Measurement of human high density lipoprotein apolipoprotein A-I in serum by radioimmunoassay

Joan B. Karlin, Dawn J. Juhn, Jerome I. Starr, Angelo M. Scanu, and Arthur H. Rubenstein[1]

Departments of Medicine and Biochemistry,
The University of Chicago Pritzker School of Medicine, Chicago, Illinois 60637

Abstract A sensitive and specific double antibody radioimmunoassay for the major apolipoprotein (apo A-I) of human serum high density lipoprotein (HDL) was developed. Initial studies indicated that direct measurement of apo A-I concentration in whole untreated sera or isolated high density lipoprotein fractions yielded variable results, which were lower than those obtained in the corresponding samples which had been subjected to delipidation. Subsequently, it was observed that heating diluted sera or HDL for 3 hr at 52°C prior to assay resulted in maximal increases in apo A-I immunoreactivity to levels comparable to those found in the delipidated specimens. This simple procedure permitted multiple sera to be assayed efficiently with full recovery of apo A-I.

Serum apo A-I in healthy normolipemic males was 130 ± 3 mg/dl (range 95–165), while the values in females were significantly higher 154 ± 6 mg/dl (range 107–199) ($P < 0.005$). The apo A-I levels correlated with the total serum cholesterol (males $r = 0.46$, $P < 0.05$; females $r = 0.58$, $P < 0.001$).

Preliminary results with sera from patients with abetalipoproteinemia, hypercholesterolemia and Tangier disease indicated that alterations in the low density lipoprotein concentration and changes in the physical chemical properties of the high density lipoproteins did not affect the optimal conditions for measuring serum apo A-I. The apo A-I concentrations were abnormally low in each of the above disorders.

Supplementary key words High density lipoprotein radioimmunoassay • apolipoprotein A-I • abetalipoproteinemia • hypercholesterolemia • Tangier disease

Serum lipoproteins are commonly separated in the ultracentrifuge into several classes which differ in their hydrated densities, hydrodynamic properties, and chemical composition (3). A major group is represented by the high density lipoproteins (HDL) which contain a large portion of the plasma cholesterol, and approximately 50% protein by weight (4). It is now established that the protein moiety of HDL (apo HDL) is composed of several polypeptides which are distinct in their physical, chemical, and immunological properties (1, 4). The two major apolipoproteins, apo A-I and apo A-II, represent by weight 70 and 20% respectively of apo HDL (5). Apo C, the other class of apoproteins, represents 3–5% of apo HDL and is composed of at least 3 major peptides, apo C-I, apo C-II, and apo C-III (4).

Rapid and precise quantification of these apoproteins in biological fluids would be valuable in studies of their structural and physiological functions. To this end we have raised antisera against human apo A-I and developed a specific and sensitive double antibody radioimmunoassay (RIA) for this polypeptide. A preliminary account of these studies has appeared (6). While this work was in progress, Schonfeld and Pfleger (7) described an assay for the determination of apo A-I, based on the same principles. However, in order to measure the total concentration of this polypeptide in plasma, a delipidation step was required. Because this procedure is time consuming, we searched for alternative ways which would permit the direct estimation of apo A-I in unextracted serum. In this report we describe the development and validity of an efficient immunoassay method which can readily be adapted to the measurement of apo A-I in a large number of samples.

MATERIALS AND METHODS

Isolation of apo-I

HDL (d 1.063–1.21 g/ml) was separated and purified by ultracentrifugation of fresh sera taken from fasting healthy human male donors (5). Delipidation of HDL was carried out at −10°C with ethanol–ether 3.2 (v:v) (8) and the apo HDL was fractionated into its major polypeptide components by Sephadex G-200 column chromatography in 8M urea (5). Peak III, containing apo A-I, was further purified by DEAE

Abbreviations: For details on nomenclature see reference (1, 2). ABL, abetalipoproteinemia; HDL, high density lipoprotein of d 1.063–1.25 g/ml; apo A-I, the major apolipoprotein A also referred to as R-Gln-I; apo-A-II, the minor apolipoprotein A also referred to as R-Gln-II; apo C-I, also referred to as R-Ser; apo C-II, also referred to as R-Glu; apo C-III, also referred to as R-Ala; LDL, low density lipoprotein of d 1.019–1.063 g/ml; VLDL, very low density lipoprotein of d < 1.006 g/ml.

[1] Reprint requests should be addressed to Dr. Arthur H. Rubenstein, Department of Medicine, The University of Chicago, Box 435, 950 East 59th Street, Chicago, Illinois 60637.

cellulose ion exchange chromatography in 6M urea (9). Fraction III-a contained pure apo A-I (5, 9), which did not react with antisera which we have raised against apo A-II, apo C-peptides and LDL in rabbits.

Preparation of antisera to apo A-I

New Zealand white rabbits were immunized against pure apoA-I (9). The immunoglobulins in the antisera were precipitated by 2M ammonium sulfate and purified further by DEAE cellulose ion exchange column chromatography.

Assay buffer

A borate buffer (0.13M) containing 0.5% bovine serum albumin (fraction V) and 0.02% sodium azide was used. It was adjusted to pH 8.0 with 12N hydrochloric acid.

Iodination of apo A-I

Apo A-I was iodinated by a modification of the chloramine-T method of Freychet and Roth (10) and was similar to that described by Schonfeld and Pfleger (7). The labeled peptide (sp act 30 mCi/mg, corresponding to approximately 0.4 atoms ^{125}I per molecule apo A-I) was purified by gel filtration on 0.8 × 15 cm Superfine Sephadex G-75 columns equilibrated in borate–albumin buffer (fraction size 0.3 ml). The ^{125}I-labeled apo A-I eluted shortly after the void volume and was completely separated from the free iodide recovered in the salt peak. The peak tubes were the most immunologically active and could be used in the assay for at least four weeks. More than 80% of the iodinated apo A-I was bound by an excess of anti-apo A-I antibody. In addition, it comigrated with unlabeled apo A-I on SDS acrylamide gel electrophoresis, and eluted coincidentally with unlabeled apo A-I after chromatography on Sephadex G-200 (5).

Protein determinations

Protein concentrations were determined according to Lowry et al. (11) using bovine serum albumin, fraction V (Pentex, Miles Laboratory, Kankakee, Ill.) as the standard. The values for apo A-I were compared to the protein concentrations determined by amino acid analysis. Since there are 18 moles of alanine per mole of apo A-I and the molecular weight of apo A-I is 28,331 (9, 12), the protein concentration in each sample was calculated on the basis of the moles of alanine detected. A comparison of the values of the apo A-I concentration determined by amino acid analyses versus the Lowry method yielded a ratio of 1.040 ± 0.017. The protein concentration of the apo A-I standard used in the assay was therefore determined directly from its Lowry value.

The assay

The assays were carried out in 10 × 75 mm disposable culture tubes (RTU #7810, Becton-Dickinson, Rutherford, N.J.). Standards or samples (0.03–1.0 ml) were made up to a total volume of 1.0 ml. The appropriate dilution (as determined in a preliminary binding assay) of 0.1 ml ^{125}I-labeled apo A-I (10,000–30,000 cpm) and 0.1 ml antibody (1:2500) were added to give approximately 50% binding of the tracer in the zero dose tubes. After a 3 day incubation at 4°C, the tubes were centrifuged at 520 *g* at 4°C for 20 min. Radioactivity levels in the precipitates were counted for 1–2 min. The results for each sample are expressed as B/B_0, where B = ^{125}I-labeled apo A-I cpm precipitated − cpm precipitated in nonspecific binding tubes and B_0 = ^{125}I-labeled apo A-I cpm precipitated in the zero dose tubes (no unlabeled apo A-I − cpm precipitated in nonspecific binding tubes.

Treatment of samples prior to assay

Delipidation of sera and HDL. Sera and HDL from fasting subjects were delipidated by ethanol–ether (8, 13). The lipid-free dry residue from serum was redissolved in 5 volumes of 0.9% NaCl, pH 7.0, and gently stirred at 4°C for 48 hr, while that from HDL was solubilized in 0.01M NH_4HCO_3 buffer, pH 8.2.

Quick freezing and thawing. Dilutions (10^{-3} thru 10^{-7}) of sera from fasting (10 hr) healthy subjects and concentrations of apo A-I in the range used in the assay, were placed in 50 ml (10 ml aliquots) and 125 ml (25 ml aliquots) Ehrlenmeyer flasks. The samples were frozen within 3 min in a bath of dry ice–acetone and then thawed in warm water in less than 6 min. This freezing and thawing cycle was repeated for a varying number of times (see Results).

Heating. Dilutions (10^{-3} thru 10^{-7}) of the control sera and concentrations of apo A-I in the range of the immunoassay were heated. The duration and incubation temperatures are given in Results. The samples were incubated in 25-ml flasks or silicone-coated test tubes and mixed every hour. Ten to 20% losses of immunoreactivity were noted in preparations heated in freshly washed flasks. Silicone-coated glassware prevented the adherence of the apo A-I to the container.

Other procedures. Triglycerides and cholesterol were determined simultaneously with an Auto Analyzer II (14) following procedures used by the Lipid Research Clinics Program (15). Serum phospholipids were extracted (16) and the phosphorus was measured according to Bartlett (17). Statistical analyses were performed according to standard methods (18). Unless otherwise indicated the values are expressed as the mean ± SEM.

Subjects. Control normolipemic subjects included hospital personnel and medical students who were within 16% of their ideal body weight (19). There were 19 females and 23 males ranging in age from 20 to 39; the mean ages of the females and males were 26 and 25 respectively. They were not taking medications and did not give a history of either diabetes or heart, thyroid, liver, or kidney disease.

The patient with abetalipoproteinemia has been described previously (20, 21). The serum of a patient with Tangier disease (22–24) was kindly provided by P. N. Herbert from the National Institutes of Health. Sera from patients with hypercholesterolemia (familial Type II homozygote) (23–25) were kindly provided by E. Stein and D. Mendelsohn from the University of the Witwatersrand, South Africa.

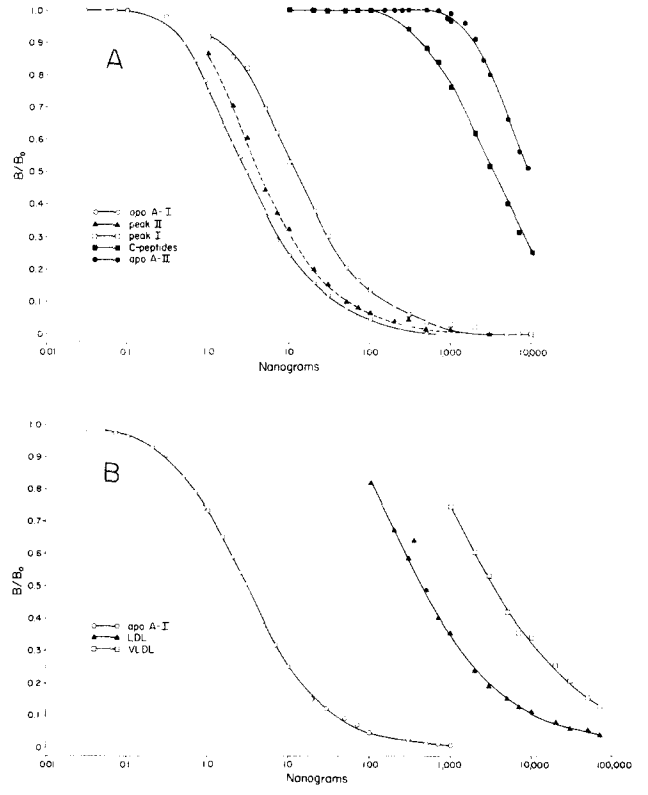

Fig. 1. Displacement of ^{125}I-labeled apo A-I from anti-apo A-I antibodies. (*A*) apo A-I (○—○); Sephadex G-200 peak I (□—□) (5); Sephadex G-200 peak II (▲—▲) (5); Sephadex G-200 peak IV, purified apo A-II (●—●) (5); and Sephadex G-200 peak V, C-peptides (■—■) (5). (*B*) apo A-I (○—○); human LDL (d 1.019–1.063) (▲—▲); human VLDL (d < 1.009) (□—□). Protein concentrations of all preparations were determined by the method of Lowry et al. (11). [The following modification was used for VLDL. After the color developed, VLDL was delipidated with two ethyl ether extractions. As a control, the albumin standards were also extracted with ether.]

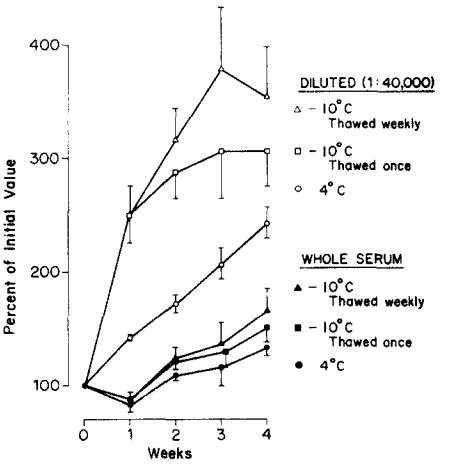

Fig. 2. Comparison of immunoassayable apo A-I in sera stored under different conditions. Sera diluted (1:40,000) in borate–albumin buffer were stored at −10°C and thawed weekly (△—△); stored at −10°C and thawed once (□—□); and stored at 4°C (○—○). Whole sera were stored and diluted in the borate–albumin buffer prior to assay: storage at −10°C and thawed weekly (▲—▲); storage at −10°C and thawed once (■—■); storage at 4°C (●—●).

RESULTS

Sensitivity and specificity of anti-apo A-I antibodies

The standard curve with purified apo A-I showed significant displacement at 0.3 ng (**Fig. 1A**). The working range of the assay lay between 1 and 10 ng of apo A-I and maximal displacement (greater than 98%) occurred with 300 ng (Fig. 1A).

Fig. 1A shows the reactivity of the anti-apo A-I antibodies with the Sephadex components of apo HDL, [peaks I, II, apo A-II, and V (C-peptides)] (5). Both peak I and peak II reacted with anti-apo A-I antibodies and the displacement curves lay parallel to that of apo A-I (Fig. 1A). Peak I contained 0.25 mg and peak II 0.64 mg of immunoassayable apo A-I per mg of protein respectively. Apo A-II and peak V (C-peptides) did not react as well and contained approximately 0.6 ng of apo A-I per mg protein (less than 0.1% apo A-I) (Fig. 1A).

Curves parallel to apo A-I were obtained with both purified human LDL and human VLDL (Fig. 1B). In the VLDL and LDL (isolated from six healthy males) apo A-I accounted for less than 0.1% and 1.0% of the protein respectively. The amount of apo A-I in LDL and VLDL is much less than one molecule of HDL per lipoprotein molecule and probably represents contamination with small amounts of HDL and/or apo A-I.

Apo A-I in stored sera

As we reported (6), values of immunoassayable apo A-I in sera did not remain constant during different conditions of storage (Fig. 2). Apo A-I was initially measured in five fresh sera. When stored as whole serum, there was a gradual increase in apo A-I values to 125–150% of the initial levels. Storage at 4°C resulted in slightly less increase than storage at −10°C (**Fig. 2**). However, when serum was stored at 4°C in a 1:40,000 dilution, there was a gradual rise to approximately 250% of the initial value and, if frozen, values of greater than 300% were detected (Fig. 2).

Apo A-I in fresh sera

Not only did the immunoassayable apo A-I level change during storage, but determinations on 10-fold serial dilutions revealed that the immunoassayable apo A-I increased with each dilution (**Fig. 3**). Instead of achieving a smooth dilutions curve [as in the case of the pure A-I protein (Fig. 1)], the apo A-I concentrations calculated from the 10^{-4}, 10^{-5}, and 10^{-6} dilutions were 40.0, 67.0, and 158.0 mg/dl, respectively. Displacement was not detected at serum concentrations less than 0.3×10^{-6} (Fig. 3). The variation with each 10-fold serial dilution was not the same for different sera, and could be influenced by the method of preparing the dilutions.

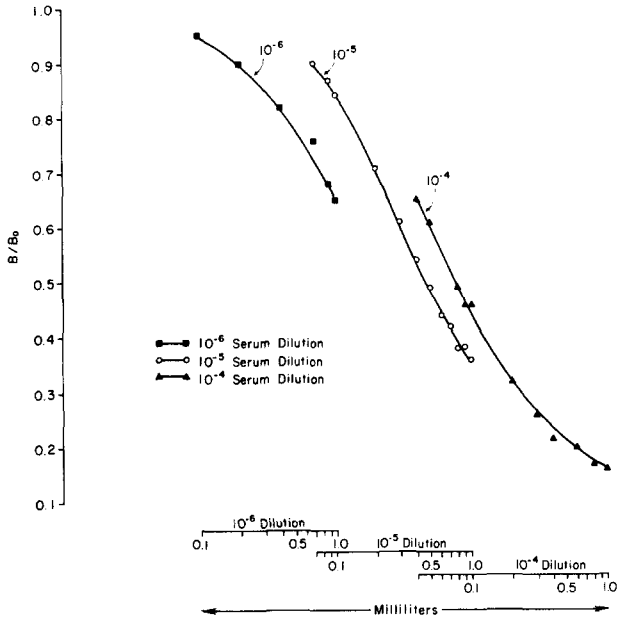

Fig. 3. Comparison of the displacement of ^{125}P-labeled apo A-I from anti-apo A-I antibodies by aliquots of ten-fold serial dilutions (10^{-6} (■—■), 10^{-5} (○—○), 10^{-4} (▲—▲)) of a normal serum. Volumes ranging from 0.020 to 1.0 ml from each serum dilution were assayed.

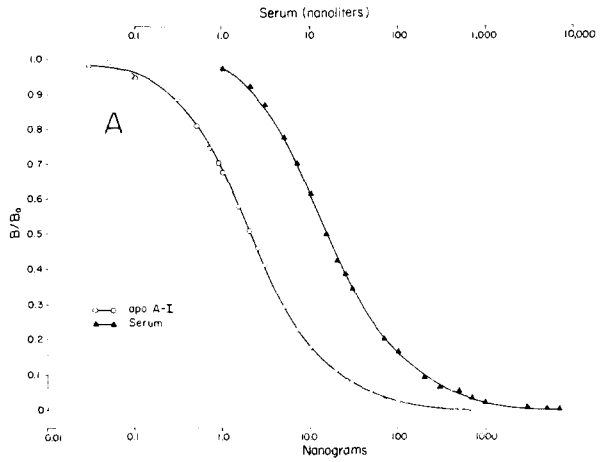

Fig. 4A. Displacement of ^{125}I-labeled apo A-I from anti-apo A-I antibodies by unheated apo A-I (○—○) and heated serum (▲—▲). Apo A-I stock solutions contained 0.1 ng/ml; 1.0 ng/ml; 10 ng/ml; 100 ng/ml; and 1000 ng/ml, while 10^{-3}; 10^{-4}; 10^{-5}; and 10^{-6} serial dilutions of serum were prepared in borate–albumin buffer. 8 ml of each solution were heated in siliconized test tubes at 52°C for 3 hr and stored overnight at 4°C. 0.1 ml to 1.0 ml aliquots were assayed in duplicate.

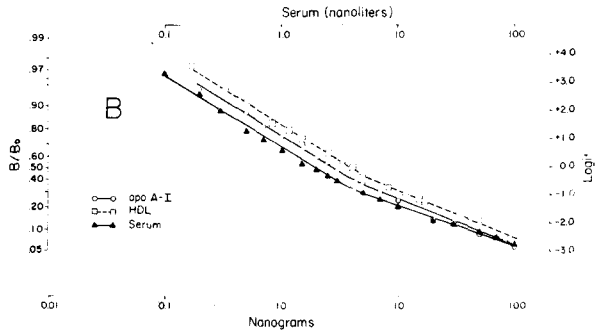

Fig. 4B. Logit transformation of the curves of apo A-I (○—○); heated diluted serum (▲—▲); and heated whole HDL (□—□). Protein concentration of whole HDL was determined by the Lowry method (11). HDL stock solutions 1.66 ng/ml; 16.6 ng/ml; 166 ng/ml and 1660 ng/ml were prepared. 8 ml of each stock dilution were heated at 52°C for 3 hr and stored overnight at 4°C. 0.1 to 1.0 ml aliquots were assayed in duplicate.

Rapid freezing and thawing of diluted fresh sera

The variable results obtained with serial dilutions of fresh sera, as well as with different methods of storage, indicated that not all the apo A-I was measured in fresh sera. Although the greatest increase in apo A-I occurred in frozen, diluted sera (Fig. 2), these storage conditions did not always maximize the apo A-I reactivity. We therefore tested the effect of rapidly freezing and thawing diluted sera on the immunoassayable apo A-I levels, and compared the results to those obtained with delipidation. The apo A-I detected after 5 and 10 freezing and thawing cycles was 85 and 90%, respectively, of that measured in delipidated sera, and this value did not increase on repeating the procedure 15 times. Therefore, it appeared unlikely that this method would allow measurement of the total apo A-I in serum.

TABLE 1. Effect of heating sera on the apo A-I levels

Preincubation Conditions	Serum #1[a]	Serum #2[a]
	mg/dl	
None	63	104
37°C–1 hr	70	98
37°C–6 hr	90	114
37°C–10 hr	94	120
37°C–22 hr	108	123
52°C–3 hr	107	133
52°C–6 hr	100	118
52°C–10 hr	99	132
52°C–22 hr	81	111
72°C–1.5 hr	96	116
72°C–3 hr	98	119
72°C–10 hr	98	111
72°C–20.5 hr	86	98

[a] 9.0 ml of 10^{-5} and 10^{-6} dilutions of fresh sera were heated in 25-ml Ehrlenmeyer flasks and stored overnight at 4° C. Each sample was assayed in five dilutions and the values represent the mean of these results.

Heating diluted fresh sera

In searching for a quick and convenient method to measure the total apo A-I in sera, we considered that warming the diluted frozen sera may have led to the observed increase in apo A-I antigenic sites. Therefore, the changes in the immunoreactivity of apo A-I in serum dilutions heated at 37°C, 52°C, and 72°C prior to assay were assessed (Table 1). Apo A-I increased with the duration of heating at 37°C and reached a maximum value at 22 hr. This level was similar to

TABLE 2. Comparison of apo A-I levels in diluted sera heated at 52°C for 3 hours versus delipidated sera

	Apo A-I		Heated sera
Sample No.	Heated sera[a]	Delipidated sera[b]	Delipidated sera
	mg/dl		
1 (D.D.)	141	138	1.02
2 (D.J.)	98	101	0.97
3 (J.K.)	141	124	1.14
4 (A.E.)	137	120	1.14
5 (J.S.)	118	102	1.16
6 (A.Z.)	125	117	1.06
7 (J.C.)	120	150	0.86
8 (T.C.)	111	113	1.10
9 (T.M.)	118	140	0.90
10 (M.M.)	106	135	0.87
11 (D.S.)	113	122	0.99
		Mean ± SEM	1.02 ± 0.03

[a] Fresh serum was diluted in the assay buffer. 5 ml of the 10^{-5} dilution was heated at 52°C for 3 hr in siliconized test tubes and stored overnight at 4°C.

[b] Sera were delipidated (13), dissolved by gentle mixing in 0.9% NaCl for 48 hr at 4° C and then diluted in assay buffer.

TABLE 3. Comparison of the logit-log slopes of apo A-I standard curves,and sera heated at 52°C for 3 hr

	Apo A-I		Sera Heated 52°C–3 hr	
	B/B_0 > 0.40	B/B_0 < 0.25	B/B_0 > 0.40	B/B_0 < 0.25
	−1.0504	−0.7155	−1.0331	−0.9056
	−0.9777	−0.7647	−1.0348	−0.5781
	−0.8915	−0.6306	−1.0454	−0.6248
	−0.9519	−0.7652	−0.9608	−0.5898
	−0.9425	−0.9910	−0.9563	−0.6716
	−1.0430	−0.6717	−0.8689	−0.6729
	−1.0063	−0.8342		
Mean ± SEM	−0.9805 ± 0.0216[a,c]	−0.7676 ± 0.0450[b,c]	−0.9832 ± 0.0279[a,d]	−0.6738 ± 0.0491[b,d]

[a] NS Upper region ($B/B_0 > 0.40$): apo A-I vs heated sera.
[b] NS Lower region ($B/B_0 < 0.25$): apo A-I vs heated sera.
[c] $P < 0.001$ Upper region vs lower region: apo A-I.
[d] $P < 0.001$ Upper region vs lower region: heated sera.

that obtained after heating for 3 hr at 52°C (**Table 1**). A decrease in apo A-I occurred after heating serum at 52°C for 22 hr (Table 1). The results of heating sera at 72°C were variable (Table 1).

Because of their convenience, we carried out additional experiments on heating serum dilutions at 52°C for 3 hr. The 10^{-3} through 10^{-6} dilutions heated at 52°C for 3 hr gave the same apo A-I value (**Fig. 4A**). Furthermore, curves made from unheated and heated apo A-I peptide standards were identical.

Comparisons of the apo A-I values obtained after heating dilutions of sera from 11 subjects at 52°C for 3 hr with delipidated sera resulted in a ratio of 1.02 ± 0.03 (**Table 2**). The close agreement between these two methods indicated that serum apo A-I maximally exposed by this heating procedure.

Apo A-I in HDL

The immunoreactivity of apo A-I in HDL was studied in a similar manner to that of serum. In five freshly isolated HDL preparations only 58 ± 2% of the immunoreactivity of the apo A-I was measured, as compared to the same dilutions of HDL heated at 52°C for 3 hr prior to being assayed. In contrast, 86% of the apo A-I reactivity was measured in an HDL preparation stored for over 2 weeks at 4°C.

Eight HDL preparations were delipidated. Immunoassayable apo A-I accounted for 65 ± 1.7% (range 58–74) and 74 ± 1.5% (range 69–80) of the protein in heated whole HDL and unheated apo HDL respectively. Both heated HDL and apo HDL gave curves parallel to the standard.

Logit transformation

The displacement curves of apo A-I, apo HDL, HDL (heated at 52°C for 3 hr), delipidated serum, unheated serum, and heated serum (52°C for 3 hr) were analyzed by logit-log transformation. This transformation should approximate a straight line when a single species of antibody reacts with a single antigen (26). The logit-log curves for the pure peptide, heated whole HDL and heated sera were resolved into two linear segments (Fig. 4B). [Not shown are delipidated sera and apo HDL which gave similar curves.] The bend usually occurred between B/B_0 0.40 and 0.25 (Fig. 4B). Because of the importance of ensuring that the slopes of heated sera and the apo A-I standard were parallel, six heated sera were assayed in multiple dilutions and compared with apo A-I standard curves by weighted regression analysis. The slopes of the sera and standards did not differ in either the upper (−0.9832 vs. −0.9805) or lower (−0.6738 vs. −0.7676) segments of the curve (**Table 3**).

Assay variability

The intraassay variation ranged from 7.7% in the region B/B_0 0.85–0.80 to 3.6% at B/B_0 0.45–0.40. The interassay variation determined over a 3 month period was 5%.

Validity of the apo A-I immunoassay

Recovery of apo A-I in serum. To ensure that the heating procedure did not lead to losses, different quantities of unheated or heated apo A-I were added to diluted, heated serum. The recovery of apo A-I was 102 ± 1.6% for the unheated peptide, and 104 ± 1.2% for the heated peptide. The recovery of the apo A-I component of HDL was also determined; HDL was added to diluted serum and the mixture heated at 52°C for 3 hr. The expected apo A-I level was calculated from measurements of separately heated serum and HDL (recovery 106 ± 2.2%). Finally, HDL and diluted sera were heated separately and then mixed prior to assay. The recovery was 105 ± 1.5%.

The apo A-I value in heated fractionated serum was compared to that determined directly in heated serum. The densities of 3 aliquots of a serum were adjusted to d 1.006 g/ml, d 1.063 g/ml, and d 1.21 g/ml, layered over 5 ml of the respective salt solutions, and spun at 114,000 *g* in a fixed angle

rotor for 24 hr and 1.0 ml fractions were collected. Each fraction was then heated for 3 hr at 52°C. Whether the HDL migrated to the bottom of the tube, with or without LDL, or was separated from the majority of serum proteins and recovered at the top of the gradient, the apo A-I value was 92, 88, and 86%, respectively, of that measured directly in the heated diluted serum. These data indicate that neither the other classes of lipoproteins nor serum proteins interfere with the direct measurements of apo A-I in serum.

Stability of apo A-I in serum. The apo A-I values of heated dilutions of freshly collected sera were determined. The apo A-I values were similar if the heated, diluted sera were stored overnight at 4°C or at room temperature prior to assay. There were no significant changes in the apo A-I value in heated 10^{-5} dilutions stored at 4°C for one week and, in one instance, for as long as two months. The heated dilutions of apo A-I were also stable when frozen for one or two months. It is of interest that the apo A-I value was the same if the heated, diluted serum, stored at −10°C was measured directly, or was reheated at 52°C for 3 hr before being assayed. The stability of apo A-I in frozen (−10°C) undiluted serum was also tested. After one or two months, undiluted serum samples were thawed, diluted and the 10^{-5} dilutions heated at 52°C for 3 hr prior to assay. The apo A-I values in the stored sera were not significantly different from that in the heated dilutions of fresh sera.

Apo A-I in human sera

Control subjects. The total serum cholesterol (166 ± 7; 182 ± 5 mg/dl) triglyceride (80 ± 7; 56 ± 4 mg/dl) and phospholipid (181 ± 7; 200 ± 11 mg/dl) levels of these healthy, young men and women were within the normal range. The apo A-I level in males was 130 ± 3.0 mg/dl (range 95–165) and in females 154 ± 6.0 mg/dl (range 107–199) ($P<0.005$). The correlation of apo A-I with total serum cholesterol, triglycerides, phospholipids and immunoassayable apo B was undertaken. The only significant correlation was between cholesterol and apo A-I (males $r = 0.46$, $P<0.005$; females $r = 0.58$, $P<0.01$).

Patients with dyslipoproteinemias:

(*a*) *Abetalipoproteinemia (ABL).* LDL has been reported to be absent from this patient's serum (20, 21) and this was confirmed in our radioimmunoassay for apo B which is similar to that of Schonfeld et al. (27). Because the HDL of ABL patients may be abnormal (20–24), it was necessary to ensure that heating the diluted sera at 52°C for 3 hr maximally exposed the apo A-I antigenic sites. The apo A-I levels in healthy and ABL sera were maximal after heating at 52°C for 3 hr, and similar percentages of the maximal apo A-I values (77 and 91%) were measured after heating both sera at 37°C for 3 hr and at 52°C for 22 hr, respectively. The apo A-I in the ABL serum was 43 mg/dl compared to 154 ± 6.0 mg/dl in the sera of healthy females.

(*b*) *Hypercholesterolemic subjects (Type II).* Three patients with markedly elevated serum cholesterol (831, 795, and 720 mg/dl) but normal triglyceride (132, 132, 111 mg/dl) concentrations were studied. They exhibited an intense band in the beta region on agarose electrophoresis and elevated concentrations of serum apo B (533, 472, and 540 mg apo B/dl serum). These apo B concentrations were five-fold greater than those found in our healthy subjects. The apo A-I level in the three patients (mean 72 mg/dl, range 54–86) was significantly less than in healthy individuals ($P < 0.001$). To ensure that the elevated LDL concentration or other alteration in the serum proteins was not interfering with the apo A-I measurement, 1.0 ml of serum from one subject was fractionated on a linear salt gradient which separated LDL from HDL. The apo A-I level in the separate fractions was 110% of that measured directly in the whole serum, thus confirming the validity of the low A-I values in these patients.

(*c*) *Tangier disease.* Because it has been shown that in patients with Tangier disease the serum HDL concentration is reduced (22–24), it was necessary to ensure that heating the diluted sera at 52°C for 3 hr maximally exposed the apo A-I antigenic sites. Diluted sera from a control subject and this patient were heated at 37°C for 3 hr or at 52°C for 3 hr. In both sera the apo A-I concentrations were maximal after heating for 3 hr at 52°C and in the Tangier patient the apo A-I level was 1.1 mg/dl which is only 1% of that measured in normal subjects.

DISCUSSION

The radioimmunoassay

The assay described for measuring apo A-I is specific, sensitive, and reproducible. Moreover, the results obtained with this method are in close agreement with those reported by Schonfeld and Pfleger (7). Both studies have shown that apo A-I antigenic sites are not maximally exposed in isolated whole HDL or serum. Schonfeld and Pfleger (7) overcame this problem by delipidating HDL and plasma samples prior to assay. Although approximately 95% of apo A-I is recovered during this procedure, it is time consuming and cumbersome for analyzing a large number of samples. An important advantage of the present method is that the lipids do not have to be extracted before the samples are assayed. Instead we have found that if the appropriate dilutions of HDL or serum are heated at 52°C for 3 hr, the apo A-I antigenic sites are maximally exposed. Heating the samples in this manner is rapid, easy, and efficient and does not alter the antigenicity of apo A-I.

After this paper had been submitted for publication, another double antibody radioimmunoassay for apo A-I was published (28). This assay (28) is similar to the one reported in this report in that the apo A-I immunogenicity in serum and HDL is maximized without a delipidation step. However, at three different times during the incubation of samples with the apo A-I antibody, the temperature was raised from 4°C to 37°C for two hours. Fainaru, Glangeaud, and Eisenberg (28) considered the possibility that the incubation at 37°C might be responsible for the differences in their results as compared to those of Schonfeld and Pfleger (7), but did not systematically investigate this point.

Analysis of the logit-log plots of the displacement curves of apo A-I standards and heated sera has important implica-

tions for the long term goal of automating the assay procedure. Most computer programs for calculating radioimmunoassay results utilize this transformation. The logit-log lines for standards and serum dilutions derived from points over the range $B/B_0 > 0.4$ are parallel and give values for unknown samples which are identical to those obtained by hand calculation from direct plots of the data. On the other hand, there is considerable interassay variation in the change of slope in the region below $B/B_0 < 0.25$, although it should be noted that there was no consistent difference between the slopes of the standards and sera.

Because apo A-I retains its full immunological activity in fresh and frozen plasma or serum, and in diluted heated serum stored at −10°C, this assay may be conveniently adapted for routine use. Appropriate dilutions of serum can first be made using automated pipetting equipment. After heating, the assay can be set up using the same equipment. The ease of preparing the samples and the use of a computer program for the calculations permits one technician to process approximately 500 serum samples in triplicate each week.

HDL

The concentration of apo A-I (74.0 ± 1.5%) in apo HDL is in good agreement with that found after chromatography on Sephadex columns (5). However, the concentration of apo A-I expressed as a percentage of the protein in whole heated HDL (65.4 ± 1.7%) was somewhat lower than that in apo HDL (7.40 ± 1.5%). Based on the good agreement (1.02 ± 0.03) between the values of apo A-I determined in diluted heated sera and delipidated sera, we do not think that the lower value of apo A-I in whole HDL reflects an inability of this radioimmunoassay to detect all the apo A-I present. The difference between whole HDL and apo HDL may rather reflect selective protein losses since small volumes of HDL (<0.5 ml) were delipidated.

The observation that the immunoassayable apo A-I is maximally increased by either heating or delipidation of sera and whole HDL, suggests that the concealment of apo A-I antigenic sites is probably due to bound lipids. Heating presumably alters the interaction of apo A-I and lipids and, by a still undefined mechanism, renders the antigenic sites accessible to the antibody. The concealment of apo A-I antigenic sites in HDL is consistent with the in vitro reassembly studies using apo HDL and lipid fractions extracted from HDL (29, 30).

Apo A-I values in serum

The serum apo A-I values in our group of healthy subjects are in the same range as those reported by Schonfeld and Pfleger (7). We have confirmed our previous evidence (6) that the levels in females are significantly higher than in males (1.54 ± 0.06 vs 1.30 ± 0.03 mg/ml). It is possible that the difference between our males and females reflects the narrow age range and stringent selection criteria for the subjects. No difference between the sexes was shown by Schonfeld and Pfleger (7), but their age range was wider (13–58 yr) and the subjects were chosen on a different basis.

Schonfeld and Pfleger (7) reported a significant correlation between the apo A-I value and HDL cholesterol, but not total serum cholesterol. Interestingly, a significant positive correlation between apo A-I and total serum cholesterol was present in both our female and male populations ($P<0.01$ and $P<0.05$ respectively). At present the significance of this correlation is uncertain. Although approximately 27% of HDL is phospholipid by weight (4) the apo A-I and serum phospholipid concentrations were not correlated.

If one makes several assumptions, the HDL concentration (mg/dl) in serum can be approximated from the apo A-I value. Using the apo A-I content (65%) of isolated HDL (d 1.063–1.21 g/ml) as representative of the ratio of apo A-I to protein in all the HDL subclasses, and a protein:lipid ratio of 51:49 (as the weighted average of HDL_2 and HDL_3, ratio 1:3 for males) (4, 23, 24). the calculated mean HDL concentration in males was 392 mg/dl (range 286–498) and females 465 mg/dl (range 324–600). These values are in agreement with the determination of HDL concentration by ultracentrifugation and heavy metal precipitation (23, 24). The significantly higher levels of apo A-I in our female subjects, as compared with the males, is in agreement with previous reports (24).

Patients with dyslipoproteinemias

Abetalipoproteinemia, hypercholesterolemic and Tangier subjects. The lower level of apo A-I in these subjects is consistent with previous reports showing lower values for whole HDL (21–25). Our preliminary results indicate that significant alterations in the LDL concentrations and changes in the physical chemical properties of HDL in the ABL patients (and Tangier patient) do not affect the optimum conditions for measuring apo A-I. Though this result will have to be substantiated for other dyslipoproteinemias, it seems probable that our apo A-I assay can readily be adapted for analyzing sera from patients with various diseases and thus improve our understanding of the role of this polypeptide and HDL in lipid transport. Important ancillary information is also expected to be derived from the quantification of the second major HDL apo protein, apo A-II, whose radioimmunoassay is being developed in this laboratory.

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