Washington School of Medicine. The apoB RIA used was developed in this laboratory in 1973 (2). New quality control pools have been substituted for older pools by running both simultaneously until a value for a new pool was established. Analysis of apoB in these quality control pools indicate that the values on these apoB pools have been consistent for the past 12 years.

An apoA-I radial immunodiffusion (RID) developed in our laboratory (3) has similarly given consistent results for the past 12 years. The RID method, however, is not well suited for large numbers of samples as it uses large amounts of antibody and it cannot be readily automated. Using the RID method as a reference, we have developed an automated apoA-I RIA to accommodate the increased demand for A-I analyses. This RIA compares well with

Abbreviations: AASP, Apoprotein and Antibody Standardization Program; RIA, radioimmunoassay; NWLRC, Northwest Lipid Research Center; RID, radial immunodiffusion; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

the established RID method and is shown to be highly reproducible.

METHODS

Samples

Plasma was obtained in approximately equal numbers from subjects in three different studies: a screening of healthy young adults, a study of Japanese-American diabetics, and from subjects undergoing coronary catheterization. The samples were comprised of 129 individuals with normal lipid concentrations, 12 hypercholesterolemic subjects, and 7 hypertriglyceridemic subjects.

ApoA-I isolation

HDL was isolated as previously described (4). A fraction enriched in A-I was obtained by guanidine-HCl treatment of HDL and centrifugation as described (4). This A-I fraction was applied to a 1.6 × 1.00 cm column of Sephacryl S-200 equilibrated with 0.03 M Tris, 5 M guanidine-HCl HDL, pH 7.4. The purified A-I exhibited a single band after SDS polyacrylamide gel electrophoresis, and contained less than 1% apoprotein A-II or albumin as determined by specific immunoassay analysis. The mass of the A-I was determined by the method of Lowry et al. (5) which previously has been shown to agree with the A-I mass determined by amino acid analysis (3). The purified A-I was dialyzed exhaustively in 5 mM NH_4HCO_3, pH 8.0, concentrated, and stored in aliquots at −70°C. Antisera to apoA-I were obtained from rabbits as previously described (3). Anti-A-I antibodies were affinity-purified by the established HDL/Sepharose procedures (6). Nonspecifically bound protein and very low affinity antibodies were removed by a wash with 0.1 M NaAc, 0.5 M NaCl, 1 mM EDTA, pH 4.5. Low affinity anti-A-I was removed with 0.1 M NaAc, 0.5 M NaCl, 1 mM EDTA, pH 3.5. The antibodies used in the RIA were eluted with 0.5 M HAc, 0.5 M NaCl, 1 mM EDTA, pH 3.0. The remainder of the anti-A-I was eluted with 0.3 M NaSCN in 0.2 M $NaPO_4$, pH 7. The last two elutions were sometimes replaced with a single 0.5 M HAc, 0.5 M NaCl, 1 mM EDTA, pH 2.6, elution.

Labeling

ApoA-I was labeled by the iodine monochloride method of McFarlane (7) as modified by Bilheimer, Eisenberg and Levy (8). After labeling, the apoA-I was dialyzed against 0.01 M Tris, 0.15 M NaCl, 1 mM EDTA, pH 7.4, hereafter called TES buffer, and an equal volume of 2% bovine serum albumin was added.

Radioimmunoassay

RIA buffer was made by adding 1% BSA, 0.05% NaN_3 to TES buffer. Samples and standards were diluted in RIA buffer + 0.04% Tween 20. Whole plasmas were diluted 1:400. Unlabeled apoA-I and ^{125}I-labeled apoA-I were mixed and diluted to 2 μg/ml in RIA buffer (0.5 μg/ml unlabeled + 1.5 μg/ml ^{125}I-labeled apoA-I). Each assay tube contained 100 μl of sample, standard, or buffer (for controls), 100 μl of ^{125}I-labeled apoA-I mixture (2 μg/ml), and 100 μl of affinity-purified rabbit anti-A-I diluted in RIA buffer, to give 50% of maximum binding. ApoA-I standards ranged from 1.5 to 5.0 μg/ml. Each assay contained controls with nonimmune rabbit IgG for nonspecific binding, and three quality control plasmas, each at a different apoA-I level. All samples were assayed in triplicate. Plasma samples were diluted using a Micromedic Accuflex diluting station (Horsham, PA) operated by a Zenith ZVM-158 or ZFL-100 computer (St. Joseph, MI). After an overnight incubation at 4°C, 100 μl of normal rabbit serum (diluted in RIA buffer) and 300 μl of sheep anti-rabbit IgG serum were added using the Accuflex diluting station. The assay mixture was again incubated overnight at 4°C. One ml of RIA buffer was added and the tubes were centrifuged in a Beckman J6-B centrifuge with a JR-32 Rack Rotor (Beckman Instruments, Palo Alto, CA). After the supernatants were decanted, 1 ml of RIA buffer was added and the tubes were recentrifuged. The supernatants were again decanted and the precipitates were counted in a Micromedic 4/600 Auto-gamma counter. The standard curve was linearized by log/logit transformation of B/B_0.

Freshly thawed samples (n = 148) were assayed by the NWLRC apoA-I RIA and RID procedures. The values obtained by the RID method were slightly higher than that obtained by the RIA method (mean 140.7 mg/dl vs. 136.9 mg/dl). Analysis by least-squares regression gave a correlation coefficient of 0.931, a slope of 0.877, and an intercept of 20.6 mg/dl. The Sy was 9.8 mg/dl (see **Fig. 1**).

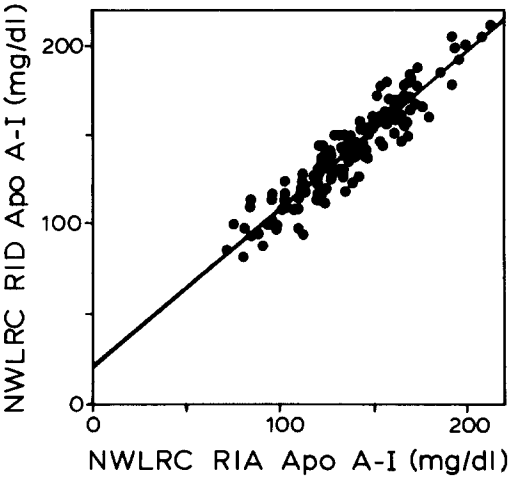

Fig. 1. Northwest LRC apoprotein A-I methods: comparison of radioimmunoassay with radial immunodiffusion.

Sy (standard error of estimate of y) is the standard deviation of the differences of the actual Y value from the Y value calculated from the least-squares equation ($Y = mx + b$) (9).

Four quality control pools with mean apoA-I levels ranging from 120 to 154 mg/dl were assayed in 61 apoA-I radioimmunoassays over a period of 15 months and showed coefficients of variation ranging between 4.6 and 6.7%.

Commercial RID kits for apoA-I and apoB determinations were obtained from Calbiochem (La Jolla, CA), Daiichi Pure Chemicals (Tokyo, Japan), and Tago (Burlingame, CA). Two sets of standards were obtained for each of the Calbiochem and Tago RID kits. These are designated as Std 1 and Std 2. A commercial RIA kit for apoA-I determinations was obtained from Ventrex Laboratories (Portland, ME).

Freshly thawed plasma samples and four serum pools used by the Northwest Lipid Research Center as quality controls were assayed according to the manufacturers' protocol. Apoprotein values were calculated using the manufacturers' standard and its assigned value. The results obtained from these samples were compared to those obtained by the apoA-I and apoB radioimmunoassays (2) developed at the NWLRC. LDL (d 1.030–1.050 g/ml) used as the apoB standard in the apoB RIA was prepared by sequential ultracentrifugation, and the protein concentration was determined as previously described (2). Non-apoB protein in the LDL standard was generally less than 3%. The LDL was then stored in the dark at 4°C under nitrogen in the presence of 1% BSA. LDL isolated and stored in this manner is stable for up to 12 weeks with no change in the immunoreactivity as determined by RIA analysis. However, reference material is generally prepared on a monthly basis.

The commercial standards associated with each kit were analyzed in the NWLRC reference assay, and also in the other commercial assays, except when precluded by the sensitivity of the assay. The results obtained by these assays were compared to those obtained in the respective NWLRC RIA assays by least-squares analysis to obtain estimates of systematic errors including proportional error or slope (m) and constant error or intercept (b) and random errors, standard error (Sy), the standard deviation (SD), and the correlation coefficient (r).

RESULTS

Evaluation of apoprotein A-I methods

One source of systematic error between methods is the difference between the reference materials. Thus we first compared the apoA-I methods by analyzing the kit reference materials in the NWLRC apoA-I RIA. We also compared means of sample values from both assays (**Table 1**) and analyzed the NWLRC quality control pools in the kit methods (**Table 2**). Least-squares regression of sample values was also performed (**Table 3**).

Sample values and NWLRC quality control pool values obtained by the Calbiochem reference standard gave values slightly higher or equal to its assigned value when analyzed by the NWLRC RIA. This relationship was confirmed when the Calbiochem standard was assayed by other RID procedures. Thus, the difference between the means of sample values cannot be attributed to the value assignment of the reference pool. Least-squares regression analysis shows a slope of only 0.617 (r = 0.685) and intercept of 63.9 mg/dl indicating a strong proportional bias (Table 3 and **Fig. 2A**). However, the bias is greater in samples with low levels of A-I.

TABLE 1. Apoprotein A-I bias of commercial kits

	ApoA-I Standards				Sample Means			
		NWLRC RIA Value						
Company	Assigned Value	Std 1	Std 2	% Bias	# Samples Run (N)	Kit Method	NWLRC RIA	% Bias
	mg/dl							
Calbiochem	62	71	76	−14	49	152	142	+7
Daiichi	22.8	24.1		−5	60	137	146	−7
Tago	8	4	4	+100	49	141	142	0
Tago	24	13	6	+85				
Tago	40	25	26	+57				
Ventrex	1.6	2.2		−27	67	130	140	−7
Ventrex	2.8	3.7		−24				
Ventrex	6.1	8.2		−26				
Ventrex	10.2	13.4		−24				

Percent bias = 100 × (kit value − NWLRC value)/NWLRC value.

TABLE 2. Bias of commercial kits on Northwest LRC quality control pools

NWLRC RIA	Assigned Value	% Bias of Commercial ApoA-I Methods			
		Calbiochem	Daiichi	Tago	Ventrex
	mg/dl				
QC 1	130	−9	−15	−1	−15
QC 2	119	+3	−12	+8	−6
QC 3	134	+6	−11	+2	−9
QC 4	158	+6	−10	+17	−5
Avg		+1	−12	+7	−9
		% Bias of Commercial ApoB Methods			
		Calbiochem	Daiichi	Tago	
QC 1	100	+1	−13	−34	
QC 2	97	+7	−6	−37	
QC 3	87	−3	−11	−35	
QC 4	150	+7	−18	−28	
Avg		+3	−12	−33	

Percent bias = 100 × (kit value − NWLRC value)/NWLRC value. Each QC pool was analyzed twice in triplicate.

The Daiichi apoA-I reference standard showed a slightly negative bias when assayed with the NWLRC apoA-I RIA. Comparison of sample value means and NWLRC quality control values obtained in both assays also showed a slightly negative bias, suggesting that the differences between methods is partly due to the assigned value of the standard (Tables 1 and 2). The proportional bias exhibited in regression analysis is somewhat greater in samples with higher levels of apoA-I (see Fig. 2B).

Tago apoA-I kit values for samples and NWLRC quality control pools showed essentially no bias when compared to NWLRC values (Tables 1 and 2). However, the Tago standards gave values almost 50% lower than their respective assigned values when assayed by the NWLRC RIA. Among the apoA-I kits examined, the Tago RID showed the highest correlation (r = 0.858) with the NWLRC RIA (Table 3). Least-squares regression showed a slope of 1.07, and an intercept of −9.7. The error of y was greatest in the samples with the highest level of apoA-I (Fig. 2C). When the Tago standards were analyzed in the NWLRC RIA, a large difference between the two mid-level Tago standards was obtained. This difference was also seen when the mid-level standards were analyzed in the other RID procedures and in the Ventrex RIA. Since all calculations were done by weighted linear regression, the standard curve generated by the second set of Tago standards was very similar to that calculated from the first set of Tago standards.

Sample means and quality control samples showed similar slightly negative biases on the Ventrex apoA-I RIA kit. The Ventrex apoA-I standard showed considerably greater negative biases when assayed by the RIA (Tables 1 and 2). Two controls provided with the Ventrex RIA kit ran similarly to their assigned values when analyzed by the NWLRC apoA-I RID method but gave considerably higher (17%) results when analyzed by the NWLRC apoA-I RIA (data not shown). Least-squares regression (Table 3) showed a relatively low correlation coefficient (0.663) and a high error of y estimate (18.5%). When Ventrex RIA values were plotted against NWLRC RIA values, several distant values were noted (Fig. 2D).

Evaluation of apoprotein B methods

We also compared the assigned values of the standards from each commercial apoprotein B RID kit to the values obtained by our RIA reference method (**Table 4**) and evaluated our quality control pools by the various kit methods (Table 2). There was a positive bias on the Calbiochem RID reference standard and on our quality con-

TABLE 3. Comparison of Northwest LRC methods and commercial kits by least squares regression analysis

Company	Graph Data				
	N	R	M	Intercept	Error of Y (Sy)
					mg/dl
ApoB methods					
Calbiochem	66	0.901	0.987	13.5	16.7
Daiichi	63	0.916	0.757	21.3	12.5
Tago	66	0.849	0.722	−2.5	15.9
ApoA-I methods					
Calbiochem	49	0.685	0.617	63.9	12
Daiichi	60	0.794	0.804	18.9	10.3
Tago	49	0.858	1.07	−9.7	12.3
Ventrex	57	0.663	0.881	6.1	18.5

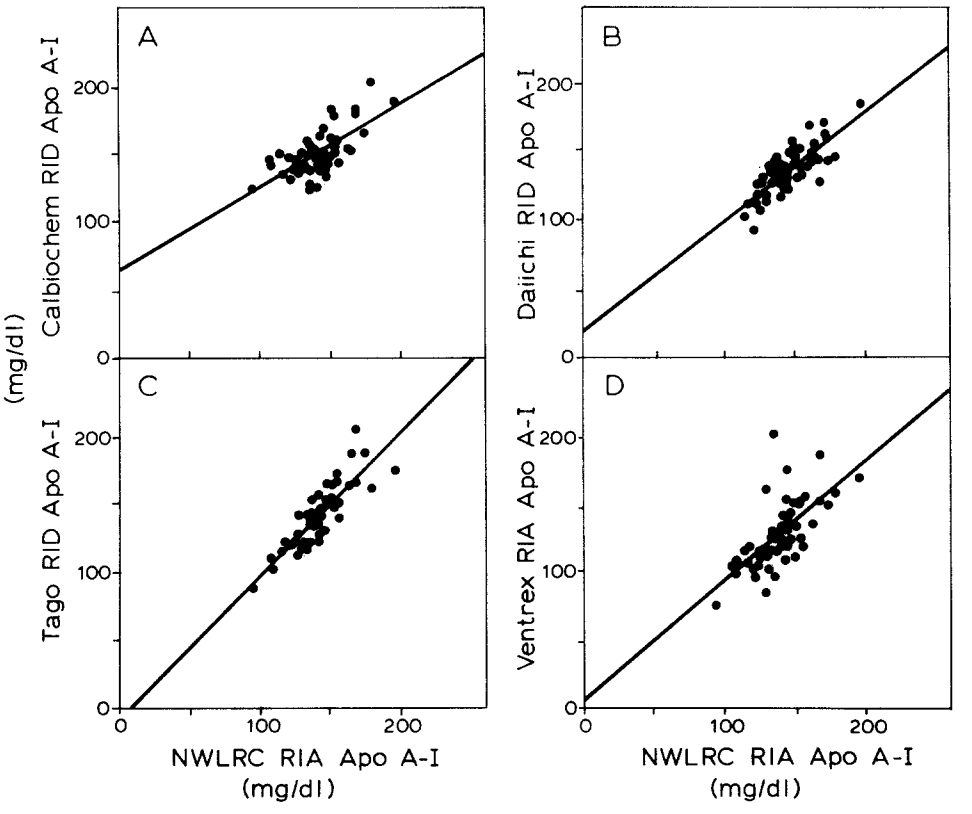

Fig. 2. Apoprotein A-I methods: comparison of Northwest LRC radioimmunoassay with commercial kits.

trol pools, suggesting that the positive bias obtained by the Calbiochem method is largely due to differences in value assignment of the reference pool. Comparison of the two methods by least-squares analysis (Table 3 and **Fig. 3A**) also indicates a positive bias in the Calbiochem procedure with an intercept of 13.5 mg/dl closely approximating the bias in the assigned value of the standard. With the Calbiochem method the proportional error appeared to be small since the slope between the methods was close to 1.0 (m = 0.987), however, the standard error of y was rather large (16.7 mg/dl).

Comparison of the Daiichi RID method with the RIA apoB method indicated that the negative bias on the assigned value of the standard and on the sample means was similar to the negative bias obtained on the quality control pools (Table 2). However, the apparent negative bias in the Daiichi procedure was not evident over the entire concentration range but primarily reflected a negative bias in the samples with higher apoprotein B values with a slope considerably less than 1.0 (0.757) and a positive intercept of 21 mg/dl (see Table 3 and Fig. 3B).

Comparison of the Tago RID method with the apoB

TABLE 4. Apoprotein B bias of commercial kits

	ApoB Standards				Sample Means			
		NWLRC RIA Value						
Company	Assigned Value	Std 1	Std 2	% Bias	N	Kit Method	NWLRC RIA	% Bias
	mg/dl							
Calbiochem	76	67	69	+ 12	66	142	129	+ 10
Daiichi	211	235		− 10	63	121	132	− 9
Tago	21	14	17	+ 35	66	91	129	− 30
Tago	42	30	35	+ 23				
Tago	84	55	61	+ 24				

Percent bias = 100 × (kit value − NWLRC value)/NWLRC value.

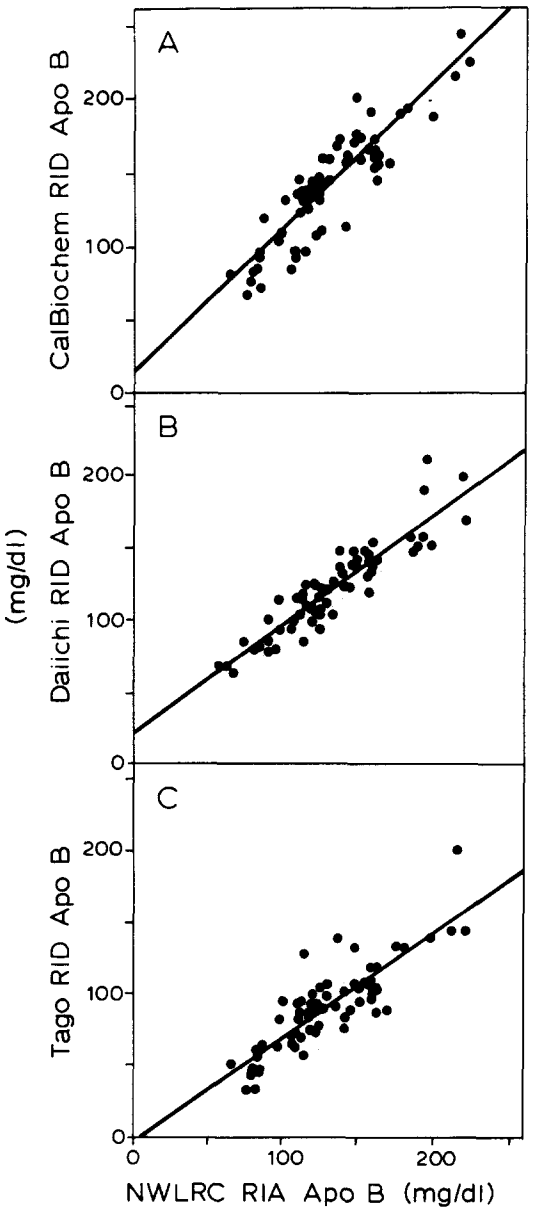

Fig. 3. Apoprotein B methods: comparison of Northwest LRC radioimmunoassay with commercial kits.

RIA indicated that there was a significant negative bias obtained with both patient samples and with NWLRC quality control pools (Tables 3 and 4) despite a positive bias in the assigned value of the Tago apoB standards (Table 1). When assayed by the Calbiochem and Daiichi RID methods, the Tago standards gave significantly higher values than those obtained by the NWLRC procedure. The reason for this apparent discrepancy is not clear but this finding suggests that the Tago RID standards are inappropriate for the RIA perhaps due to an interfering substance added to the Tago RID standard. The procedure for assaying purified LDL in the Tago apoB kit requires the addition of Tago diluent to the LDL suggesting that the Tago standard may contain a reagent that affects the apoB reactivity. The sample values obtained by the Tago RID procedure are proportional to the sample concentration, with the greatest negative bias obtained with the samples with the highest concentration, as the intercept is near zero but the slope is considerably less than 1 (Table 3) and the Sy was quite large, 15.9 mg/dl.

DISCUSSION

Most of the apoA-I RID procedures including the NWLRC RID gave slopes less than 1.0 suggesting that this difference may be method-related. Samples of low or average apoA-I levels give comparable values by both methods. Samples with high apoA-I levels show relatively lower values when analyzed by an RID method than when analyzed by the RIA method. This tendency is very marked in the Daiichi RID method in which elimination of only a few high level samples from the regression analysis results in a slope of 0.95 and intercept of only −0.6 mg/dl.

The only apoA-I RID method examined that does not exhibit a greater proportional bias at higher apoA-I levels is the Tago RID procedure. However, the Tago method also differed markedly from the other apoA-I RID methods in the performance of its standards on the NWLRC RIA. Although the sample means for both assays were similar, NWLRC apoA-I RIA values for Tago standards were only 50–63% of the assigned Tago value. Values for the Tago standards obtained by the Daiichi RID and Ventrex RIA methods were similar to the Tago assigned value while NWLRC RID values for Tago standards were 38–50% of the assigned value, indicating that this difference is not due to methodological differences between the RID and RIA but perhaps due to variations in the RID methods. Because not all of the apoA-I antigenic determinants are exposed in plasma, many of the immunoassays for apoA-I employ the addition of a denaturing agent (10). The NWLRC RID uses both TMU and urea as dissociating reagents, whereas the NWLRC RIA procedure uses Tween 20 and HDL affinity-purified antibodies. While none of the commercial apoA-I RID methods evaluated here employed the addition of denaturants directly to the plasma, reagents such as sodium decyl (or dodecyl) sulfate could have been incorporated into the agarose. The Tago apoA-I RID brochure states that when purified apoA-I is to be assayed, it must first be diluted to a final concentration of 10 mM sodium decyl sulfate. It appears that purified apoA-I, the Tago standards, and samples all react somewhat differently in the Tago method.

Several commercial apoB RID methods also showed a greater proportional bias in samples with high apoB levels. A similar bias was reported when comparing

lipemic samples in the NWLRC apoB RID and the NWLRC apoB RIA (1). RID procedures are unable to measure apoB in large particles such as VLDL because the pore size of the agarose is too small for effective migration (11). Hyperlipidemic subjects have a greater percentage of their total apoB in larger particles, thus a greater percentage of the total B is underestimated.

In conclusion, considerable differences were observed between the commercial kit methods and the Northwest Lipid Research radioimmunoassay methods. Some of the differences between methods were related to differences in the assigned value of the reference materials. Other differences between methods were clearly method-dependent.

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