papers and notes on methodology

Gradient gel electrophoresis–immunoblot analysis (GGEI): a sensitive method for apolipoprotein profile determinations

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Abstract A method is described which will determine the distribution of individual apolipoproteins within the HDL subclasses. This method requires 1–2 μl of plasma per determination and involves six steps: *1*) electrophoresis of samples on non-denaturing 2–30% concave acrylamide gradient gels; *2*) electrophoretic transfer of the lipoproteins to charge-modified nylon membranes; *3*) fixation of the transferred lipoproteins with glutaraldehyde; *4*) immunolocalization of the apolipoproteins with iodinated monospecific antibodies; *5*) autoradiography followed by densitometry; and *6*) reduction of the data to provide a plot of percent distribution versus particle size. When this method was applied to the analysis of rat apolipoproteins, differences were noted in the distribution of apoA-I, apoA-IV, and apoE. The majority of apoA-I was localized to HDL particles between 9 and 12 nm in diameter, with a median diameter of 10.0 nm, while apoE resided on substantially larger particles with a median diameter of 12.5 nm. ApoA-IV could be localized to three distinct areas: an HDL particle with a median diameter approximately 0.4 nm larger than apoA-I HDL, a particle smaller than albumin (lipoprotein-free apoA-IV), and a particle of 7.6 nm that does not appear to contain apoA-I or apoE.—**Lefevre, M., J. C. Goudey-Lefevre, and P. S. Roheim.** Gradient gel electrophoresis–immunoblot analysis (GGEI): a sensitive method for apolipoprotein profile determinations. *J. Lipid Res.* 1987. **28:** 1495–1507.

Supplementary key words high density lipoprotein • rat apolipoprotein • non-denaturing gradient gel electrophoresis • protein blotting

With the recognition that apolipoproteins are important determinants of lipoprotein metabolism (reviewed in 1, 2), considerable effort has been directed towards the determination of apolipoprotein profiles. Apolipoprotein profile can be defined as the mass distribution of a given apolipoprotein among the different lipoprotein density classes.

Traditionally, apolipoprotein profile determinations have been made following sequential ultracentrifugation to isolate the lipoprotein classes. However, ultracentrifugation has significant disadvantages. The procedure typically requires large sample amounts, minimally 1–2 ml of plasma, and there is significant sample manipulation associated with the density adjustments. Perhaps most importantly, the method itself introduces artifacts in apolipoprotein profiles. Certain apolipoproteins, particularly apoE, apoA-I, and apoA-IV, can be removed from the surface of the lipoproteins and appear artifactually in the lipoprotein-free fraction (3–6). Consequently, other methods have been developed to determine apolipoprotein profiles.

Gel filtration chromatography is a commonly used alternative method for apolipoprotein profile determinations (3, 7). When applied to whole plasma, apolipoprotein profiles, free of artifacts, can be obtained. Gel filtration chromatography, however, still requires relatively large sample sizes. In addition, the final separated sample is diluted approximately tenfold. Finally, because of the requirements for post-separation assays and chromatographic equipment, only a limited number of samples can be processed at one time.

A method that has gained considerable popularity in the last 5 years is that of non-denaturing gradient gel electrophoresis (8, 9). This method provides excellent resolu-

Abreviations: HDL, high density lipoprotein; HDL, low density lipoprotein; VLDL, very low density lipoprotein; apo, apolipoprotein; GGEI, gradient gel electrophoresis–immunoblot; CS, calf serum; PBS, phosphate-buffered saline; PBST, PBS with 0.05% Tween 20; CMNM, charge-modified nylon membranes.

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tion of lipoprotein subclasses. However, the lipoproteins must first be isolated before application to the gel. Furthermore, only a total lipoprotein protein profile can be determined, not the profiles of the individual apolipoproteins. In some recent studies (10, 11) non-denaturing gradient gel electrophoresis has been coupled with protein blotting methodology to provide qualitative assessments of apolipoprotein distribution.

In this report, we describe a method based upon non-denaturing gradient gel electrophoresis which provides the apolipoprotein distribution using small amounts (2–25 μl) of plasma (not ultracentrifuged). This method, which we have designated as gradient gel electrophoresis–immunoblot analysis (GGEI) employs six steps: *1*) electrophoresis of samples on non-denaturing polyacrylamide gradient gels; *2*) electrophoretic transfer of the lipoproteins to a blotting membrane; *3*) fixation of the transferred lipoproteins with glutaraldehyde; *4*) immunolocalization of the apolipoproteins with iodinated monospecific antibodies; *5*) autoradiography followed by densitometry; and *6*) reduction of data to provide a plot of percent distribution versus lipoprotein size.

METHODS

Plasma and lipoprotein preparation

Blood was obtained from both adult humans and male rats (300–350 g) after an overnight fast. Blood was collected into tubes containing disodium EDTA (1 mg/ml) and immediately centrifuged (1500 *g*) at 4°C. Following centrifugation, sodium azide was added to plasma at a final concentration of 0.01%. Plasma lipoproteins ($d < 1.21$ g/ml) were isolated by ultracentrifugation in a Beckman SW-40 rotor (12).

In selected studies, plasma lipoproteins were isolated by ultracentrifugation in a Beckman Airfuge employing an A-100 rotor. In these studies, 100 μl of plasma adjusted to a density of 1.25 g/ml was placed in each tube and overlayed with 75 μl of d 1.21 g/ml KBr solution. The plasma was centrifuged for 4 hr at 165,000 *g*. Following centrifugation, the top 100 μl containing between 85–90% of the total plasma cholesterol was carefully aspirated.

Lipoproteins were iodinated by the iodine monochloride method (13) as modified by Bilheimer, Eisenberg, and Levy (14). Labeled human lipoproteins had a specific activity of 20 cpm/ng of protein of which less than 1% of the ^{125}I was TCA-soluble and 15% was extractable by chloroform–methanol (2:1). Labeled rat lipoproteins had a specific activity of 40 cpm/ng of which less than 1% of the ^{125}I was TCA-soluble and 17% was extractable by chloroform–methanol.

Antisera and antibodies

Rat apoA-I, E, and A-IV were isolated from a total lipoprotein fraction using a combination of gel filtration chromatography and heparin affinity chromatography (15). Antisera to these apolipoproteins were produced in goats as previously described (16). The antisera were further purified by affinity chromatography against immobilized antigens. All antibody preparations were monospecific as judged by immunoblots against both total lipoproteins and plasma.

Affinity-purified rabbit anti-goat IgG (Cooper Biomedical, Inc.) was iodinated by the chloramine-T method (17). The iodinated IgG had a specific activity of 5–10 $\times 10^6$ cpm/μg protein.

Non-denaturing gradient gel electrophoresis

In initial studies, lipoproteins or whole plasma were subjected to non-denaturing gradient gel electrophoresis using Pharmacia PAA 4/30 precast gels as described by Blanche et al. (9). As documented in the Results section, these gels were found to be unsuitable for our studies because of poor transfer efficiency. In subsequent experiments, we used gradient gels of our own making.

Gradient gels (2.7 × 80 × 140 mm) were cast in a Pharmacia GSC-8 gel slab casting apparatus. The protocol and solution used for casting the gels were similar to those described by Pharmacia (18) except that TEMED (115 μl/100 ml) was substituted for 3-dimethylaminopropionitrile and the amount of ammonium persulphate was halved to prevent premature polymerization. Gels were poured with a concave gradient of 2 to 30% acrylamide. This was accomplished by mixing two parts of 2% acrylamide with one part 30% acrylamide in a modified gradient former (Bio-Rad model 395) in which one chamber had been vertically partitioned to provide equal fluid heights. Gels were allowed to polymerize for at least 3 hr and were used within 72 hr.

Sample preparation and electrophoresis conditions were similar to those described by Blanche et al. (9) and by Pharmacia (18) with the following exceptions. To compensate for the longer gel format, electrophoresis was carried out at 220 V instead of 125 V. The gels were pre-electrophoresed for 1 hr instead of 15 min to remove traces of persulfate. Electrophoresis was stopped at 5000 V • hr (after approximately 24 hr). At this point, the migration of the individual lipoproteins relative to the high molecular weight standards (Pharmacia) approached zero allowing for calculation of lipoprotein size.

Electrophoretic transfer

Upon completion of electrophoresis, the gel cassette assembly was carefully disassembled and a narrow strip of gel containing the protein standard lane was removed and

stained with Coomassie R-250. The remainder of the gel was soaked for a total of 1 hr in three changes of transfer buffer (20 mM Tris, 150 mM glycine, pH 8.4).

Electrophoretic transfer of proteins was carried out essentially as described by Burnette (19) with modifications as noted in the Results section. In initial studies, proteins were transferred to 0.2 μm nitrocellulose (S&S #BA83). In subsequent studies, charged-modified nylon membranes (Zetaprobe, Bio-Rad) were used in place of nitrocellulose because of the higher protein-binding capacity and more efficient retention of transferred proteins (see Results). Electrophoretic transfer proceeded for 70–90 (V/cm • hr (30 V for 24 hr) at 10°C. The transfers were placed between two sheets of filter paper, wrapped in plastic, and stored at 4°C for up to 6 months.

Fixing, blocking, and immunolocalization

The transfer media were cut into 7-mm wide strips and placed into 15 × 150 mm screw-cap tubes. To stabilize the lipoprotein–nylon membrane complex, the transfers were incubated in phosphate-buffered saline (PBS) containing 0.03% glutaraldehyde for 1 hr at room temperature with constant agitation (rotation on a tube rotator). Following fixation, the transfers were rinsed twice with PBS. Residual protein binding sites on the nylon membrane transfers were blocked by overnight incubation at 50°C in PBS containing 10% calf serum (CS), 3% BSA, 0.05% Tween 20, and 0.04% sodium azide. This blocking agent gave the lowest overall background of those tested (10% BSA only, 10% CS only, or 10% CS with 3% gelatin) and could be reused several times.

Apolipoproteins were immunolocalized by incubation with appropriate affinity purified primary antibody diluted (1:200–1:1000) in PBS with 0.05% Tween 20 (PBST) containing 10% CS. Incubations were for 1 hr with constant agitation. The transfers were then washed five times for 10 min each in PBST. The transfers were subsequently incubated with the iodinated secondary antibody reagent (10^6 cpm ^{125}I-labeled IgG/tube diluted in PBST, 10% CS) for 1 one hr followed by five washes. The transfers were autoradiographed by overnight exposure to X-ray film (Kodak XRP-5) at −70°C with the use of intensifying screens (Dupont Cronex).

Quantitation and normalization of apolipoprotein distribution

Quantitation of the distribution of the bound ^{125}I-labeled secondary antibody was accomplished by either direct counting of 2-mm sections of the transfer strip or by densitometric scanning of the autoradiogram with a Bio-Rad Video Densitometer (Model 620) interfaced to a microcomputer (IBM AT). Preliminary experiments indicated that the amount of iodinated secondary antibody bound to the transfer media was not directly proportional to the amount of apolipoprotein present. To circumvent this problem, a standard curve was constructed as described below.

Rat d < 1.21 g/ml lipoproteins were incorporated into cooled (56°C) molten 2% agarose at varying concentrations and the agarose was poured into 100 mm × 100 mm × 1.5 mm templates. After solidification, the agarose sheets with the incorporated rat lipoproteins were cut into 10 × 100 mm long strips and the lipoproteins were transferred to charge-modified nylon membranes as described for the gradient gels. The concentration of the rat lipoproteins in the agarose was varied such that when transferred, the concentration of lipoproteins would range from 0.1 to 10 μg of protein/cm^2 of blotting membrane. Following transfer, the agarose strips were removed and the transfer media were cut into a series of 7-mm wide strips, with each standard strip containing transfers of lipoproteins of varying concentrations.

By including a standard strip containing various known concentrations of lipoproteins during the immunolocalization procedure, the counts or absorbance of a given region on a test strip could be directly related to relative apolipoprotein concentration. **Fig. 1** demonstrates that the relationship between relative absorbance and lipoprotein concentration (in terms of μg of protein/cm ^{2} transfer membrane) was reproducible. Also shown is the fact that the working range for these standards extended over approximately a tenfold change in apolipoprotein concentration.

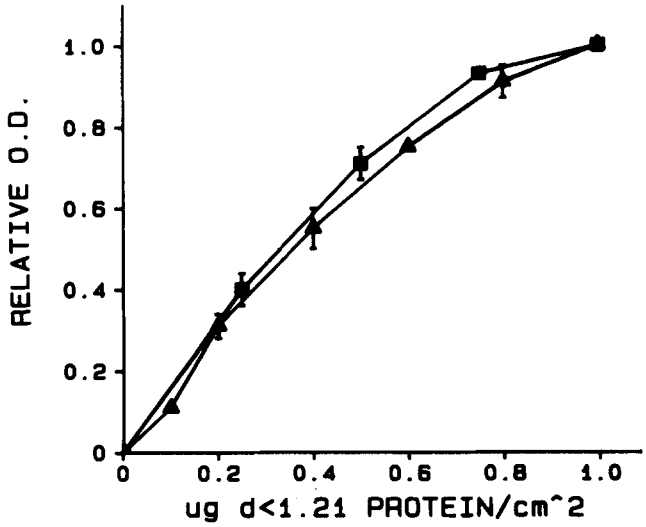

Fig. 1. Representative standard curves used for relating absorbance to relative apolipoprotein concentration. Two different preparations of standard curves (■, ▲) were prepared as described in the text from a single pool of rat d < 1.21 g/ml lipoproteins. Each strip was immunodecorated for rat apoA-I as described in the text. Standard curves were generated on separate days ranging over a 1-year period. Data are expressed relative to the absorbance obtained at 1 μg of protein/cm^2 transfer membrane so that differences in ^{125}I-labeled IgG specific activity, autoradiograph exposure time, etc., could be normalized.

By comparison of lipoprotein R_f with the R_f of the protein size standards, the data were subsequently reduced to provide a report of percent distribution as a function of particle size.

RESULTS

Selection of non-denaturing gradient gel/electrophoretic transfer system

Our initial studies were designed to determine conditions that would provide maximal transfer and recovery of lipoproteins separated by non-denaturing gradient gels. Studies with the pre-cast gradient gels (Pharmacia PAA 4/30) demonstrated their unsuitability for this method. Using the procedure described by Burnette (19) (transfer buffer: 20 mM Tris, 150 mM glycine, and 20% methanol; pH 8.4), we were able to recover on the nitrocellulose membrane only 35.8% of an iodinated lipoprotein preparation applied to the gradient gel (% bound) after 48 hr of transfer **(Table 1,** Exp. #1; **Fig. 2)**.

In subsequent studies with pre-cast gels, we varied transfer conditions in an attempt to improve transfer and recovery of the lipoproteins (Table 1). We eliminated the use of methanol in the transfer buffer, since this has been reported to inhibit transfer. By changing transfer media from nitrocellulose to charge-modified nylon membranes (CMNM), we were able to increase our percent capture (percent of lipoproteins transferred out of the gel that were captured by the membrane) by 20%. Therefore, in subsequent experiments (Table 1, Exps. #2-5), CMNM was used exclusively.

Because the percentage of lipoproteins transferred out of the gradient gels (% transfer) was low, we investigated the use of various detergents to facilitate transfer (Table 1, Exp. #2-4). In general, the detergents failed to significantly improve transfer efficiency and/or decreased capture efficiency, resulting in little improvement in the percentage of lipoproteins bound to the blotting membrane.

Failing to obtain acceptable results with the precast gels, we investigated the possibility of casting our own gradient gels. Based upon preliminary experiments, we employed an 8 × 14 cm gel with a 2-30% concave acrylamide gradient. These gels allowed us to size not only HDL subclasses, but LDL subclasses as well **(Fig. 3)**.

TABLE 1. Summary of transfer experiments with precast and homemade gels

Experiment	Transfer Conditions	% Transfer[a]	% Capture[b]	% Bound[c]
1	Precast gel transferred according to Burnette (19) (20 mM Tris, 150 mM glycine, 20% methanol; pH 8.4) 48 hr transfer onto nitrocellulose.	57.0	62.8	35.8
2a	As in Exp. #1 except methanol was omitted from the transfer buffer and the blotting membrane was changed to CMNM.	43.9	76.3	33.5
2b	2a + cathodal filter paper was soaked with 1.0% SDS in transfer buffer.	73.8	27.2	20.1
3a	As in Exp. #2a.	51.3	84.0	43.1
3b	3a + gels equilibrated with transfer buffer for 30 min.	43.1	77.6	33.4
3c	3a + gels equilibrated with transfer buffer + 0.1% SDS for 30 min.	48.8	86.2	42.1
3d	3a + gels equilibrated with transfer buffer + 1.0% SDS for 30 min.	45.9	54.4	25.0
4a	As in Exp. #2a + gels equilibrated with transfer buffer 3 × 20 min.	48.5	93.4	45.2
4b	4a + gels equilibrated with transfer buffer + 1.0% NP-40 for 3 × 20 min.	67.5	85.1	56.9
4c	4a + gels equilibrated with transfer buffer + 1.0% Tween 20 for 3 × 20 min.	30.7	79.8	24.9
4d	4a + gels equilibrated with transfer buffer + 1.0% Triton X-100 for 3 × 20 min.	58.8	89.2	52.5
5	As in Exp. #2a except: *1*) homemade gels were used instead of precast gels; *2*) gels were equilibrated with transfer buffer 3 × 20 min; and *3*) gels were transferred for only 24 hr (mean ± SD of six transfers).	87.2 ± 4.1	96.9 ± 3.1	84.4 ± 2.3

^{125}I-labeled rat (Exp. 1-4) or rat and human (Exp. 5) lipoproteins were mixed with carrier homologous plasma (ca. 20,000 cpm ^{125}I-labeled Lp + 1 μl of plasma per lane) and subjected to non-denaturing gradient gel electrophoresis as described. The cassettes were disassembled and one lane was removed and counted to determine the ^{125}I-labeled Lp content of the gel before transfer. Approximately 90% of the counts applied to the gel could be recovered in the gel following electrophoresis. Lipoproteins were transferred as described by Burnette (19) with modifications to the procedure as outlined in the table. Upon completion of the transfer, the gel and the blotting membrane were removed and counted separately.

[a] % Transfer, 100 × (cpm in gel before transfer − cpm in gel at end of transfer) ÷ cpm in gel before transfer.

[b] % Capture, 100 × (% bound ÷ % transfer).

[c] % Bound, 100 × (cpm on blotting membrane at end of transfer ÷ cpm in gel before transfer).

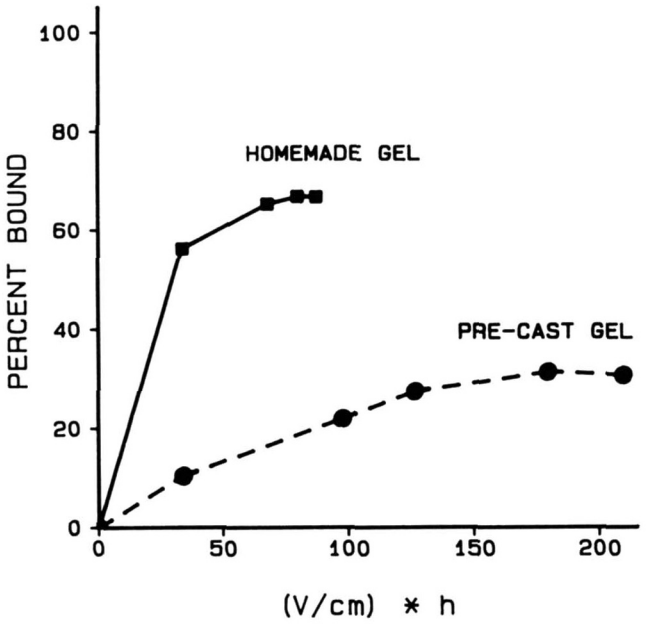

Fig. 2. Rate of lipoprotein transfer to blotting membranes. ^{125}I-labeled d < 1.21 g/ml rat lipoproteins were mixed with carrier homologous plasma (ca. 20,000 cpm of ^{125}I-labeled Lp + 1 μl of plasma per lane, six lanes per gel) and subjected to non-denaturing gradient gel electrophoresis on pre-cast gels (3000 V • hr) or homemade gels (5000 V • hr). The cassettes were disassembled and one lane was removed and counted to determine the ^{125}I-labeled Lp content of the gel before transfer. The remainder of the gel was transferred as described in Table 1, Exp. #1 for the pre-cast gels and Exp. #2a for the homemade gels. At varying times, the transfer was stopped and the transfer assembly was removed and disassembled. One lane of the gel was carefully removed along with its corresponding section of blotting membrane and then counted. The transfer assembly was then reassembled and the transfer was continued. Data are plotted as the percent of ^{125}I-labeled Lp bound (100 × cpm on blotting membrane ÷ cpm in gel before transfer) versus length of transfer (field strength × time).

Preliminary studies with the homemade gradient gels were encouraging. With these gels, transfer and subsequent binding of the lipoproteins to the membranes were significantly increased (Fig. 2). Furthermore, transfer time could be cut to 24 hr. There was little potential for the lipoprotein to transfer through the membrane with extended transfer times. By equilibrating the gels in transfer buffer prior to transfer (3 × 20 min), we further improved our transfer efficiency. Over 80% of the total lipoprotein radioactivity applied to the gel was recovered on the membrane after 24 hr of transfer (Table 1, Exp. #5).

To determine whether all the lipoproteins transferred with equal efficiency, the CMNM was cut into 1-cm segments after transfer and the distribution of ^{125}I-labeled lipoproteins on the CMNM was compared to the distribution in the gel prior to transfer **(Fig. 4)**. It can be seen that recovery of ^{125}I-labeled VLDL (segment 0–1 cm) and ^{125}I-labeled LDL (segment 1–3 cm) on the blotting membrane was less than it was for ^{125}I-labeled HDL which was almost completely transfered to the CMNM. In **Table 2**, the recoveries of the individual lipoprotein fractions are given. Recoveries for ^{125}I-labeled VLDL and ^{125}I-labeled LDL were approximately 20% and 50%, respectively, while recoveries for the ^{125}I-labeled HDL fractions all exceeded 90%.

In the region of lipoprotein-free apoA-IV and albumin, recoveries were in the range of 85%. Since albumin was a minor component in the preparation (see Fig. 3) and since albumin saturates the protein binding sites on the transfer membrane (see below), this value is likely to be a slight underestimate of the true recovery for lipoprotein-free apoA-IV.

Selection of immunolocalization protocol

Since the lipoproteins are transferred under relatively non-denaturing conditions, the potential existed for the transferred lipoproteins to retain possible antigenic differences. Furthermore, there also existed the possibility that the transferred apolipoproteins might exchange with apolipoproteins present in the calf serum used for blocking. To guard against this, we investigated the use of a post-transfer treatment of the CMNM with glutaraldehyde followed by detergent treatment. The results of this study are shown in **Table 3**.

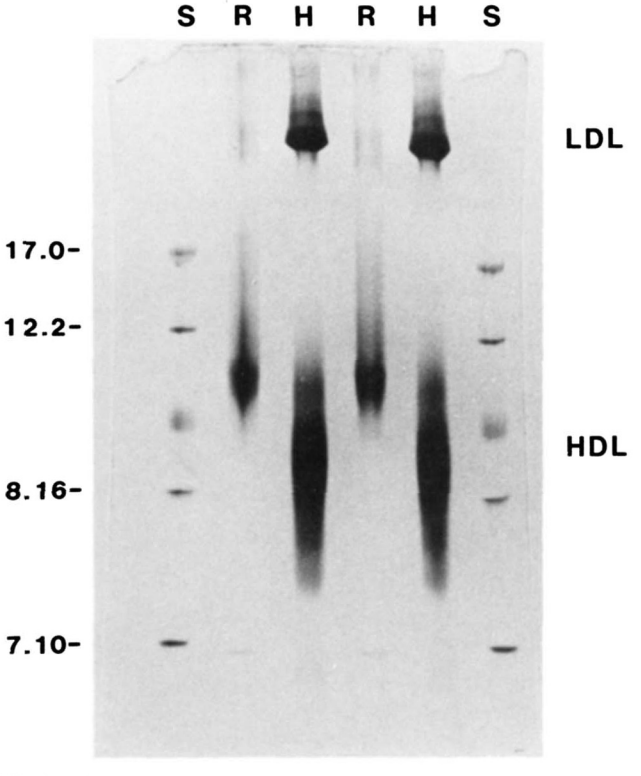

Fig. 3. Coomassie staining of d < 1.21 g/ml human (H) and rat (R) lipoproteins separated on a homemade 2–30% concave acrylamide gradient gel. Lipoproteins were electrophoresed under non-denaturing conditions for 5000 V • hr. Size standards are thyroglobulin, 17.0 nm; ferritin, 12.2 nm, catalase, not used; lactate dehydrogenase, 8.16 nm; and bovine serum albumin, 7.10 nm.

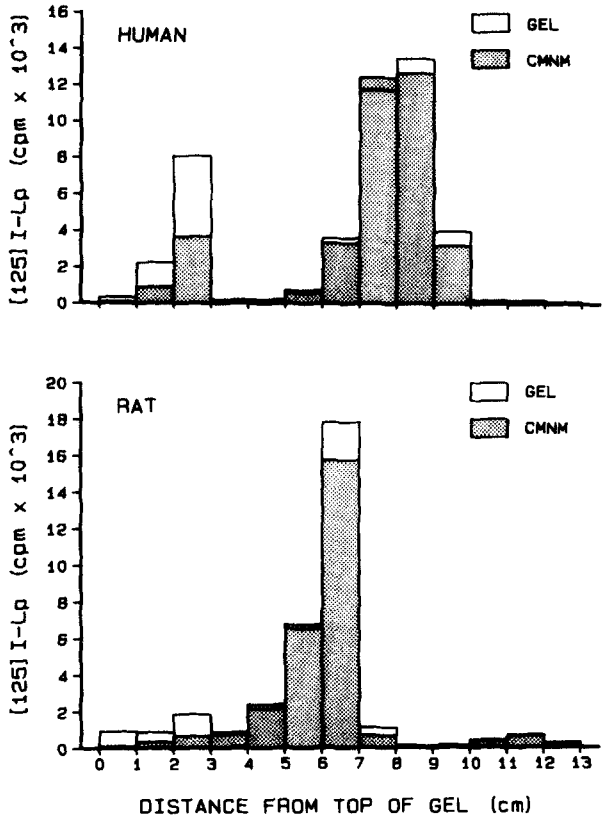

Fig. 4. Comparison between lipoprotein distribution on blotting membrane after transfer and the distribution in the original gel. ^{125}I-labeled rat and human lipoproteins were electrophoresed on homemade gradient gels and transferred as described in Table 1, Exp. #5. Upon completion of the transfer, the blotting membrane was segmented into serial 1-cm sections and counted. The distribution of the ^{125}I-labeled Lp on the blotting membrane (shaded bars) is superimposed over the distribution obtained in the original, untransferred gel (open bars). Data are the average of three experiments each for human and rat lipoproteins.

Overnight incubation of CMNM in blocking reagent as opposed to PBS increased the loss of ^{125}I-labeled lipoproteins from 3.6% to 6.7%. Pretreating the CMNM with glutaraldehyde reduced this loss to 4.9%. When the CMNM was treated with detergents without prior fixation, over 50% of the ^{125}I-labeled lipoproteins was lost from the membrane. Prior fixation with glutaraldehyde significantly reduced this loss.

In preliminary studies with rat plasma using polyclonal antibodies, we were unable to detect differences between detergent-treated and non-detergent-treated transfers. Therefore, in the following studies, all the transfers were fixed with glutaraldehyde but were not detergent-treated.

Following fixation, the transfers were blocked overnight. After investigating a number of blocking agents and protocols, we found that a combination of 3% bovine serum albumin, 10% calf serum, 0.05% Tween 20, 0.04% sodium azide in PBS gave us the lowest overall background and the best peak to background ratio.

Application to rat plasma

Rat plasma (10 μl) was layered across the top of a homemade gel, electrophoresed, and transferred as described above. The transfers were subsequently cut into 7-mm wide strips, fixed, and the apolipoproteins were immunolocalized. **Fig. 5** shows a typical autoradiograph, and **Fig. 6** shows the corresponding plot of apolipoprotein distribution. **Fig. 7** shows a summary of rat apolipoprotein distribution obtained from a study involving nine rats.

Several points can be made concerning the distribution of rat apolipoproteins. It can be seen that the distributions of HDL-associated apoA-I, apoA-IV, and apoE differ. Rat apoA-I is distributed on smaller HDL particles (median diameter, 10.0 ± 0.1 nm) than either apoA-IV (median diameter, 10.4 ± 0.2 nm) or apoE (median diameter, 12.5 ± 0.3 nm). Approximately 30% of plasma apoA-IV can be found on two particles with diameters smaller than 8 nm. The larger particle has an apparent diameter of 7.60 ± 0.03 nm, is not associated with either apoA-I or apoE, and accounts for between 2 and 5% of total plasma apoA-IV. The smaller apoA-IV particle is smaller than albumin and presumably is lipoprotein-free apoA-IV.

TABLE 2. Recoveries of lipoprotein fractions on CMNM following transfer

Lipoprotein Region	Gel Region	N (Species)	% Bound
	cm		
VLDL	0–1	6 (R + H)	19.7 ± 9.4
LDL	1–3	6 (R + H)	50.6 ± 6.6
HDL_1	3–5	3 (R)	95.3 ± 8.1
HDL_2	5–7	6 (R + H)	93.9 ± 7.3
HDL_3	7–10	3 (H)	96.5 ± 3.5
Free A-IV and albumin	10–12	3 (R)	86.2 ± 3.0

^{125}I-labeled lipoproteins isolated from humans (H) and rats (R) were electrophoresed, transferred, and segmented as described in Fig. 3. Lipoprotein regions were identified by their apparent sizes. Values given under gel regions correspond to the boundaries used in segmenting the gels. Values for % bound are mean ± SD of three analyses each of human and rat lipoprotein regions except where insufficient counts were present in a given region for a species (i.e., human HDL_1).

TABLE 3. Effect of post-transfer treatment on the stability of lipoproteins on CMNM

Treatment	18 hr with PBS	18 hr with Blocking Reagent[a]			
	No Detergent	PBS	NP-40	TX-100	SDS
		% ^{125}I-labeled Lp lost			
No fixation (PBS)	3.6	6.7	52.0	51.9	84.3
0.03% Glutaraldehyde	2.4	4.9	6.0	6.6	22.9

Five μl of ^{125}I-labeled human lipoproteins were carefully spotted on a 2-cm^2 strip of CMNM. The strip was then incubated with agitation with either 0.03% glutaraldehyde or PBS for 1 hr. One set of strips was incubated at 50°C overnight with PBS. Following a 1-hr treatment with either PBS or detergent (1.0% in PBS), the remaining strips were subsequently incubated at 50°C overnight with blocking reagent, rinsed, and counted. The data are expressed as percent of counts lost from the blotting membrane relative to the untreated controls.

[a] Blocking reagent, 10% calf serum, 3% BSA, 0.05% Tween 20, and 0.04% sodium azide in PBS; PBS, phosphate-buffered saline; NP-40, Nonidet P40; TX-100, Triton X-100; SDS, sodium dodecyl sulfate.

A nadir of immunoreactivity is evident in the small HDL region of all rat apolipoproteins. The nadir coincides with the location of a major plasma protein in the rat (Fig. 5) and most likely is the result of competition for protein binding sites on the CMNM. Attempts to circumvent this competition by decreasing the plasma load were not completely successful.

Because of this competition problem, we investigated using brief ultracentrifugation in conjunction with the gradient gel electrophoresis–immunoblot analysis method. One hundred μl of rat plasma was ultracentrifuged at d 1.21 g/ml for 4 hr in a Beckman Airfuge to isolate a total lipoprotein fraction. The lipoprotein fraction was dialyzed, electrophoresed, and blotted. The results are shown in **Fig. 8**. In the d < 1.21 g/ml fraction, the problems with plasma protein competition were eliminated. However, the distribution of apolipoproteins in the d < 1.21 g/ml fraction and the original plasma, while similar, was not identical.

In particular, a population of small apoA-I particles present in the original plasma was missing in the d < 1.21 g/ml fraction. The distribution of apoE in the d < 1.21 g/ml fraction was less well defined than in the original plasma with apoE-containing particles extending over a wider size range. Brief ultracentrifugation may also disrupt apoA-IV-containing particles, causing apoA-IV to dissociate from HDL during electrophoresis. In addition, complexes of apoA-I in association with apoA-IV and/or apoE could be found in the d > 1.21 g/ml fraction. All these changes in apolipoprotein distribution appeared to persist after recombination of the d < 1.21 g/ml fraction and the d > 1.21 g/ml fraction.

Reproducibility

To test the reproducibility of the GGEI method, a single sample of rat plasma was applied to three different batches of gels produced at different times. Each batch of gels was electrophoresed and transferred under identical conditions. The resulting transfers from each batch of gels were sliced into five strips and carried through the immunolocalization procedure for rat apoA-IV as described. ApoA-IV was chosen because its distribution is more

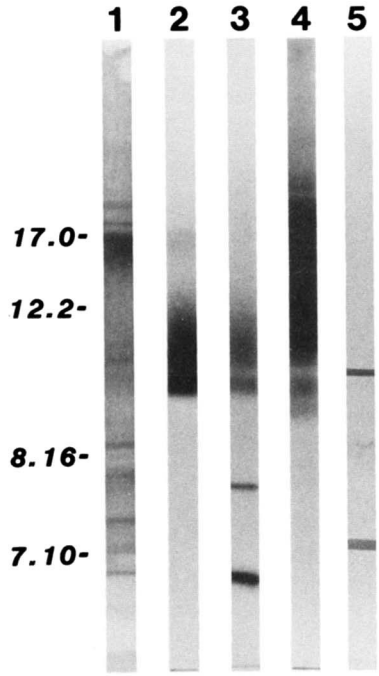

Fig. 5. Distribution of rat apolipoproteins as determined by gradient gel electrophoresis–immunoblot analysis. Rat plasma (10 μl) was layered across the top of a homemade gradient gel and electrophoresed and transferred as described. The blotting membrane was sliced into 7-mm wide strips and the apolipoproteins were immunolocalized using affinity-purified goat antibodies followed by ^{125}I-labeled rabbit anti-goat IgG. The distribution of ^{125}I-labeled rabbit anti-goat IgG was visualized by autoradiography. Lane 1, protein staining of transfer membrane; lane 2, immunolocalization of apoA-I; lane 3, immunolocalization of apoA-IV; lane 4, immunolocalization of apoE; lane 5, protein staining of reverse side of transfer membrane showing saturation by albumin (bottom band) and unidentified protein in the size range of rat HDL. Numbers at left indicate migration and Stoke's diameters of protein size standards.

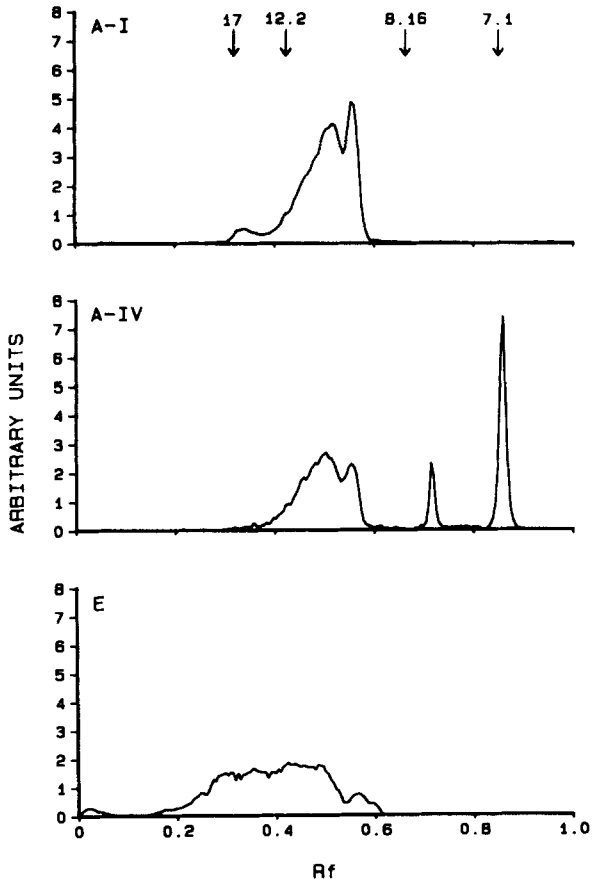

Fig. 6. Normalized distribution of rat apolipoproteins. Following immunolocalization, the autoradiograph was scanned and the distribution of the apolipoprotein was normalized as described in the text, providing a plot of arbitrary units versus *Rf*. Numbers at top indicate migration and Stoke's diameters of protein size standards.

complex than either apoA-I or apoE. The immunolocalization procedures for each batch of gels were conducted on different days. The percent distribution of apoA-IV as a function of particle size was calculated and the data were blocked into 1.0-nm intervals for comparisons of intra- and interassay variation. The data are presented in **Table 4**.

The intra- and interassay coefficients of variation for percent apoA-IV distribution varied as a function of particle size. For particles smaller than 13 nm, the intra- and interassay coefficients of variation averaged 7% and 12%, respectively. At larger particle diameters, where less than 8% of the apoA-IV was localized, the coefficients of variation were considerably larger. The intra- and interassay coefficients of variation for apoA-IV median and modal particle diameter were on the order of 1%.

To determine the effect of the amount of plasma applied to the gels on the resulting apolipoprotein profiles, gels were loaded with either 7.5 μl or 15 μl of plasma. The resulting apoA-IV profiles were quite similar **(Fig. 9)**. With the lower plasma load, there was, however, a relative inability to detect and quantify the small percentage of the larger apoA-IV-containing particles (>13 nm). As a result, median particle size was shifted to a smaller diameter (from 10.38 ± 0.04 nm to 10.23 ± 0.63 nm) while modal diameter was unchanged.

DISCUSSION

In this paper, we describe a method for determining HDL apolipoprotein profiles in whole unultracentrifuged plasma. This method takes advantage of the high resolution of non-denaturing gradient gels and couples it with protein-blotting techniques. Our aim in developing this method was to provide as near a quantitative assessment of HDL apolipoprotein distribution as possible. To achieve this goal, several obstacles had to be overcome. The major difficulty in the development of the GGEI method was the selection of a proper transfer protocol that would provide near quantitative recovery of HDL

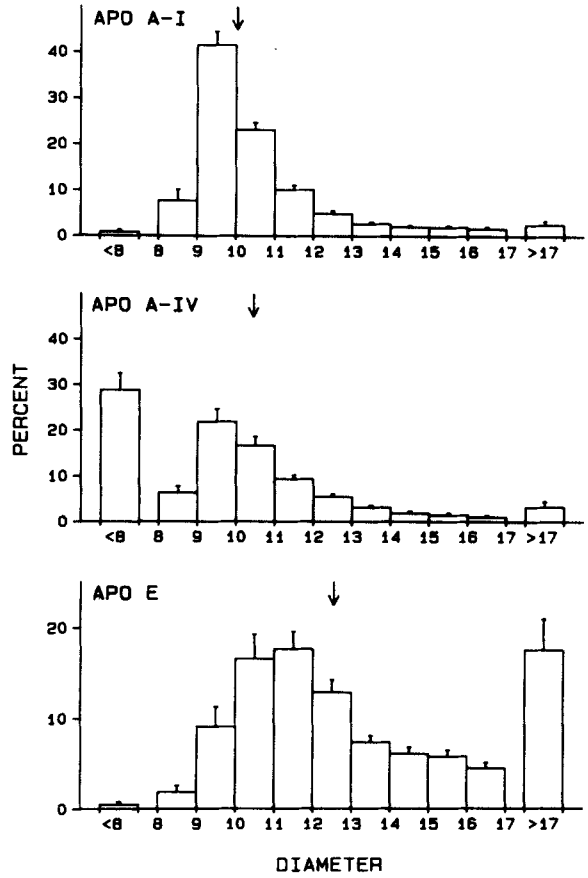

Fig. 7. Summary of rat apolipoprotein distribution. Data represent the average distribution of rat apoA-I, apoA-IV, and apoE from nine experiments. For apoA-IV distribution, data for lipoprotein-free apoA-IV and its dimer (7.6 nm particle; see Discussion) have been combined in the "<8 nm" group. Arrows at top show median lipoprotein diameter. (For apoA-IV, Lp-free apoA-IV and its dimer were not included in the calculation.) Data are mean ± SEM.

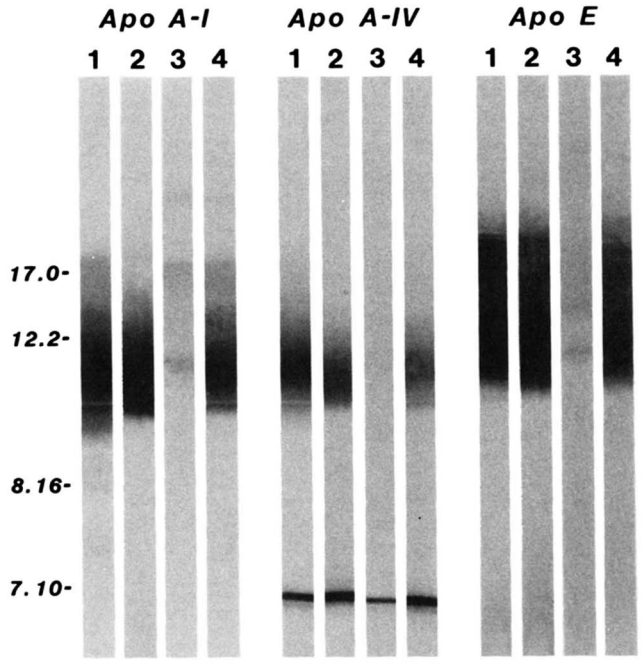

Fig. 8. Effect of brief ultracentrifugation on rat apolipoprotein distribution. One hundred μl of rat plasma was ultracentrifuged at d 1.21 g/mL for 4 hr in a Beckman Airfuge. The distributions of apoA-I, apoA-IV, and apoE are compared before and after ultracentrifugation. Lane 1, original plasma; lane 2, d < 1.21 g/ml fraction; lane 3, d > 1.21 g/ml fraction; lane 4, combined d < 1.21 and d > 1.21 g/ml fractions. All ultracentrifuged samples were loaded at amounts equivalent to that of plasma. Numbers at left indicate migration and Stoke's diameters of protein size standards.

apolipoproteins on the blotting media. We had originally hoped to use pre-cast, commercially available gradient gels because of their high quality and uniformity. However, after considerable experimentation, we found that these gels were unsuitable for our application. The reasons behind the poor transfer efficiency associated with the pre-cast gels are not fully understood. This may be due to gel ageing or to the use of proprietary crosslinkers and/or catalysts in their production.

In contrast to the pre-cast gels, we found that homemade gels of similar thickness and porosity behaved much more efficiently in the transfer process. With the aid of a modified gradient former, we could easily pour eight 2–30% acrylamide gels with a concave gradient within an hour. Each gel within a given batch of eight gels gave virtually identical apolipoprotein profiles. However, when using gels obtained from different batches, it was necessary to express the apolipoprotein profile relative to the migration of the protein size standards (i.e., convert R_f to apparent diameter) before comparisons could be made. It was concluded, therefore, that the problems with nonuniformity associated with the homemade gels were minimal and were far outweighed by the improvement in transfer efficiency.

Once transferred to the membrane, we wanted to be assured that the lipoprotein remained bound to the transfer media during the subsequent quenching and immunolocalization steps. Furthermore, since it was envisioned that detergent treatment might be necessary to express all epitopes equally during the immunolocalization step, we wanted to be sure that the lipoprotein-transfer media complexes would also survive this step. Relatively minor losses of ^{125}I-labeled lipoproteins occurred when the strips were incubated with the blocking agent alone, but the addition of a detergent treatment step resulted in an over 50% loss of the bound lipoproteins. By prior treatment of the strips with glutaraldehyde, the loss of bound lipoproteins during the subsequent steps was greatly reduced. Additional studies indicated that detergent treatment of the strips did not significantly alter the apolipoprotein profile obtained with rat plasma, and, therefore, the detergent treatment step was not routinely included in the procedure. However, since even a small loss of apolipoproteins may significantly influence the resulting profile, especially if confined to a population of highly exchangeable apolipoproteins (i.e., C apoproteins), the glutaraldehyde step was retained.

The final obstacle to overcome was the conversion of the final autoradiographic image into a report detailing the percent apolipoprotein distribution as a function of size. It was recognized that the relationship between the amount of apolipoprotein transferred to the media and the associated absorbance on the autoradiogram was curvilinear. It was therefore necessary to construct a standard curve to relate relative apolipoprotein mass with absorbance. This was achieved by incorporating varying concentrations of d < 1.21 g/ml lipoproteins into 2% agarose and then electrophoretically transferring the lipoproteins to the CMNM. The resulting sheet was then cut up into individual standard lanes. By including a standard lane along with the samples during the immunolocalization protocol, a standard curve could be constructed relating

TABLE 4. Analysis of error for apoA-IV distribution

Parameter	Mean	Intra-Assay CV[a]	Inter-Assay CV
	%	%	%
Lipoprotein region[b]			
Lp-free apoA-IV	27.4	8.0	9.2
9–10 nm	12.3	4.3	15.9
10–11 nm	29.4	4.1	8.1
11–12 nm	16.7	7.1	10.5
12–13 nm	7.0	11.4	14.4
13–14 nm	3.7	27.2	28.9
> 14 nm	3.5	54.3	94.3
	nm		
Modal size	10.39	0.5	1.1
Median size	10.37	0.7	0.8

[a] CV, coefficient of variation.
[b] In this plasma preparation, no apoA-IV-containing particles were found in the 8–9 nm region. Values for Lp-free apoA-IV include contribution of the 7.6 nm apoA-IV dimer (See Discussion).

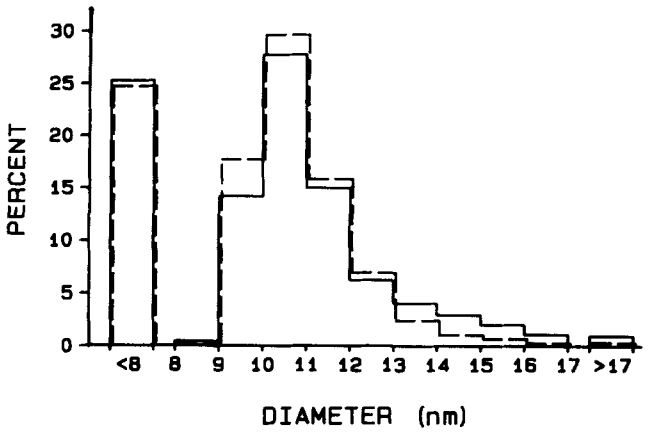

Fig. 9. Effect of different plasma loads on apolipoprotein profiles. Two gels were loaded with either 7.5 (-----) or 15.0 (——) μl of plasma/gel and electrophoresed as described. Following electrophoretic transfer and fixation, the transfers were probed for apoA-IV. The resulting autoradiographs were scanned and the data were reduced to provide plots of percent apoA-IV distribution as a function of particle size at 1-nm intervals.

relative apolipoprotein concentration to absorbance and, thus, allow for an accurate assessment of apolipoprotein distribution.

As evidenced by repeated analysis of a single plasma sample, the apolipoprotein profiles obtained by the GGEI method were found to be reasonably reproducible. Within the size range that includes approximately 95% of the apoA-IV distribution, the intra- and interassay coefficient of variations averaged 7% and 12%, respectively. These values are approximately twice as high as can be expected for typical apolipoprotein assays. Similar coefficients of variation were obtained during analysis of human plasma apoA-I distribution (unpublished observation).

We felt that the analysis of the distribution of apoA-IV would be the most stringent test of our method for reproducibility, since Lp-free apoA-IV would have the highest local concentration of any apolipoprotein and thus would put the greatest demands on our ability to quantitate its relative contribution. Within the range of 5–25 μl of plasma/gel, the percent of apoA-IV appearing as Lp-free apoA-IV remained constant, while at higher plasma loads (50 μl/gel), the optical absorbance of the autoradiogram in the region of Lp-free apoA-IV exceeded the useful portion of the standard curve.

In our study, a twofold change in plasma loads (7.5 vs. 15 μl/gel) resulted in minor changes in apoA-IV distribution. This was characterized by a relative decrease in the amount of apoA-IV-containing particles migrating in the region of 13–17 nm at the lower plasma load. Wider variations (>twofold) in apolipoprotein loads could therefore lead to potential problems in analysis. The standard curves generated have a working range of approximately a tenfold change in apolipoprotein concentration. In a separate study, we found that by decreasing the amount of plasma applied to a gel by fivefold, significant portions of the apolipoprotein profile were below the detection limits of our system. Consequently, at the large and smaller boundaries of our size profiles, where the concentrations of the individual apolipoproteins were low, there was a substantial loss of immunoreactive material. The net result was a narrowing of the apolipoprotein profiles to a more restricted range of sizes. Thus, for accurate comparisons of apolipoprotein profiles, the amounts of apolipoprotein applied to the gels should be similar and probably should not exceed a twofold difference.

Given that each strip generates a minimum of 100 data points, that calculations are required to correct for nonlinearity in autoradiographic response, and that each R_f must be converted to apparent diameter for comparisons between experiments, it can be seen that several hundred calculations are required to process a single profile. This issue has been overcome in our laboratory through the development of specialized software that will perform these calculations unattended.[2] Directly interfacing the densitometer to the microcomputer so that the digitized scan can be stored on disk further decreases the time required to process a profile.

In analyzing the distribution of rat plasma apolipoprotein distribution by GGEI, we noted a competition for binding on the transfer media between lipoproteins and the remaining plasma proteins. The rat presents a unique situation, since its plasma is particularly abundant in a single protein that migrates in the size range of rat HDL_2. Similarly sized proteins in human plasma are much less abundant and do not cause problems in the analysis of human apolipoprotein distribution.

Attempts to circumvent the competition problem by prior isolation of the lipoproteins with brief ultracentrifugation in a Beckman Airfuge provided mixed results. While the resulting apolipoprotein profiles were free from the effects of competing proteins, the distributions of the apolipoproteins were not identical to those found in original plasma. ApoA-I was lost from a population of small HDL particles, and the amount of apoA-IV present as lipoprotein-free apoA-IV increased. While changes in the distributions of apoA-I and apoA-IV due to ultracentrifugation have been reported by others (4, 5), it should be pointed out that in our study the duration of ultracentrifugation was only 4 hr. Thus, even brief ultracentrifugation can lead to a distortion in apolipoprotein distribution.

Interestingly, apoA-I and apoE that appeared in the $d > 1.21$ g/ml fractions were not found as free apolipoproteins but as complexes with each other and with apoA-IV. One study has reported the isolation of an apoA-IV–apoA-I lipoprotein complex from the $d > 1.21$ g/ml fraction of human plasma (20). Whether this complex is, in fact, a unique, naturally occurring lipoprotein particle or an artifact of ultracentrifugation remains to be unequivocally es-

[2]A copy of the data reduction program is available upon request.

tablished. Other methods of lipoprotein isolation, including heparin affinity and immunoaffinity-chromatography, are currently being explored in conjunction with the GGEI method.

Potential problems with the GGEI method may arise from the generation of artifacts during the initial electrophoresis in the non-denaturing gradient gel. Using methods similar to those described here, Bisgaier et al. (11) suggested that apoA-IV could dissociate from HDL during the initial electrophoresis. We have also found that rat apoA-IV appears to dissociate from isolated HDL samples during electrophoresis (Fig. 8 and Lefevre, M., et al., unpublished results). Whether this is due to an inherent instability in the association of apoA-IV with isolated HDL or due to electrophoretic stripping of apoA-IV from HDL cannot as yet be answered. In preliminary studies using iodinated lipoproteins, we were unable to demonstrate any differences in the distribution of the lipoproteins (including free apoproteins) when the initial electrophoresis was allowed to proceed at 500V as opposed to 220V or allowed to continue for 8000 V • hr as opposed to 5000 V • hr. Moreover, the value obtained for the percentage of rat apoA-IV appearing in the lipoprotein-free fraction is comparable to that found by column chromatography (21) and considerably less than that found by conventional ultracentrifugation. Finally, in the rat, we could not find any evidence for either apoA-I or apoE to become dissociated from the lipoproteins during electrophoresis. Thus, any electrophoretic "stripping" appears to be minimal and may be confined to apoA-IV.

When analyzed by non-denaturing gradient gel electrophoresis and stained for protein, at most two populations of rat HDL can be visualized (Fig. 3 and references 22, 23). The majority of protein staining material migrates within a narrow size range between 9 and 12 nm; a second population of larger-sized HDL is variably present in normal rats but can be greatly increased by dietary manipulations (22, 23). Analysis of rat HDL by heparin affinity chromatography has shown that the apoE-rich HDL subfractions are larger in size than the apoE-poor HDL subfractions (23, 24). Similar observations in the rat have been made using gel filtration chromatography (25) and rate zonal ultracentrifugation (26).

Data obtained by GGEI analysis of rat plasma confirm and extend these previous findings. Approximately 80% of rat apoA-I was associated with particles with diameters between 9 and 12 nm. The median diameter for these apoA-I-containing particles was 10.0 nm. In contrast, apoE was localized on considerably larger particles with over 50% of the apoE associated with particles greater than 12 nm in diameter. Thus, the size distributions of rat apoA-I and apoE, as determined by GGEI, are in keeping with the size ranges previously reported for rat HDL_2 and HDL_1, respectively (26).

ApoA-IV had a more complex distribution. ApoA-IV could be localized to two discrete regions with apparent diameters smaller than rat HDL. The smallest of these particles accounted for approximately 25% of plasma apoA-IV, was smaller than albumin, and is assumed to be lipoprotein-free apoA-IV. A second, larger particle, with an apparent diameter of 7.6 nm, accounted for between 2 and 5% of plasma apoA-IV and did not comigrate with either apoA-I or apoE. Thus, this particle is distinct from a similarly sized apoA-I-containing apoA-IV particle found in rat mesenteric lymph (27), and may, in fact, be an apoA-IV dimer or oligomer (28). That this 7.6-nm band is a dimer would be consistent with the observation that simple dilution of rat plasma resulted in a decrease in the appearance of the 7.6-nm apoA-IV band with a concomitant increase in the amount of lipoprotein-free apoA-IV (Lefevre, M., et al, unpublished observation).

In addition to the lipoprotein-free forms of apoA-IV, a substantial percentage of rat apoA-IV was localized in the HDL size range. On the average, HDL-associated apoA-IV resided on particles that were 0.4 nm larger (median diameter) than the apoA-I-containing HDL. This is in agreement with data provided by Dallinga-Thie, Van 't Hooft, and Van Tol (29), who demonstrated by gel filtration chromatography that apoA-IV was distributed on particles larger than those containing apoA-I. The larger size of the apoA-IV particles is also consistent with the findings of DeLamatre et al. (21), which suggested that apoA-IV preferentially binds to larger, LCAT-modified HDL particles.

While the GGEI method can tell us whether two apolipoproteins are localized to the same sized lipoproteins, it cannot tell us whether the two apolipoproteins are, in fact, on the same particle. Thus, based on our GGEI data alone, we cannot be certain whether the apoA-IV-containing HDL also contain apoA-I or apoE. However, preliminary studies in our laboratory using immunoaffinity-isolated apoA-I, apoA-IV, and apoE-containing particles have provided evidence for complexes of apoA-I and apoA-IV, complexes of apoA-I and apoE, and complexes of apoA-IV and apoE, as well as for particles containing apoA-I but devoid of both apoA-IV and apoE (Lefevre, M., et al., unpublished observation). Unlike human HDL, which can be resolved by nondenaturing gradient gel electrophoresis into relatively discrete subclasses (9, 30), these complexes of rat HDL appear to be present as a rather continuous spectrum of particles differing in size and apolipoprotein composition. The functional significance of these HDL complexes is currently under investigation.

In conclusion, we feel that GGEI analysis is a potentially powerful method for the determination of apolipoprotein profiles. Its strengths include: *1*) its sensitivity, requiring less than 25 μl of plasma for up to ten apolipoprotein pro-

files; *2*) its resolving power: based on the well-established non-denaturing gradient gel method, it can discriminate several HDL subclasses as well as lipoprotein-free apolipoproteins; *3*) its ability to handle large numbers of samples: using standard well formers, up to forty individual apolipoprotein profiles can be determined simultaneously; and *4*) its ability to use non-ultracentrifuged biological material: extensive sample manipulation and artifacts introduced by ultracentrifugation are therefore eliminated. We find that the GGEI method is particularly suited for applications where limited sample is available or where the sample is present as a dilute solution. We believe that it has the potential to become an important tool in the study of lipoprotein metabolism.

Note Added in Proof: We have recently discovered that some individual lots of charge-modified nylon membranes were unsuitable for use because of low overall transfer efficiencies. We therefore recommend that each lot of membranes be tested prior to use.

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