

# Sodium dodecyl sulfate-glycerol polyacrylamide slab gel electrophoresis for the resolution of apolipoproteins

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**Summary** We describe the resolution of the plasma apolipoproteins with molecular weights from 8,800 to greater than 550,000, using a 3.5% sodium dodecyl sulfate-glycerol polyacrylamide slab gel system. The simplicity of this system and the resolution of proteins over a broad range of molecular weights will make it particularly useful in investigations of apolipoprotein composition of plasma lipoproteins.—**Maguire, G. F., M. Lee, and P. W. Connelly.** Sodium dodecyl sulfate-glycerol polyacrylamide slab gel electrophoresis for the resolution of apolipoproteins. *J. Lipid Res.* 1989. 30: 757-761.

**Supplementary key words** apo[a] • apoB-100 • apoB-48 • apoE • apoA-I • apoA-II • apoC-III • apoC-II • chylomicrons • lipoproteins

Investigation of the apolipoproteins of the plasma lipoproteins presents a unique analytical challenge. The apolipoproteins span a broad range of molecular weights and isoelectric points (1) and exhibit very different solubilities in aqueous buffers or mixtures of aqueous buffers and organic solvents (2). The apolipoproteins can be separated by sodium dodecyl sulfate (NaDodSO<sub>4</sub>) polyacrylamide electrophoresis. This currently is accomplished using two different systems, one of a low percentage of acrylamide to resolve the B apolipoproteins (3, 4) and a second of a high percentage of acrylamide to resolve the remaining apolipoproteins (5, 6), or the use of gradient gels (7, 8). The B apolipoproteins have been separated using either tube gels (3) or agarose-polyacrylamide slab gels (4). We developed the method of NaDodSO<sub>4</sub>-glycerol polyacrylamide gel electrophoresis for the resolution of apolipoproteins with molecular weights between 6,000 and 80,000 (5). We now report the application of this system to the separation of apolipoproteins with molecular weights from 8,000 to greater than 550,000. Optimal resolution can be achieved with the use of a 3.5% acrylamide, 18% glycerol vertical slab gel. The gel is also suitable for immunoblot experiments.

## METHODS

### Materials and reagents

Electrophoresis grade acrylamide, N,N-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), NaDodSO<sub>4</sub>, dithiothreitol (DTT), and ammonium persulfate

were obtained from Bio-Rad Laboratories. Glycerol (Analar Grade) was obtained from BDH. Pyronin-Y, bovine serum albumin (BSA),  $\beta$ -galactosidase ( $\beta$ -GAL), and fructose-6-phosphate kinase (F6P) were obtained from Sigma Chemical Co. Coomassie Brilliant Blue G-250 was obtained from Kodak.

### Sample preparation

Blood was collected into tubes containing solid Na<sub>2</sub>EDTA as anticoagulant and kept on wet ice. Plasma was isolated by centrifugation at 2,000 rpm for 30 min at 4°C. Lipoprotein fractions were obtained by standard methods (9) using a Beckman 50.3 Ti rotor and L8-80 ultracentrifuge. Chylomicrons were isolated from a lipoprotein lipase-deficient subject by ultracentrifugation of plasma for 30 min at 20,000 rpm; the particles were suspended in NaCl solution d 1.006 g/ml, 0.1% Na<sub>2</sub>EDTA, pH 7.5 and reisolated by ultracentrifugation for 30 min at 20,000 rpm. The very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL<sub>2</sub> and HDL<sub>3</sub>) were isolated from a normal subject at densities of 1.006 g/ml, 1.006 to 1.019 g/ml, 1.019 to 1.063 g/ml, 1.063 to 1.125 g/ml, and 1.125 to 1.21 g/ml, respectively. Each fraction was dialyzed against 0.01% Na<sub>2</sub>EDTA, pH 7.5, and the protein concentration was determined (10).

Aliquots of chylomicrons containing 50  $\mu$ g of protein were transferred to 16  $\times$  125 mm screw-top conical-bottom test tubes, lyophilized, and delipidated with diethylether-ethanol (11). The other lipoprotein fractions did not require delipidation prior to electrophoresis. Aliquots of these samples containing 50  $\mu$ g of protein were transferred to polypropylene tubes (Bio-Rad), lyophilized, and stored at -70°C.

The amount of protein applied to the gel was chosen to optimize the separation of apolipoproteins and varied as follows: chylomicrons, 25  $\mu$ g; VLDL, 15  $\mu$ g; IDL, LDL, HDL<sub>2</sub>, and HDL<sub>3</sub>, 10  $\mu$ g.

Each 50- $\mu$ g sample was dissolved in 50  $\mu$ l of 0.15 M NaCl, 0.04% Na<sub>2</sub>EDTA, 2% NaDodSO<sub>4</sub>, pH 8.6, that had been heated to 37°C. After adding the solubilization buffer, the sample was vortexed for 10 sec and incubated at room temperature for 30 min with vortexing at 10-min intervals. The samples were reduced by the addition of freshly prepared 0.4 M DTT, to give a final concentration of 40 mM DTT. The samples were then incubated at 100°C in a boiling water bath for 2 min, after which they were allowed to cool to room temperature. An aliquot of a 0.02% stock so-

Abbreviations: BSA, bovine serum albumin;  $\beta$ -GAL,  $\beta$ -galactosidase; HDL, high density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; NaDodSO<sub>4</sub>, sodium dodecyl sulfate; VLDL, very low density lipoproteins.

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lution of pyronin Y was added to each sample to give a final concentration of 0.002%. Glycerol was added to give a final concentration in each sample of 10% (w/v), to facilitate layering the samples under the electrophoresis buffer.

### NaDodSO<sub>4</sub>-glycerol gel electrophoresis

Electrophoresis was performed using a Bio-Rad Protean II vertical slab gel apparatus, 1.5-mm-thick gels and a 10-tooth well former. The gel consisted of 3.5% acrylamide (with an acrylamide-bisacrylamide ratio of 20:1), 18% glycerol (v/v), 0.1% NaDodSO<sub>4</sub> (w/v), 0.075% TEMED (v/v), 0.01% ammonium persulfate (w/v), 0.1 M Tris/phosphate, pH 6.8, and was prepared according to the following protocol.

A 0.8 M Tris/phosphate gel buffer stock solution was prepared using 4.98 ml of 85% H<sub>3</sub>PO<sub>4</sub>, 0.6 ml TEMED, adjusted to pH 6.8 by the addition of about 15.6 g of solid Tris, and brought to a final volume of 100 ml with distilled H<sub>2</sub>O. Acrylamide-bisacrylamide in a ratio of 20:1 was prepared as a 22% (w/v) stock solution. NaDodSO<sub>4</sub> was prepared as a 20% (w/v) stock solution. Ammonium persulfate was freshly prepared as a 10% (w/v) solution. The gel solution was prepared in a 250-ml vacuum flask by mixing 5.64 ml of 0.8 M Tris/phosphate, 0.6% TEMED buffer, 7.2 ml of 22% acrylamide-bisacrylamide, 8.1 ml of glycerol, and 24.06 ml distilled H<sub>2</sub>O. This solution was degassed, transferred to a beaker, and gently mixed with 250  $\mu$ l of 20% NaDodSO<sub>4</sub> and 50  $\mu$ l of 10% ammonium persulfate. This solution was immediately poured between the glass plates. The wells were formed using a 10-tooth comb which was removed from its support to allow insertion into the gel. No airspace was left between the well former and the acrylamide solution. The gel was allowed to polymerize at room temperature for 90 min, when it was to be used on the same day, or it was allowed to polymerize overnight when it was to be used on the following day. The well former was removed with care. As it was removed from the gel, the well former was flooded with distilled water to avoid distortion of the sample slots.

The lower buffer chamber was filled with 4 l of 0.1 M sodium phosphate, pH 7.0, and the upper buffer chamber was filled with 0.1 M sodium phosphate, pH 7.0, 0.1% NaDodSO<sub>4</sub>. The lower and upper running buffers were cooled to 16–18°C before use and cold tap water was circulated through the central core to maintain the lower buffer at 16–18°C. Immediately before applying the samples, the bottom of each sample well was thoroughly rinsed with upper buffer. A 10–15  $\mu$ l aliquot of each sample was layered in each well under the upper buffer.

Electrophoresis was performed at a constant current of 20 mA for 30 min and then at 60 mA for 4.5 h.

The gel was removed from the apparatus as follows. The glass plates were carefully separated and the plate to which the gel adhered was held over a tray of 4% formaldehyde

(v/v) with the gel facing down and the gel was gently removed from the plate. The gel was soaked in this solution for 30 min, transferred and stained overnight in 0.025% Coomassie Brilliant Blue G-250 in 45.4% methanol, 9.2% acetic acid. The gel was destained in 7.5% acetic acid, 5% methanol, and stored in 5% acetic acid.

The Bio-Rad Mini-Protean II system was also tested. One half of the volume of gel solution used to prepare a single large slab gel provided sufficient solution for two mini-slab gels (8 cm width  $\times$  6 cm length  $\times$  1.5 mm thickness). The acrylamide was allowed to polymerize for 2 h before removing the 10-well comb and running the samples. One liter of lower buffer was used in the outer chamber and 200 ml of upper buffer was used in the inner chamber.

For chylomicrons, VLDL, and LDL samples, the optimum separation was achieved with 5  $\mu$ g of protein in 5  $\mu$ l of solubilization buffer. For HDL samples, 2.5  $\mu$ g of protein in 5  $\mu$ l of buffer was suitable.

Electrophoresis was performed at room temperature without cooling, with an initial constant current of 25 mA for 10 min followed by 50 mA for 2 h. After electrophoresis the gel was transferred to a petri dish and fixed for 30 min in 4% formaldehyde, stained for 90 min in staining solution, and destained as described above. Manipulation of either the large gels or the small gels was facilitated by using a fiberglass screen.

### Identification of apolipoproteins by immunoblot

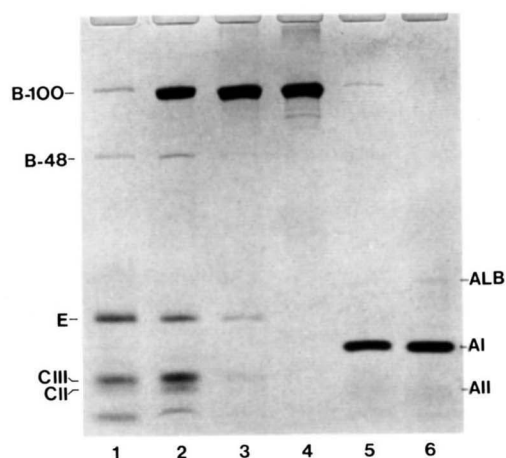
Apolipoproteins [a], A-I, A-II, B, C-II, C-III, and E were identified by immunoblot essentially as described by Towbin, Staehelin, and Gordon (12) using monospecific rabbit antisera produced in our laboratory, with the exception of antibody to apo[a] that was kindly provided by Dr. W. Carl Breckenridge (Dalhousie University).

Electrophoresis was carried out as described above. The apolipoproteins were transferred from the gel to a nitrocellulose membrane (0.45  $\mu$ m, Bio-Rad) in 25 mM glycine, 192 mM Tris, pH 8.3, containing 20% methanol. A Hoefer Transfor apparatus was used with a current of 100 mA for 18 h, or 500 mA for 4 h in a 10°C room.

All incubations and detection of apolipoproteins were performed using the horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and 4-chloro-1-naphthol as substrate, following the protocol described by the manufacturer.

### Molecular weights of proteins

The molecular weights of the apolipoproteins were taken from the literature (1, 6): apoB-100, 549,000; apoB-48, 264,000; human albumin, 64,000; apoE, 35,000; apoA-I, 28,100; apoC-II, 8,800; and apoC-III, 8,800. The following proteins were included as standards in some runs:  $\beta$ -GAL, mol wt 116,000; F6P, mol wt 84,000; and BSA, mol wt 68,000.

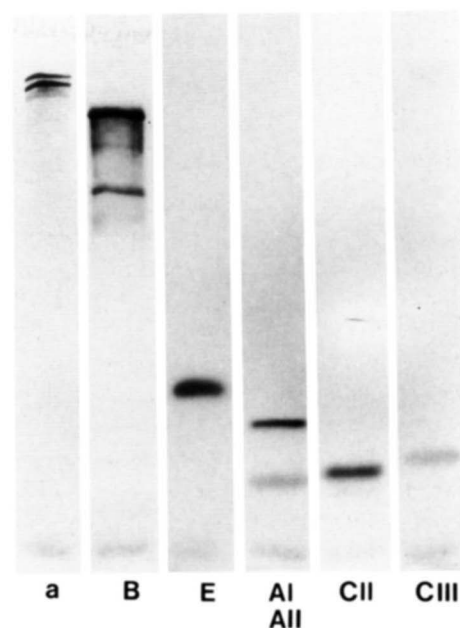


**Fig. 1.** NaDodSO<sub>4</sub>-glycerol polyacrylamide gel electrophoresis of apochylomicrons (1), apoVLDL (2), apoIDL (3), apoLDL (4), apoHDL<sub>2</sub> (5), and apoHDL<sub>3</sub> (6). Each sample was applied in a 15- $\mu$ l volume containing the following amounts of protein: 1, 25  $\mu$ g; 2, 15  $\mu$ g; 3, 10  $\mu$ g; 4, 10  $\mu$ g; 5, 10  $\mu$ g; and 6, 10  $\mu$ g.

## RESULTS AND DISCUSSION

The separation of the apolipoproteins of chylomicrons, VLDL, IDL, LDL, HDL<sub>2</sub>, and HDL<sub>3</sub> is shown in **Fig. 1**. Identification of the apolipoproteins by immunoblot is shown in **Fig. 2**. Chylomicrons (**Fig. 1**, lane 1) contained two bands, identified as B apolipoproteins by immunoblot (**Fig. 2**). Assignment of identity as B-100 and B-48 was made on the basis of comparison with the apoB-100 of the LDL fraction. The other chylomicron apolipoproteins resolved were: apoE, the C-III apolipoproteins, and apoC-II. ApoB-100, apoE, apoC-III, and apoC-II were identified in VLDL (**Fig. 1**, lane 2). A minor band was observed with an apparent molecular weight corresponding to apoB-48 and was identified as a B apolipoprotein by immunoblot (data not shown). The IDL fraction contained apoB-100 and apoE as the major apolipoproteins (**Fig. 1**, lane 3). Proteins corresponding to apoB-48 and apoC-III were also detected. ApoB-100 and minor quantities of smaller molecular weight forms of apoB were observed in LDL (**Fig. 1**, lane 4). HDL<sub>2</sub> apolipoprotein was found to consist primarily of apoA-I (**Fig. 1**, lane 5). Minor amounts of apoB-100 and apoA-II were also observed in this HDL<sub>2</sub>. The sample shown in **Fig. 1** did not contain detectable amounts of apo[a]. In other HDL<sub>2</sub> samples that did contain apo[a], it could be detected by stain (data not shown) and identified by immunoblot (**Fig. 2**). HDL<sub>3</sub> contained primarily apoA-I (**Fig. 1**, lane 6). ApoA-II and albumin were detected as minor components of HDL<sub>3</sub> in this subject.

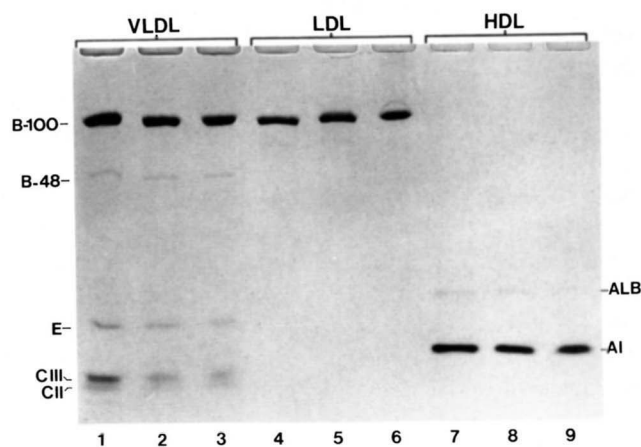
Since this system does not use a stacking gel, we tested the effects of sample volume on the resolution of the apolipoproteins (**Fig. 3**). We found that the resolution of the apolipoproteins was compromised at sample volumes of 50  $\mu$ l. This was particularly evident for the resolution of the



**Fig. 2.** Identification of apolipoproteins by immunoblot. The lipoprotein fraction used as a source of apolipoproteins and the amounts applied to each lane are shown in parentheses. Apo[a], lane 1 (2.5  $\mu$ g of apoHDL<sub>2</sub>); apoB-100 and apoB-48, lane 2 (10  $\mu$ g of apoChylomicron); apoE, lane 3 (5  $\mu$ g of apoVLDL); apoA-I and apoA-II, lane 4 (25  $\mu$ g of apoChylomicron); apoC-II, lane 5 (7.5  $\mu$ g apoVLDL); apoC-III, lane 6 (25  $\mu$ g of apoChylomicron).

C apolipoproteins of VLDL (**Fig. 3**, lanes 1-3). A distortion of some protein bands was observed when relatively large sample volumes were applied (**Fig. 3**, lane 6).

The effect of the load of protein in each sample is shown in **Fig. 4**. The apoB-100 of VLDL and LDL showed distortion with increasing amount of protein, while the apo-



**Fig. 3.** The effect of the volume of the sample on the resolution of the apolipoproteins. ApoVLDL (lanes 1-3) 20  $\mu$ g of protein; apoLDL (lanes 4-6) 10  $\mu$ g of protein; and apoHDL (lanes 7-9) 10  $\mu$ g of protein. Each sample was applied in the following volumes: lanes 1, 4, and 7, 10  $\mu$ l; lanes 2, 5, and 8, 25  $\mu$ l; lanes 3, 6, and 9, 50  $\mu$ l.

lipoproteins with lower molecular weights were resolved at each of the concentrations of protein tested.

A standard for use in each run was prepared by mixing appropriate quantities of the apolipoproteins of chylomicrons and HDL. The migration distance of the apolipoproteins was plotted versus the  $\log_{10}$  of the known molecular weights. Linear regression analysis using the data for apoB-100, apoB-48, albumin, apoE, and apoA-I gave an  $r^2$  of 0.996.

The analysis of normal apoVLDL (Fig. 5, lanes 2 and 3) and of the apoVLDL from a patient with apoC-II deficiency (Fig. 5, lane 4) demonstrates that it is possible to distinguish between the presence and absence of apoC-II using this system.

The resolution of the apolipoproteins using a Bio-Rad Mini-Protean II system was also tested. It was similar to that obtained with a standard size gel, except for the loss of the separation of apoC-III and apoC-II.

It was found that for VLDL, IDL, LDL, and HDL, delipidation was not a necessary step in sample preparation and could often produce apolipoprotein aggregates that did not enter the gel. It was also found that samples could be stored in a lyophilized form for several weeks before analysis with essentially no change in the apolipoprotein pattern.

During the evaluation of gel conditions, several concentrations of acrylamide and different ratios of acrylamide to bisacrylamide were tested and the combination of 3.5% acrylamide with a 20:1 acrylamide-bisacrylamide ratio was found to be optimal. It was observed that gels consisting of less than 3% acrylamide were significantly softer and adhered readily to surfaces and were thus unsuitable for immunoblotting.

Some of the practical advantages of this system are that a gel of a single concentration of acrylamide is easier to

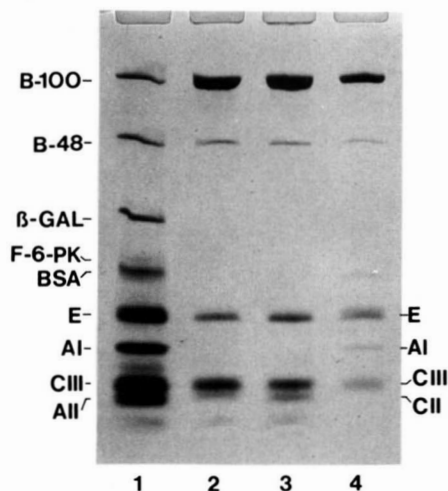


Fig. 5. Electrophoresis of the standard protein mixture (lane 1), normal apoVLDL (lanes 2 and 3), and apoVLDL from a patient with a deficiency of apolipoprotein C-II (lane 4). The standard was prepared from 25  $\mu$ g of chylomicron apolipoprotein, 3  $\mu$ g of HDL apolipoprotein, 1.5  $\mu$ g of  $\beta$ -GAL, 1.5  $\mu$ g of F6P, 1.5  $\mu$ g of BSA, and 2  $\mu$ g of apoA-II in 15  $\mu$ l. Each apoVLDL consisted of 15  $\mu$ g protein in a volume of 15  $\mu$ l.

produce than a gradient acrylamide gel, no stacking gel is required, and it is suitable for use in a mini-gel apparatus. In addition, the method of sample preparation is straightforward and eliminates the need for delipidation. This method should be particularly useful in the investigation of apolipoproteins and generally applicable to proteins from other sources. ■

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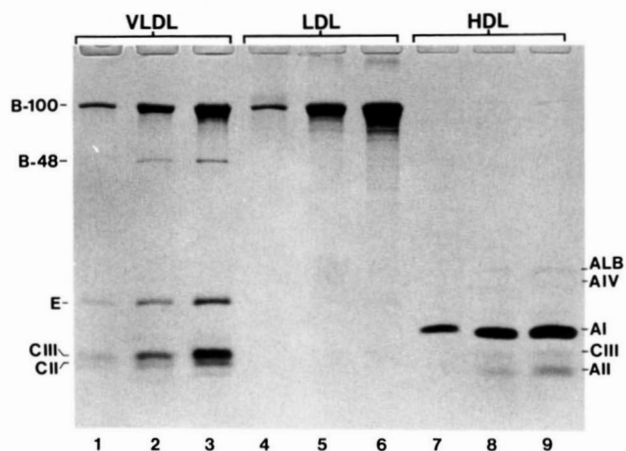


Fig. 4. The effect of the amount of protein on the resolution of the apolipoproteins. ApoVLDL (lanes 1-3), apoLDL (lanes 4-6), and apoHDL (lanes 7-9) protein were applied in 15- $\mu$ l volumes. The amount of protein in each sample was as follows: lanes 1, 4, and 7, 5  $\mu$ g; lanes 2, 5, and 8, 15  $\mu$ g; and lanes 3, 6, and 9, 30  $\mu$ g.

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