Measurement of small high density lipoprotein subclass by an improved immunoblotting technique

Ragheb F. Atmeh[1,*,†] **and Horst Robenek**[†]

Department of Chemical Sciences,* University of Science and Technology, Irbed, Jordan, and Institute for Arteriosclerosis Research at the University of Münster, Department of Cell Biology and Ultrastructural Research,† Münster, Germany

Abstract An improved method of immunoblotting of plasma onto agarose gel matrix containing antiapolipoprotein A-I is described. Fresh plasma samples were subjected to gradient polyacrylamide gel electrophoresis (4–25%) followed by electrotransfer onto agarose gel layer containing antiapolipoprotein A-I. This method was compared with immunoblotting onto nitrocellulose where the transfer onto agarose gel matrix has been shown to be more convenient, quantitative, and can be kept permanently. Plasma apolipoprotein A-I was found to be distributed among regions of varying molecular weights ranging from 43,000 to 800,000. A small size fraction of molecular weight range of 43,000–50,000 (small HDL) was found in normolipidemic and hyperlipidemic subjects. The proportion of the latter fraction varied considerably among subjects (range: 0.0–32.0%), being lower in normolipidemic subjects (mean ± SEM: 11.6 ± 1.4%), and higher in hyperlipidemic subjects (mean ± SEM: 23.7 ± 1.7%, $P < 0.001$). Physiological increase in the level of the small HDL was observed in normolipidemic subjects 4 h after fat ingestion (difference: 5.0%, $P < 0.001$); moreover, the level was higher in normolipidemic subjects who consumed moderate amounts of alcohol (mean ± SEM: 17.9 ± 1.2%, $P < 0.001$) compared with normolipidemic subjects who do not drink alcohol at all.—**Atmeh, R. F., and H. Robenek.** Measurement of small high density lipoprotein subclass by improved immunoblotting technique. *J. Lipid Res.* 1996. **37:** 2461–2469.

A large proportion of plasma apolipoprotein A-I (apoA-I) resides in the high density lipoprotein (HDL) fraction of the density range 1.063–1.21 g/mL, while smaller amounts reside in lipoproteins of lower and higher flotation densities (1, 2). HDL is heterogeneous and comprises several subfractions whose number depends on the method of separation (3–7). According to the apolipoprotein content, HDL can be classified into two main classes, e.g., (A-I)HDL and (A-I + A-II)HDL (3, 4, 6, 8), where the latter subclass has a fixed apoA-I/apoA-II molar ratio of approximately 1:1 (2, 8, 9). These main subclasses have been shown by

Atmeh, Shepherd, and Packard (8) to be distinct physiological entities where minimal exchange of their apoA-I was observed both in vitro and in vivo; moreover, these two subclasses showed different responses to nicotinic acid and probucol therapies. In accordance with that, Rader et al. (10) have reported that these subclasses have divergent metabolic pathways. Consequently, they are expected to have different roles in the lipoprotein metabolism. (A-I)HDL is the best activator of the enzyme LCAT and it facilitates the movement of cholesterol from cells into plasma (11, 12), and is able to promote cholesterol efflux from cultured adipose cells, while the (A-I + A-II)HDL particles have no effect (13). Both subclasses show particle size heterogeneity (2, 8, 9, 14, 15). Size heterogeneity was studied by Nichols et al. (16) and Blanche et al. (7) by using gradient polyacrylamide gel electrophoresis (gPAGE) where they reported the presence of five main HDL subclasses designated as HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b}, and HDL_{3c}.

The expected role of HDL in the reverse cholesterol transport requires the presence of small discrete particles having the HDL characteristics in order to initiate the removal of excess cholesterol from extrahepatic cells. In accordance with this concept Barter and Connor (17) concluded, from in vivo kinetic studies, that all plasma esterified cholesterol is produced in a small, rapidly turning over subfraction of HDL. At present the methods used to study the HDL subfractions may not detect low levels of HDL particles of molecular weight less than 70,000. Prolonged ultracentrifugation and concentration of the separated fractions by ultrafiltration through membranes with relatively large pores may

---

Abbreviations: HDL, high density lipoprotein; (A-I)HDL, high density lipoprotein containing apolipoprotein A-I as the major protein; (A-I + A-II)HDL, high density lipoprotein containing both apolipoproteins A-I and A-II; SHDL, small high density lipoprotein; gPAG, gradient polyacrylamide gel; gPAGE, gradient polyacrylamide gel electrophoresis; NC, nitrocellulose.
[1]To whom correspondence should be addressed.

cause loss of the small HDL. Along with that, the methods are not applied directly on fresh plasma for the quantitation of HDL subfractions.

We here describe a method, utilizing agarose gel matrix containing anti-apoA-I, for detection and quantitation of the distribution of the plasma apoA-I, the major apolipoprotein of HDL, and we report the level of small HDL particles in fresh plasma from normolipidemic and hyperlipidemic subjects. Moreover, we found that the level of these particles in normolipidemic subjects was affected by the ingestion of a fatty meal and by the consumption of alcohol.

MATERIALS AND METHODS

Subjects

Twenty four normolipidemic, healthy nonsmokers, and asymptomatic subjects (thirteen men and eleven women), and twelve hyperlipidemic patients of mixed hyperlipoproteinemia (six men and six women, receiving no drug therapy) were studied (**Table 1** summarizes the subjects' data). Ten of the normolipidemic subjects (five men and five women) consumed moderate amounts of alcohol (about 30 g of alcohol per day) while the others did not drink alcohol at all. Venous blood was withdrawn into EDTA-containing tubes from the normolipidemic and the hyperlipidemic subjects after a 14-h fast. Plasma was isolated promptly by low-speed centrifugation at 4°C and gentamycin (0.08 mg/mL), sodium azide (0.1 mg/mL), and chloramphenicol (0.08 mg/mL) were added immediately. Plasma was applied on the gradient polyacrylamide gel (gPAG) and run within 3 h of sample collection.

Eight normolipidemic, healthy non-smokers, non-obese, and asymptomatic subjects (five men and three women) were given 100 g of butter with a small piece of bread and traces of marmalade after a 14-h fast. A fasting blood sample was collected just before the ingestion of fat and blood was withdrawn 4 and 6 h after that. Blood samples were treated as described above.

Preparation of HDL and lipoprotein fraction of d < 1.21 g/mL

HDL (d 1.063–1.21 g/mL) was prepared by sequential ultracentrifugation at 10°C according to Havel, Eder, and Bragdon (18) in a Beckman model L8-70M ultracentrifuge using a Beckman 50.3 Ti rotor (Beckman, Palo Alto, CA). Similarly, lipoprotein fraction of d < 1.21 g/mL was prepared by a single spin of plasma overnight. Both fractions were dialyzed extensively for a few hours.

Nondenaturing gradient polyacrylamide gel electrophoresis(gPAGE)

A 4–25% gPAG was prepared and run as was described previously (2, 19). In brief, the gPAG was cast and run in the LKB 2001 vertical electrophoresis system (LKB, Sweden). Five microliters of the fresh plasma was applied to each well of the gPAG and run at 125 V for 24 h in 14 mM Tris and 110 mM glycine buffer, pH 8.3. Molecular weight markers (Sigma, St. Louis, MO) were applied on each gel and after the run the part of the gPAG containing the markers was cut, stained, destained, and dried (19). The rest of the gel was subjected to immunoblotting on agarose gel as will be described below.

Immunoblotting on agarose gel matrix

Antibody specific for apolipoprotein A-I (Boehringer Mannheim, Mannheim, Germany) was used; it did not react with apoA-II, apoB, apoC, apoE, or human albumin. One percent agarose gel solution containing 1.5% of anti-apoA-I of , 0.3 titer was prepared and treated as described elsewhere (19). In brief, the antibody was mixed with the warm agarose solution (56°C) containing 4% polyethylene glycol (PEG 6000) and poured (1.5 mm thick) on glass plates of the required area. Immediately after the end of the electrophoretic run, the gPAG was placed on a wet filter paper supported by a piece of sponge (e.g., Scott Brite® used in kitchen cleaning). The solidified agarose gel was slipped down from its support slowly over the ready gPAG, covered

TABLE 1. Chemical data of the subjects

Subjects	Total Cholesterol	HDL-Cholesterol	Triglycerides	ApoA-I	ApoA-II
All normal (I, II)	173 ± 5	48 ± 2	74 ± 5	131 ± 3	40 ± 1
Normal (I)	177 ± 7	44 ± 2	76 ± 6	131 ± 5	41 ± 2
Normal (II)	168 ± 6	51 ± 3	69 ± 5	131 ± 4	38 ± 1
Hyperlipidemic	357 ± 35	45 ± 3	150 ± 16	133 ± 5	39 ± 2

All concentrations are in mg/dL and all values are expressed as the mean ± SEM. Normal subjects were taken as one group (n = 24; age 30 ± 1), then separated into two groups. One group included those who did not drink alcohol [normal I] (n = 14; age 30 ± 2), and the other group included those who consumed moderate amounts of alcohol [normal II] (n = 10; age 29 ± 2). Hyperlipidemic subjects were taken as one group (n = 12; age 32 ± 5).

by a sheet of wet filter paper and a piece of sponge. Both gels, agarose, and gPAG, that were already sandwiched between two layers of filter paper and sponge, were placed in the cell holder that is put in the transfer cell (Trans-Blot cell, Bio-Rad, CA). The agarose gel layer faced the anode. The transfer was done overnight, at 100 mA constant current, in the Tris-glycine buffer used in the previous step. After completion of the transfer, the agarose layer was carefully removed and placed on a glass plate or a Gel-Bond film (Pharmacia, Sweden), wrapped with a piece of wet soft medical gauze and washed for 24 h with several changes of 0.15 M saline to remove the unreacted antibody. The agarose gel was dried and stained with 1% Coomassie brilliant blue R-250 in 50% methanol and 7% acetic acid, then destained with 10% methanol and 7% acetic acid solution. After the completion of the electrotransfer step, the remaining gPAG slab was fixed with sulfosalicylic acid and stained with 0.04% Coomassie brilliant blue R-250 in 3.5% perchloric acid and destained with 5% acetic acid (19). No stained bands were detected.

Measurement of apolipoprotein A-I distribution

The stained bands on the agarose gel and gPAG were scanned at 595 nm using a Quick Scan Densitometer (Helena, TX). The area under the peaks was used for the calculation of the percentage of each fraction. The amount of apoA-I present in each fraction was calculated from the percentage of the individual fraction multiplied by the plasma concentration of apoA-I.

HDL labeling with [125]I

HDL (d 1.063–1.21 g/mL) was labeled with ^{125}I according to the method of Bilheimer, Eisenberg, and Levy (20).

Immunoblotting on nitrocellulose (NC)

Iodinated HDL was separated on 4–25% gPAGE and transferred onto NC followed by immunoblotting as was described previously (2, 21).

Assay methods

Apolipoproteins A-I and A-II were assayed by immunoprecipitation and turbidimetry using antibodies from Boehringer Mannheim (Mannheim, Germany) according to the manufacturer's instructions. HDL-cholesterol, plasma cholesterol, and triglycerides were assayed by enzymatic methods (Boehringer Mannheim, Germany).

RESULTS

Immunoblotting of plasma lipoproteins on agarose gel containing anti-apoA-I, after their separation by the
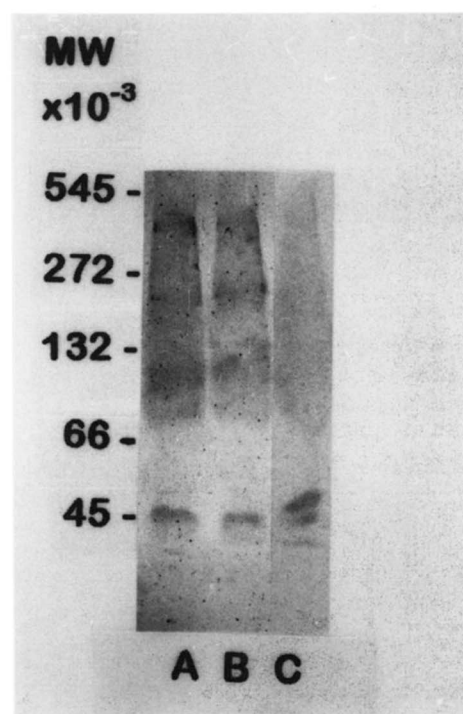

Fig. 1. Immunoblot of apolipoprotein A-I distribution in human fresh plasma after separation on nondenaturing gPAGE 4–25%. Lanes A and B, plasma (5 μL) from a normolipidemic subject 4 h after a fatty meal and in the fasting state, respectively; lane C, plasma (5 μL) from a hyperlipidemic subject.

4–25% nondenaturing gPAG, showed that apoA-I is present in several regions of varying molecular weights ranging from 43,000 to 800,000. Two main regions of molecular weights of 43,000–50,000 (designated as fraction A; small HDL (SHDL)) and 60,000–800,000 (designated as fraction B) will be considered in this study (**Fig. 1**). Although fraction B contains several components, it is considered as one group in this study because the components are well defined in some samples while they are not in others. An observation worth mentioning is that the components are reasonably separated in samples from subjects with low level of SHDL while they are not well separated in samples from subjects with high level of SHDL.

Capture efficiency of agarose gel matrix compared to NC

Known counts of iodinated HDL (3 μg) were applied several times to a 4–25% gPAG and electrophoresed. After the run was over, the gels were sliced longitudinally and divided into three groups. The first group was considered as a control and the gel slices were counted for radioactivity. The second and third groups were transferred onto NC and agarose gel containing anti-apoA-I, respectively. The average (n = 7) percentage of

radioactivity entered the gel was calculated from the first group (95.2% ± 0.6; mean ± SEM). Similar percentage was considered for the second and third groups. In the NC membrane, 6.5% ± 0.5 (mean ± SEM, n = 7) of the radioactivity was found, and 2.0% ± 0.1 (mean ± SEM) in the residual gPAG slice. On the other hand, 91.5% ± 0.6 (mean ± SEM, n = 7) of the radioactivity was found in the agarose gel and 2.2% ± 0.1 (mean ± SEM) in the residual gPAG slice.

Capacity of agarose gel to hold the transferred apoA-I

Two layers of agarose gel (1.5 mm thickness each) containing 1.5% anti-apoA-I, separated by a wet filter paper, were placed on a gPAG slab (containing 5 μL of plasma) after electrophoretic separation. At the end of the transfer step, the two layers were removed and washed separately, dried, stained, and destained. Stained bands were seen on the first layer only while the second layer showed no stained bands.

Reproducibility of apoA-I distribution in plasma

Plasma sample was run several times (n = 8) on separate gradient polyacrylamide gels followed by immunoblotting on agarose gel containing anti-apoA-I. The positions of the apoA-I regions were similar and the average percentage of fraction A was 8.4% with a CV of 9.1%.

Immunoblotting of lipoproteins on agarose gel containing anti-apoA-I

Lipoprotein fraction of d < 1.21 g/mL was isolated from the plasma of twelve normolipidemic subjects and run on 4–25% gPAGE. Part of the gel was stained and the other part, which contained the same samples, was transferred onto agarose gel containing anti-apoA-I. The percentage of the SHDL calculated by the two methods was correlated with r = 0.91 (**Fig. 2**). The percentages calculated from the stained gPAG and immunoblotted were 6.8% ± 0.6 and 7.7% ± 0.6 (mean ± SEM), respectively.

Distribution of apoA-I in the plasma of normolipidemic subjects compared with that of hyperlipidemic subjects

The distribution of apoA-I in the fresh plasma of normolipidemic subjects (n = 24) after immunoblotting on agarose gel containing anti-apoA-I is summarized in **Table 2**. The normolipidemic subjects were segregated into two groups according to the alcohol consumption. One group that includes those who did not drink alcohol at all [normal I, n = 14] and the other group that includes those who consumed moderate amounts of alcohol (about 30 g/day) [normal II, n = 10]. Higher proportion of the SHDL was found in the fresh plasma
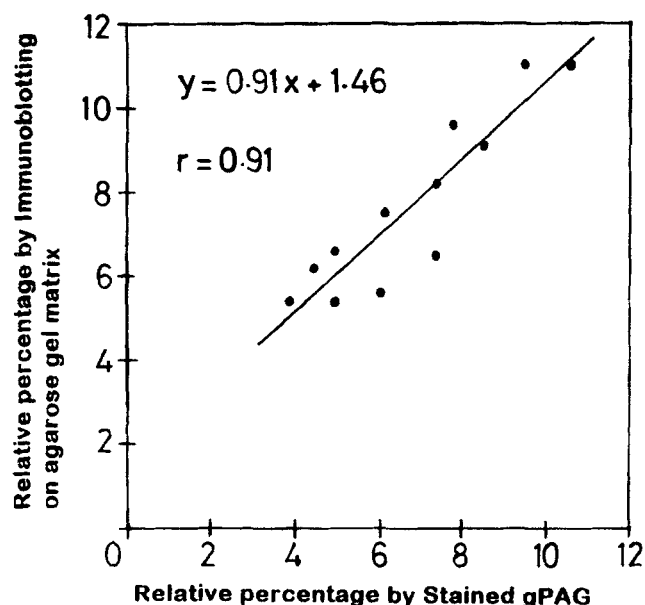

Fig. 2. Comparison of the relative percentages of apolipoprotein A-I in the SHDL fraction of plasma determined by immunoblotting on agarose gel matrix with those determined by stained gPAG.

of the group of moderate alcohol drinkers compared with the other group of no alcohol consumption, and the differences were statistically significant (Table 2). Statistically significant differences were obtained in the percentage of the small fraction between the hyperlipidemic (n = 12) and the normolipidemic (n = 24) subjects (P < 0.001) and between the hyperlipidemic subjects and the normolipidemic subjects who did not consume alcohol at all (n = 14) (P < 0.001) (Table 2). Significant differences remained (P < 0.05) when the results of the hyperlipidemic subjects were compared with those of the normolipidemic subjects who consumed moderate amounts of alcohol (Table 2).

Effect of fat ingestion on the apoA-I distribution in normolipidemic subjects

The relative percentage and the mass of apoA-I associated with the small HDL subfraction were increased (P < 0.001) 4 h after the fat load in all subjects (**Table 3**). Six hours after fat ingestion, the proportions have decreased relative to the 4 h value (−4.0, P < 0.05), and in six subjects the levels were still higher than the fasting level.

DISCUSSION

HDL plays an important role in the reverse cholesterol transport. It is reasonable to assume the presence

TABLE 2. Distribution of apoA-I in plasma of normolipidemic and hyperlipidemic subjects

Fraction	All Normal (n = 24)	Hyperlipidemic (n = 12)	Difference	Normal I[a] (n = 14)	Normal II[a] (n = 10)	Difference between I and II	Difference between I and Hyperlipidemic	Difference between II and Hyperlipidemic
Fraction A								
Rel%	11.6 ± 1.4	23.7 ± 1.7	−12.1[d]	7.1 ± 1.2	17.9 ± 1.2	−10.8[d]	−16.6[d]	−5.8[c]
Range	0.0–24.9	13.6–32.0		0.0–14.5	11.3–24.9			
ApoA-I	15.3 ± 1.9	32.2 ± 3.1	−16.9[d]	9.4 ± 1.7	23.6 ± 1.9	−14.2[d]	−22.8[d]	−8.6[b]
Range	0.0–31.6	16.4–49.6		0.0–21.5	12.7–31.6			
Fraction B								
Rel%	88.4 ± 1.4	76.3 ± 1.7	12.1[d]	92.9 ± 1.2	82.1 ± 1.2	10.8[c]	16.6[d]	5.8[c]
Range	75.1–100	68.0–86.4		85.5–100	75.1–88.7			
ApoA-I	115.6 ± 3.2	101.0 ± 3.1	14.6[d]	121.6 ± 4.5	107.1 ± 3.0	14.5[c]	20.6[d]	6.1[c]
Range	94.6–146.4	75.0–115.6		94.6–146.4	95.4–125.6			

Five microliters of plasma were run on 4–25% nondenaturing gPAGE followed by immunoblotting on agarose gel containing anti-apoA-I and staining. The relative percentage of the stained subfractions was calculated from the area under the curve of each subfraction after densitometric scanning. The distribution of apoA-I (mg/dL) among the subfractions was calculated by multiplying the individual relative percentage of the subfraction by the concentration of apoA-I in plasma. Values are expressed as mean ± SEM. Fraction A represents SHDL and fraction B represents all HDL subfractions other than SHDL.

[a]As in Table 1.
[b]$P < 0.05$.
[c]$P < 0.01$.
[d]$P < 0.001$.
[e]NS.

of very small, newly formed HDL particles as being the initial stage in the reverse cholesterol transport, because such small particles are expected to be relatively poor in cholesterol (22–26) and are able to pass through the capillary wall to the interstitial fluid where they can interact directly with peripheral cells and be converted into larger HDL (22, 27–29) by incorporation of unesterified cholesterol. The presence of small apoA-I-containing lipoprotein particles was reported in the human interstitial fluid (27) in the plasma of normolipidemic subjects (2, 30), and in vitro (26, 31).

In order to understand the initial events associated

TABLE 3. Plasma lipids and apoA-I concentrations and apoA-I distribution after a fatty meal in eight normolipidemic subjects

	0 Hour	4 Hour	6 Hour
Plasma			
Triglycerides (mg/dL)	69 ± 4	111 ± 6[d]	97 ± 6[d]
	(100%)[a]	(164 ± 6.6%)	(143 ± 6.5%)
Cholesterol (mg/dL)	153 ± 8	157 ± 8[d]	158 ± 8[c]
	(100%)	(102 ± 0.3%)	(103 ± 0.6%)
ApoA-I (mg/dL)	135 ± 6	138 ± 6[d]	137 ± 6[d]
	(100%)	(103 ± 0.5%)	(101 ± 0.2%)
Fraction A			
Relative %	10.4 ± 1.0	15.4 ± 1.5[d]	11.4 ± 1.6
	(100%)	(149 ± 5.6%)	(112 ± 11.2%)
Range	6.9–14.5	9.8–21.5	5.3–21.0
ApoA-I (mg/dL)	14.3 ± 1.8	21.7 ± 2.7[d]	15.8 ± 2.6
	(100%)	(153 ± 5.2%)	(114 ± 12.3%)
Range (mg/dL)	8.3–21.5	12.1–32.3	8.3–31.5
Fraction B			
Relative %	89.6 ± 1.0	84.6 ± 1.5[d]	88.6 ± 1.6
	(100%)	(94 ± 0.8%)	(99 ± 1.5%)
Range	85.5–93.1	78.5–90.2	79.0–94.7
ApoA-I (mg/dL)	120.9 ± 5.0	116.7 ± 4.7[b]	121.2 ± 5.8
	(100%)	(97 ± 1.1%)	(100 ± 1.5%)
Range (mg/dL)	100.2–140.3	94.3–134.8	98.4–147.7

Values are means ± SEM.
[a]Percentages (mean ± SEM) in parentheses represent the plasma concentration expressed as a percentage of zero time concentration.
[b]Significantly different from zero time value by paired t-test; $P < 0.05$.
[c]$P < 0.01$.
[d]$P < 0.001$.

with the reverse cholesterol transport and the expected role of the small apoA-I-containing lipoprotein particles, these particles should be identified, quantitated, and isolated by methods that are not expected to change their composition and structure. The main methods used at present for the isolation and quantitation of HDL subfractions depend on lengthy ultracentrifugation or affinity chromatography. Both methods may affect the size distribution and composition of the HDL particles (1, 32); moreover, the individual subfractions are not detected simultaneously but are obtained at different stages of separation. Ultrafiltration was successfully used (2) for the isolation of small HDL particles directly from fresh plasma. A promising method for detection and quantitation of HDL subfractions is the use of nondenaturing gPAGE technique for fresh plasma followed by transfer onto nitrocellulose or similar membranes, and finally the treatment of the membrane by a sequence of antibodies (31, 32). Electrophoresis is a mild method and does not strip apoA-I from the lipoprotein particles as was reported by Lefevre, Goudey-Lefevre, and Roheim (32). However, the use of such membranes includes some serious drawbacks that render the method a semiquantitative one (33). The aim of this study was to improve a method that can be applied directly to plasma and overcomes the major drawbacks of the present methods. One of the major drawbacks in the membranes used is that the transfer of particles with different molecular weights occurs at different rates from the gPAG onto the binding membrane where the small ones escape through the membrane to the transfer buffer before the larger ones are transferred and bound to the membrane (34). Another major drawback is the limited capacity of nitrocellulose and other similar membranes (19, 31, 34, 35). Lefevre et al. (32) reported improved transfer efficiency of HDL on charge-modified nylon membranes, but their improved method suffers from drawbacks. A major problem was the use of glutaraldehyde for fixation of transferred apolipoproteins on the nylon membrane before the immunoblotting step. Glutaraldehyde acts as a crosslinking agent where it might crosslink lysine residues of the apolipoproteins and causes intermolecular and/or intramolecular linking. This linking may affect the antigenic properties of the apolipoproteins and, consequently, may affect the quantitative binding of the antibodies to the sample lipoproteins. Indeed, Lefevre et al. (32) noticed that the amount of labeled antibody bound to the transfer media was not directly proportional to the amount of apolipoprotein present; moreover, they reported large differences from lot to lot in the overall transfer efficiency of the charge-modified nylon membranes. Another drawback is the incubation of the nylon membrane transfers with calf serum (10%)

overnight; this treatment may cause an exchange of the transferred apolipoproteins with those present in the calf serum. These and other drawbacks were discussed in detail in the work of Atmeh and Abuharfeil (19) where they successfully overcame many of the drawbacks by using agarose gel matrix containing specific antibodies as a transferring medium. The method was proved to be quantitative, reproducible, sensitive, and can be used for the study of protein aggregates in complex protein mixtures such as plasma.

In this study we used agarose gel containing anti-apoA-I as a transfer medium for the detection and quantitation of apoA-I distribution on lipoprotein particles in fresh plasma with minimal manipulations. Agarose in itself is not as efficient in binding proteins as NC, but the incorporation of specific antibodies in its matrix during preparation offers an invaluable medium for capturing proteins specifically within the gel matrix during their electrotransfer. The advantage of using agarose gel is that the IgG fraction of the antibody moves slightly towards the cathode in agarose under the running conditions of alkaline pH (19). Binding of lipoproteins with NC and other membranes is weaker than that with our agarose matrix because the binding with these membranes is only electrostatic and therefore relatively reversible (34). Indeed, up to ten copies of the protein pattern were obtained by transfer on several layers of NC (19, 34, 35) while no lipoproteins escaped, during electrotransfer, through the first into a second layer of agarose gel. The present method includes neither the use of glutaraldehyde or other crosslinking agents nor the treatment of the transfer medium with protein blocking agents.

When fresh plasma from normolipidemic subjects was analyzed by the present method using anti-apoA-I, several bands of molecular weights ranging from 43,000 to 800,000 could be identified in some subjects, while in other subjects some bands were not well identified. In this work we considered two main regions for study, one region included particles of molecular weights from 43,000 to 50,000 (SHDL), and the other region (50,000–800,000) included the rest of the apoA-I-containing particles. If these small HDL subclasses contain apoA-I as the sole protein, then they are expected to comprise one apoA-I molecule per particle and the minimum protein percentage in the fractions would be in the range of 57–66%. This high protein content is similar to that in very high density lipoprotein (VHDL) particles reported by Vezina et al. (31). The presence of small HDL subfractions was reported in normal subjects (1, 2, 31, 36–38), in hyperlipoproteinemic patients (33), and in other pathologic conditions (23, 33, 37, 39–44).

The quantitative aspect of the present method is evident where 92.0% of the radioactivity present in the

gPAG slice containing HDL was recovered in the agarose gel after electrotransfer, compared to 5.8% recovery in the case of NC. Moreover, the coefficient of variation of 9.1% (n = 8) for the SHDL particles in plasma denotes the reproducibility of the method. In agreement with that, all the transferred lipoproteins were captured by the agarose gel where nothing escaped to a second layer of agarose after electrotransfer and nothing remained in the gPAG after its staining. A good correlation exists between the present method and that of directly stained gPAG (Fig. 2).

The detection of these SHDL particles in fresh plasma is not expected to be an artifact as the fresh plasma was subjected to the electrophoretic separation within a short time of collection and with minimum manipulation, and the proportion of these particles differed from subject to subject. Physiological relevance of small HDL particles was reported in different conditions (37, 45) and many factors were found to disturb their plasma level (8, 14, 37, 46–51).

In order to study the physiological relevance of these small particles: 1) The distribution of apoA-I in the plasma of hyperlipidemic patients was compared with that of normolipidemic subjects and was statistically significantly higher (Table 2). 2) The normolipidemic subjects were separated into two groups according to alcohol consumption and the distribution of apoA-I in the plasma of the patients was compared with each group of the normal subjects, separately. Statistically significant differences remained and the difference as well as the level of significance were greater with the nonalcoholic group (normal I, Table 2). In accordance with that, many workers (1, 33, 47) reported differences in the small molecular weight fractions between hyperlipidemic and normolipidemic subjects. When the distribution of apoA-I in the plasma of both groups of normal subjects was compared, there were statistically significant higher values of the SHDL in the members of the second group who consumed alcohol moderately (Table 2). These differences are in accordance with the known effect of alcohol where it induces the synthesis of nascent HDL of small molecular weight (52), increases the synthesis of apoA-I (53), and may lower the CETP activity (54). 3) Eight normolipidemic subjects were given a fat load and the apoA-I distribution was found to be disturbed postprandially. These results indicate that the SHDL particles are not artifacts and they are physiological entities.

When the data obtained in this study were analyzed and correlated we found that the percentage of apoA-I in SHDL was 7.1% ± 1.2, 17.9% ± 1.2, and 23.7% ± 1.7 (mean ± SEM; in nonalcoholic normals, normals who drank moderately, and hyperlipidemic patients, respectively). A positive correlation was observed between the percentage of apoA-I in SHDL and plasma triglycerides in all the subjects ($r = 0.41$, $P < 0.05$, n = 36) and in the hyperlipidemic subjects ($r = 0.43$, $P < 0.05$, n = 12). In accordance with that, Schonfeld, Bailey, and Steelman (1) found that a subfraction with molecular weight of 50,000 comprised 5–24% of plasma apoA-I in hyperlipidemic patients and its level was strongly positively correlated with plasma triglycerides level. An interesting strong positive correlation existed between apoA-I content of SHDL and HDL-cholesterol in the group of normals who consumed moderate amounts of alcohol ($r = 0.70$, $P < 0.05$) and in the hyperlipidemic subjects ($r = 0.79$, $P < 0.05$); but a negative correlation was found in the nonalcoholic normal subjects ($r = -0.69$, $P < 0.05$). This positive correlation between SHDL and HDL-cholesterol suggests that the observed increase in HDL-cholesterol associated with alcohol intake (52, 55–57) can be mainly ascribed to the increase in the SHDL subfraction. Similarly, the increase in plasma apoA-I in some cases of hyperlipidemia (1, 33) can be related to the increase in the SHDL.

In the light of the reported results (1, 33, 52, 55–57) and our findings that the proportion of the SHDL fraction is low and inversely correlated with HDL-cholesterol in nonalcoholic normals and the higher proportion and positive correlation in the moderate drinkers and hyperlipidemic subjects, the validity of the well-accepted statement that "HDL is protective against coronary artery disease" can be questioned. In support of that, Hirano et al. (54) reported cases of alcohol drinkers who had hyperalphalipoproteinemia associated with corneal opacities and coronary heart disease. Moreover, Williams et al. (58) and Syvanne et al. (59) have shown that high levels of HDL$_{3b}$ were associated with coronary heart disease risk factors in both men and women, and Johansson et al. (48) reported a positive association between HDL$_{3b}$ and progression of coronary atherosclerosis. It seems reasonable to further investigate which of the various HDL subfractions may have a "protective effect" from those that may not, or, unexpectedly, may be atherogenic.

The present work offers a new and rapid method for the quantitative studies of the HDL subfractions distribution in different physiologic and pathologic conditions. Fresh plasma samples are analyzed directly with few steps and minimal manipulations.█

REFERENCES

1. Schonfeld, G., A. Bailey, and R. Steelman. 1978. Plasma apolipoprotein A-I and A-II levels in hyperlipidemia. *Lipids.* **13:** 951–959.
2. Atmeh, R. F. 1990. Isolation and identification of HDL particles of low molecular weight. *J. Lipid Res.* **31:** 1771–1780.
3. Albers, J. J., and F. Aladjem. 1971. Precipitation of ^{125}I-labeled lipoproteins with specific polypeptide antisera. Evidence for two populations with differing polypeptide compositions in human high density lipoproteins. *Biochemistry.* **10:** 3436–3442.
4. Suenram, A., W. J. McConathy, and P. Alaupovic. 1979. Evidence for the lipoprotein heterogeneity of human plasma HDL isolated by three different procedures. *Lipids.* **14:** 505–510.
5. Patsch, W., G. Schonfeld, A. M. Gotto, Jr., and J. R. Patsch. 1980. Characterization of human high density lipoproteins by zonal ultracentrifugation. *J. Biol. Chem.* **255:** 3178–3185.
6. Norfeldt, P-I. P., O. S. Olofsson, G. Fager, and G. Bondjers. 1981. Isolation and partial characterization of the lipoprotein families A and A-I from high density lipoproteins of human serum. *Eur. J. Biochem.* **18:** 1–8.
7. Blanche, P. J., E. L. Gong, T. M. Forte, and A. V. Nichols. 1981. Characterization of human high density lipoproteins by gradient gel electrophoresis. *Biochim. Biophys. Acta.* **665:** 408–419.
8. Atmeh, R. F., J. Shepherd, and C. J. Packard. 1983. Subpopulations of apolipoprotein A-I in human high-density lipoproteins, their metabolic properties and response to drug therapy. *Biochim. Biophys. Acta.* **751:** 175–188.
9. Leroy, A., K. L. H. Toohill, J-C. Fruchart, and A. Jonas. 1993. Structural properties of high density lipoprotein subclasses homogeneous in protein composition. *J. Biol. Chem.* **268:** 4798–4805.
10. Rader, D. J., G. Castro, A. Zech, J-C. Fruchart, and H. B. Brewer, Jr. 1991. In vivo metabolism of apolipoprotein A-I on high density lipoprotein particles LpA-I and LpA-I, A-II. *J. Lipid Res.* **32:** 1849–1859.
11. Fielding, C. J., and P. E. Fielding. 1981. Evidence for a lipoprotein carrier in human plasma catalyzing sterol efflux from cultured fibroblasts and its relationship to lecithin:cholesterol acyltransferase. *Proc. Natl. Acad. Sci. USA.* **78:** 3911–3914.
12. Fielding, P. E., and C. J. Fielding. 1980. A cholesteryl ester transfer complex in human plasma. *Proc. Natl. Acad. Sci. USA.* **77:** 3327–3330.
13. Barbaras, R., P. Puchois, J-C. Fruchart, and G. Ailhaud. 1987. Cholesterol efflux from cultured adipose cells mediated by LpA-I particles but not by LpA-I:A-II particles. *Biochem. Biophys. Res. Commun.* **1420:** 63–69.
14. Ohta, T., Y. Ikeda, R. Nakamura, and I. Matsuda. 1992. Lipoprotein-containing apolipoprotein A-I: sex-related quantitative and qualitative changes in this lipoprotein subspecies after ingestion of fat. *Am. J. Clin. Nutr.* **56:** 404–409.
15. James, R. W., D. Hochstrasser, J-D. Tissot, M. Funk, R. Appel, F. Barja, C. Pellegrini, A. F. Muller, and D. Pometta. 1988. Protein heterogeneity of lipoprotein particles containing apolipoprotein A-I without apolipoprotein A-II and apolipoprotein A-I with apolipoprotein A-II isolated from human plasma. *J. Lipid Res.* **29:** 1557–1571.
16. Nichols, A. V., P. J. Blanche, R. M. Krauss, and E. L. Gong. 1979. Gradient gel electrophoresis of HDL within the ultracentrifugal d < 1.200 fraction from human serum. Report of the HDL Methodology Workshop, San Francisco (K. Lippel, editor) NIH publication 79-1661: 303–309.
17. Barter, P. J., and W. E. Connor. 1976. The transport of esterified cholesterol in plasma high density lipoproteins of human subjects: a mathematical model. *J. Lab. Clin. Med.* **88:** 627–639.
18. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
19. Atmeh, R. F., and N. Abuharfeil. 1993. Electroblotting of proteins on agarose gel containing specific antibodies. *Electrophoresis.* **14:** 69–72.
20. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. Metabolism of low density lipoproteins. *Biochim. Biophys. Acta.* **260:** 212–221.
21. Abuharfeil, N. M., R. F. Atmeh, M. N. Abo-Shehada, and S. N. El-Sukhon. 1991. Detection of proteins after immunoblotting on nitrocellulose using fluorescent antibodies. *Electrophoresis.* **12:** 683–684.
22. Dory, L., L. M. Boquet, R. L. Hamilton, C. H. Sloop, and P. S. Roheim. 1985. Heterogeneity of dog interstitial fluid (peripheral lymph) high density lipoproteins: implications for a role in inverse cholesterol transport. *J. Lipid Res.* **26:** 519–527.
23. Deckelbaum, R. J., S. Eisenberg, Y. Oschry, M. Cooper, and C. Blum. 1982. Abnormal high density lipoproteins of abetalipoproteinemia: relevance to normal HDL metabolism. *J. Lipid Res.* **23:** 1274–1282.
24. Ohta, T., S. Hattori, S. Nishiyama, and I. Matsuda. 1988. Studies on the lipid and apolipoprotein compositions of two species of apoA-I-containing lipoproteins in normolipidemic males and females. *J. Lipid Res.* **29:** 721–728.
25. Lundberg, B. B. 1988. Incorporation of cholesterol into lipoprotein A-I-dimyristoylphosphatidylcholine recombinants. *Biochim. Biophys. Acta.* **962:** 265–274.
26. McCall, M. R., T. M. Forte, and V. G. Shore. 1988. Heterogeneity of nascent high density lipoproteins secreted by the hepatoma-derived cell line, HepG2. *J. Lipid Res.* **29:** 1127–1137.
27. Vessby, B., S. Gustafson, M. J. Chapman, K. Hellsing, and H. Lithell. 1987. Lipoprotein composition of human suction-blister interstitial fluid. *J. Lipid Res.* **28:** 629–641.
28. Reichl, D., D. N. Rudra, N. B. Myant, and J. J. Pflug. 1982. Further evidence for the role of high density lipoprotein in the removal of tissue cholesterol in vivo. *Atherosclerosis.* **44:** 73–84.
29. Rudra, D. N., N. B. Myant, J. J. Pflug, and D. Reichl. 1984. The distribution of cholesterol and apoprotein A-I between the lipoproteins in plasma and peripheral lymph from normal human subjects. *Atherosclerosis.* **53:** 297–308.
30. Castro, G. R., and C. J. Fielding. 1988. Early incorporation of cell-derived cholesterol into pre-β-migrating high density lipoprotein. *Biochemistry.* **27:** 25–29.
31. Vezina, C. A., R. W. Milne, P. K. Weech, and Y. L. Marcel. 1988. Apolipoprotein distribution in human lipoproteins separated by polyacrylamide gradient gel electrophoresis. *J. Lipid Res.* **29:** 573–585.

32. Lefevre, M., J. C. Goudey-Lefevre, and P. S. Roheim. 1987. Gradient gel electrophoresis-immunoblot analysis (GGEI): a sensitive method for apolipoprotein profile determinations. *J. Lipid Res.* **28:** 1495–1507.

33. Ishida, B. Y., J. Frolich, and C. J. Fielding. 1987. Prebeta-migrating high density lipoprotein: quantitation in normal and hyperlipidemic plasma by solid phase radioimmunoassay following electrophoretic transfer. *J. Lipid Res.* **28:** 778–786.

34. Andrews, A. T. 1986. Electrophoresis: Theory, Techniques, and Biochemical and Clinical Applications. 2nd ed. Clarendon Press, Oxford. 59–74.

35. Manabe, T., and T. Okuyama. 1984. Multiple replica technique and quantitation of human serum proteins on the immunoreplicas. *In* Electrophoresis '84, VCH. V. Neuhoff, editor. Weinheim. 59–74.

36. Forte, T., K. R. Norum, J. A. Glomset, and A. V. Nichols. 1971. Plasma lipoproteins in familial lecithin:cholesterol acyltransferase deficiency: structure of low and high density lipoproteins as revealed by electron microscopy. *J. Clin. Invest.* **50:** 1141–1148.

37. Chen, C., K. Applegate, W. C. King, J. A. Glomset, K. R. Norum, and E. Gong. 1984. A study of the small spherical high density lipoproteins of patients afflicted with familial lecithin:cholesterol acyltransferase deficiency. *J. Lipid Res.* **25:** 269–282.

38. Cheung, M. C., J. P. Segrest, J. J. Albers, J. T. Cone, C. G. Brouillette, B. H. Chung, M. Kashyap, M. A. Glasscock, and G. M. Anantharamaiah. 1987. Characterization of high density lipoprotein subspecies: structural studies by single vertical spin ultracentrifugation and immunoaffinity chromatography. *J. Lipid Res.* **28:** 913–929.

39. Norum, K. R., J. A. Glomset, A. V. Nichols, and T. Forte. 1971. Plasma lipoproteins in familial lecithin:cholesterol acyltransferase deficiency: physical and chemical studies of low and high density lipoproteins. *J. Clin. Invest.* **50:** 1131–1140.

40. Solaas, M. H. 1974. Structural studies on serum lipoproteins in homozygotes and heterozygotes for the lecithin:cholesterol acyltransferase deficiency gene. *Scand. J. Clin. Lab. Invest.* **33 Suppl 137:** 133–134.

41. Soutar, A. K., B. L. Knight, and N. B. Myant. 1982. The characterization of lipoproteins in the high density fraction obtained from patients with familial lecithin:cholesterol acyltransferase deficiency and their interaction with cultured human fibroblasts. *J. Lipid Res.* **23:** 380–390.

42. Assman, G., P. H. Herbert, D. S. Fredrickson, and T. Forte. 1977. Isolation and characterization of an abnormal high density lipoprotein in Tangier disease. *J. Clin. Invest.* **60:** 242–252.

43. Carlson, A. 1982. Fish eye disease: a new familial condition with massive corneal opacities and dyslipoproteinaemia: clinical and laboratory studies in two afflicted families. *Eur. J. Clin. Invest.* **12:** 41–53.

44. Cheung, M. C., A. V. Nichols, P. J. Blanche, E. L. Gong, G. Franceschini, and C. R. Sirtori. 1988. Characterization of A-I-containing lipoproteins in subjects with A-I Milano variant. *Biochim. Biophys. Acta.* **960:** 73–82.

45. Benvengas, S. 1989. The 28-kilodalton thyroxine (T₄)-binding protein is human apolipoprotein A-I: identification of a 68-kilodalton high density lipoprotein that binds T₄. *Endocrinology.* **124:** 1265–1269.

46. Bagdade, J. D., D. Kaufman, M. C. Ritter, and P. V. Subbaiah. 1990. Probucol treatment in hypercholesterolemic patients: effect on lipoprotein composition, HDL particle size, and cholesteryl ester transfer. *Arteriosclerosis.* **84:** 145–154.

47. Dobiasova, M., J. Stribrna, D. L. Sparks, P. H. Pritchard, and J. J. Frohlich. 1991. Cholesterol esterification rates in very low density lipoprotein- and low density lipoprotein-depleted plasma. *Arterioscler. Thromb.* **11:** 64–70.

48. Johansson, J., A. Carlson, C. Landou, and A. Hamsten. 1991. High density lipoproteins and coronary atherosclerosis: a strong inverse relation with the largest particles is confined to normotriglyceridemic patients. *Arterioscler. Thromb.* **11:** 174–182.

49. Cheung, M. C., B. Gregg-Brown, A. C. Wolf, and J. J. Albers. 1991. Altered particle size distribution of apolipoprotein A-I-containing lipoproteins in subjects with coronary artery disease. *J. Lipid Res.* **32:** 383–394.

50. Weidman, S. W., J. B. Ragland, and S. M. Sabesin. 1982. Plasma lipoprotein composition in alcoholic hepatitis: accumulation of apolipoprotein E-rich high density lipoprotein and preferential reappearance of "light" HDL during partial recovery. *J. Lipid Res.* **23:** 556–569.

51. Clifton, P. M., P. J. Barter, and A. M. Mackinnon. 1988. High density lipoprotein particle size distribution in subjects with obstructive jaundice. *J. Lipid Res.* **29:** 121–135.

52. Valimaki, M., M. R. Taskinen, R. Ylikahri, R. Roine, T. Kuusi, and E. A. Nikkila. 1988. Comparison of the effects of two different doses of alcohol on serum lipoproteins, HDL-subfractions and apolipoproteins A-I and A-II: a controlled study. *Eur. J. Clin. Invest.* **18:** 472–480.

53. Bertiere, M. C., D. Betoulle, M. Apfelbaum, and A. Girard-Globa. 1986. Time course, magnitude and nature of the changes induced in HDL by moderate alcohol intake in young non-drinking males. *Atherosclerosis.* **61:** 7–14.

54. Hirano, K-L., Y. Matsuzawa, N. Sakai, H. Hiraoka, S. Nozaki, S. Yamashita, M. Kubo, and S. Tarui. 1992. Polydisperse low-density lipoproteins in hyperalphalipoproteinemic chronic alcohol drinkers in association with marked reduction in cholesteryl ester transfer protein activity. *Metabolism.* **41:** 1313–1318.

55. Hully, S. B., and S. Gordon. 1981. Alcohol and high-density lipoprotein cholesterol. Causal inference from diverse study designs. *Circulation.* **64 Suppl. III:** 57–63.

56. Castelli, W. P., T. Gordon, M. C. Hjortland, A. Kagan, J. T. Doyle, C. G. Hames, S. B. Hully, and W. J. Zukel. 1977. Alcohol and blood lipids. The cooperative lipoprotein phenotyping study. *Lancet.* **i:** 153–155.

57. Barboriak, J. J., A. J. Anderson, and R. G. Hoffmann. 1979. Interrelationship between coronary artery occlusion, high density lipoprotein cholesterol, and alcohol intake. *J. Lab. Clin. Med.* **94:** 348–353.

58. Williams, P. T., R. M. Krauss, K. M. Vranizan, M. L. Stefanick, P. D. Wood, and F. T. Lindgren. 1992. Associations of lipoproteins and apolipoproteins with gradient gel electrophoresis estimates of high density lipoprotein subclasses in men and women. *Arterioscler. Thromb.* **12:** 332–340.

59. Syvanne, M., M. Ahola, S. Lahdenpera, J. Kahri, T. Kuusi, K. S. Virtanen, and M-R. Taskinen. 1995. High density lipoprotein subfractions in non-insulin-dependent diabetes mellitus and coronary artery disease. *J. Lipid Res.* **36:** 573–582.