Agarose–starch gel electrophoresis of rat serum lipoproteins

A. CHALVARDJIAN

Research Institute, The Hospital for Sick Children, Toronto 101, Ontario, Canada

ABSTRACT Rat serum lipoproteins were separated into at least four fractions by agarose–starch gel electrophoresis. The system used was discontinuous in that glycine and sodium barbitone buffer was used in the reservoirs and Tris buffer was used for the gels. The four major bands could be related to the pattern obtained by ultracentrifugation. The high density lipoproteins consisted of at least two poorly resolved bands and were not separated from albumin.

The vertical gel apparatus was further modified to accept 0.4 ml of rat plasma, which was prestained with Sudan black. After electrophoresis the different lipoprotein bands could conveniently be cut out and the lipid phosphorus determined. The addition of Sudan black B decreased the recovery of the low and high density lipoproteins by 5–9%. However, the recovery of phospholipids was reproducible (80 ± 2%) and the high density lipoproteins contained over two-thirds of the plasma lipid phosphorus.

SUPPLEMENTARY KEY WORDS discontinuous system · analytical, preparative, ultracentrifugal correlation · prestained

RAT SERUM or plasma lipoproteins have been separated by electrophoresis in several media, including acrylamide gel (1) and agarose gel (2, 3). The main advantages of agarose gel electrophoresis are that it is more rapid and provides better resolution than paper electrophoresis, it is easier to perform than electrophoresis on acrylamide or starch, more permanent records are obtained than with acrylamide and starch gels, and the staining of the bands with lipid dyes is less cumbersome to perform than with cellulose acetate membranes.

An apparatus for vertical gel electrophoresis was designed and used in the separation of human serum lipoproteins (4). The advantage of this apparatus was that minimal handling of the gels was required before electrophoresis, and known amounts of samples could be layered onto the gel without the prior need of mixing them with some molten agarose. When electrophoresis of rat sera was performed using the apparatus and the buffer systems described, although the high density lipoprotein band was clearly seen, the low density lipoprotein bands were diffuse and indistinct. A better system was therefore sought and will be described in the following paragraphs. Also the apparatus was modified to accept 0.4 ml of plasma, thus enabling the isolation of individual bands and their quantification by chemical procedures.

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.

EXPERIMENTAL PROCEDURES

Materials

The rats were young male adults of the Wistar strain and weighed 300–400 g each. The rats were fed ad lib. and then were anesthetized with ether between 8 and 10 AM. The inferior vena cava was clamped with a hemostat above the entrance of the renal veins and blood was withdrawn from the vena cava. The blood was allowed to clot at room temperature for 1–2 hr. The clot was then freed from the tube walls and removed. After centrifugation to precipitate the remaining red cells, the serum was recovered.

Agarose–Starch Gel Electrophoresis and Equipment

The apparatus used has been described (4). It can handle regular microscope slides and the gel layers are 1.5 mm thick, and even. To obtain a combined agarose–starch gel concentration of 1.34%, 0.2 g of agarose (Seravac, obtained from Gallard-Schlesinger Chemical Mfg. Corp., Long Island, N.Y.) and 0.2 g of partially

hydrolyzed starch (Connaught Laboratories, Toronto, Ontario) are weighed in a 200-ml Berzelius beaker, and 30 ml of 0.45 M Tris (Trizma, Sigma Chemical Co., St. Louis, Mo.), brought to pH 8.9 with HCl, is added. The beaker is placed in a household pressure cooker which is filled with the appropriate amount of distilled water and the regulator is set to provide 15 psi. Once this pressure is attained the cooker is removed from the source of heat, cooled in tap water, and, after releasing the pressure, the beaker is transferred to a hot water bath (60–80°C). This procedure provides a very homogeneous gel with no scorching at the bottom of the beaker. The slide frames of the vertical gel electrophoresis apparatus are filled with the molten agarose and the sample molds are introduced. After the gel has firmly set (about 45 min at room temperature), the molds are pried loose. The upper and lower reservoirs are now filled with the same buffer solution, consisting of 43 mM glycine and 24 mM sodium barbitone brought to pH 8.6 with HCl.

The rat serum can be used unstained, or bromphenol blue can be added as a marker for the albumin band. The serum is layered in the gel pocket with a fine-tipped Lang-Levy pipette; the volume of serum can be between 10 and 20 μliters with adequate resolution and staining of the bands.

A current of 15 mamp per slide will cause a 2-cm migration of the albumin band in about 40 min, and will provide adequate separation of the lipoprotein bands. The voltage developed for electrophoresis of six slides is about 140 v. After termination of the electrophoretic run, the slides with the covering gels are removed and placed in 5 vol of 1.5% picric acid in 12% acetic acid, in a Petri dish. Protein bands are precipitated and can be seen within a few minutes. After 30 min, the slides are taken out of the fixing solution and the gels, centered on the slides, are covered with 3MM Whatman paper cut to the size of the slides. They are then dried under a hot lamp. The filter paper covers can be removed easily just before the slides are completely dry. If allowed to dry completely, no attempt should be made to remove the filter papers. The slides or the slides with filter paper still stuck on are placed in running tap water and after a few minutes the filter papers or any adherent lint can easily be removed. Once the background of agarose gel has been cleared of picric acid (10–15 min), the slides are removed from the water bath and left to dry either at room temperature or for a few minutes under a hot lamp. It is essential that the slides are thoroughly dry before staining is attempted. The albumin band will still retain the yellow color.

The staining solution is prepared as follows: 7 g of Fat Red 7B (Ciba) and 3.5 g of Oil Red O are dissolved in 1 liter of propylene glycol with constant stirring (magnetic stirrer-hot plate). The mixture is brought to 100°C and kept at that temperature for 5–10 min. The solution is then filtered while still hot through a No. 2 Whatman filter with suction, allowed to cool, and stored in a brown bottle. This stock staining solution is stable for several months. Just before staining, 10 ml of distilled water is added to 40 ml of the stock staining solution and allowed to mix for a few minutes. The working staining solution is then poured without filtration into a Coplin jar in which the slides with the covering gels are placed. The gels are stained overnight. Once stained, they can be stored without any special precautions.

The lipoprotein classes separated by ultracentrifugation can be correlated readily with the classes obtained by agarose–starch gel electrophoresis (Fig. 1). Serum is centrifuged overnight at 35,000 rpm in the 40.3 rotor of the Spinco model L ultracentrifuge at 4°C. The upper 1 ml is recovered and termed the lipoprotein fraction of density <1.006 (fraction *B*). The next upper 1 ml from the centrifuge tube is removed and the remaining infranatant (fraction *C*) is brought up to density 1.063 by the addition of NaCl–KBr solution (5). The resulting solution is centrifuged again overnight at 35,000 rpm and the upper 1 ml is recovered and designated the lipoprotein fraction of density 1.006–1.063 (fraction *D*). The next upper 1 ml from the centrifuge tube is removed and the remaining infranatant solution (fraction *E*) is brought up to density 1.210 by the addition of KBr solution. The resulting solution is centrifuged for 24 hr at 40,000 rpm and the upper 1 ml is recovered and designated the lipoprotein fraction of density 1.063–1.21 (fraction *F*). The infranatant is

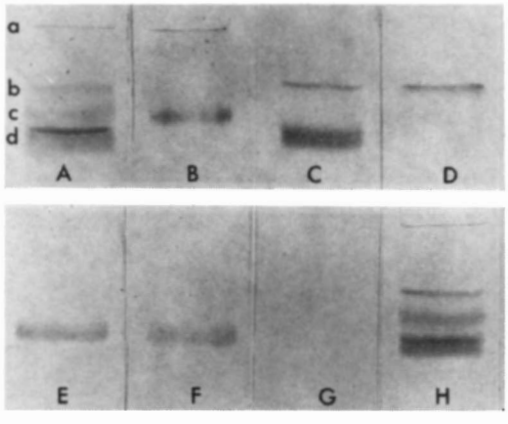

FIG. 1. Electrophoresis of rat serum lipoproteins separated by ultracentrifugation. *A* and *H*, whole serum; *B*, supernatant of solution d = 1.006 (serum); *C*, infranatant of d = 1.006; *D*, supernatant of solution d = 1.063; *E*, infranatant of d = 1.063; *F*, supernatant of solution d = 1.21; *G*, infranatant of d = 1.21, no bands are stained with the lipid dyes. Protein bands could still be visualized after staining with ponceau S. In *A*, band *a* = chylomicrons; band *b* = low density protein (LDL); band *c* = very low density lipoprotein (VLDL); and band *d* = high density lipoproteins (HDL) and albumin.

designated fraction *G*. Fractions *B–E* can be electrophoresed directly without further manipulation of the solutions. However, because of the high salt concentration, which distorts the electrophoretic pattern, fractions *F* and *G* are first diluted 5-fold and the resulting solutions are then concentrated by placing them in a collodion bag (Sartorius, obtained from Brinkmann Instruments, Westbury, N.Y.) and applying a vacuum. Within half an hour, the fractions are concentrated to their original volume and the salt concentration is reduced enough to permit electrophoresis.

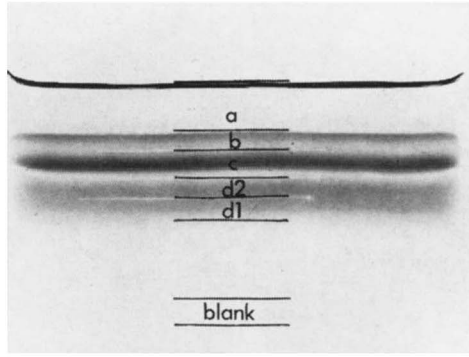

FIG. 2. Preparative electrophoresis of rat plasma lipoproteins. The plasma (0.4 ml) has been prestained with Sudan black. The black horizontal lines when extended indicate the cut zones. Area *a*, chylomicrons; area *b*, LDL; area *c*, VLDL; area *d2*, HDL-band 2; area *d1*, HDL-Band 1; *blank*, area carried th ough extraction procedure and used as blank.

Preparative Electrophoresis

The frame carrier (*C* in Ref. 4) was modified to accommodate two $3^1/_4 \times 4$ inch lantern slides and the size of the frames was increased to provide gels 5 mm thick. Gels are prepared by adding 90 ml of 0.45 M Tris buffer, pH 8.9, to 0.45 g of agarose and 0.2 g of starch. No molds to form sample pockets are used and leveling of the gel surface is achieved by placing the frame carrier into the leveled empty lower reservoir before filling the frames with the molten agarose. The gels are allowed to set and then placed in the refrigerator at 2°C for 30 min before the apparatus is assembled and the reservoirs filled with the same reservoir buffers as in the analytical procedure. Plasma was obtained from young male adult albino rats and clotting was prevented by addition of EDTA. After removal of the blood cells by centrifugation, 0.25 ml of Sudan black B in propylene glycol (6) was added to 1 ml of plasma and mixed. The mixture was kept in the dark at room temperature for 1 hr and the precipitated dye was removed by centrifugation. The supernatant prestained plasma was then layered over the agarose with a fine-tipped Lang-Levy pipette. As a further check on linearity, 0.4 and 0.55 ml of prestained sample (corresponding to 0.33 and 0.44 ml of plasma) were layered over each gel frame.

Electrophoresis is performed at a current of 200 mamp and 170 v and usually lasts for 30–45 min. At that time the albumin band has migrated about 2 cm. The slides are removed with their covering gels and placed on top of frosted glass over a light source. A single-edged razor blade is held vertically above the gel and sharp cuts are made. The gels are divided to provide six fractions (Fig. 2). The section from the origin to the trailing edge of band *b* is designated, for convenience, the chylomicron fraction (band *a*). The purity and accurate recovery of this fraction is questionable and will be discussed later. The next fraction separated is band *b* (LDL). Band *c* (VLDL) is separated from HDL-Band 2 by cutting halfway between the leading edge of VLDL and the trailing edge of HDL-Band 2. Likewise, HDL-Band 2 (*d2*) and HDL-Band 1 (*d1*) are cut and recovered separately. The cut slices are gently slid into 12-ml centrifuge tubes with the tip of a microspatula. Blank areas of the gel beyond the last band toward the anode are cut and carried through the extraction procedures. The gels are centrifuged into a compact mass at the bottom of the tubes, frozen in dry ice, and lyophilized overnight. After lyophilization the dry gels are easily powdered and are then extracted by constant agitation for 1 min with 2–3 ml of chloroform–methanol 2:1; the extraction procedure is carried out three times. The lipid extract is recovered after each extraction by centrifuging the tubes. The extracts are transferred to marked 150 × 20 mm test tubes and the volume made up to 10 ml with chloroform–methanol 2:1. Following this, 2 ml of 0.034% $MgCl_2$ is added to the chloroform–methanol solution (7) and the tubes are shaken vigorously. Two phases are readily formed after centrifugation, and the aqueous phase is removed by suction and discarded. The volume is made up to 10 ml and appropriate aliquots are removed from the extracts for the determination of phosphorus (8).

To test for the effect of propylene glycol on the recovery of the lipoprotein bands (no chylomicrons present), pooled rat serum was centrifuged at 35,000 rpm in the 40.3 rotor for 30 min. The top 0.5 ml of the centrifuged serum, with any visible fat pellicle, was removed and discarded. The removed volume of serum was replaced with 0.9% NaCl and the tube was recentrifuged at 35,000 rpm at 4°C for 16 hr. The top 0.5 ml of the centrifuged serum was removed and labeled the VLDL fraction; the remaining infranate constituted the combined LDL–HDL fraction. To aliquots of each of the two fractions recovered, Sudan black B in propylene glycol was added as described above. Preparative electrophoresis of the VLDL and the LDL–HDL fractions, with and without prestaining, was performed. The prestained bands were used to locate their un-

stained counterparts. However, because of the sample overload in this experiment, the unstained bands were faintly visible when the gels were viewed against a dark background.

RESULTS

Analytical Agarose–Starch Gel Electrophoresis of Rat Serum Lipoproteins

Electropherograms *A* and *H* in Fig. 1 are of whole serum and show at least four bands, designated as *a*, *b*, *c*, and *d*. Electropherograms *B–G* in Fig. 1 correspond to fractions *B–G* isolated by ultracentrifugation (see Experimental Procedures).

Two bands can be seen in Fig. 1*B*. The upper band is very narrow and has not penetrated the gel; this corresponds to the chylomicron fraction (band *a*, Fig. 1*A*). The lower band in Fig. 1*B* is wider, shows minimal trailing, and corresponds to the VLDL fraction (band *c*, Fig. 1*A*). Fraction *C* (Fig. 1*C*) also shows at least two bands. The upper band is sharp and narrow and corresponds to the LDL fraction (fraction *D* consisting of a single band in Fig. 1*D* and band *b* in Fig. 1*A*). The lower band in Fig. 1*C* corresponds to HDL (fractions *E* and *F*, and band *d* in Fig. 1*A*). This band migrates with and cannot be separated from albumin, and in contrast to the other fractions appears to be composed of more than one band. Its heterogeneous pattern is most readily recognized during the preparative procedure using plasma prestained with Sudan black (Fig. 2). The two HDL bands in the latter instance appear distinct and are stained blue, while albumin stains brownish grey (9). HDL-Band 1 (*d1* in Fig. 2) migrates slightly faster than albumin and is wider than HDL-Band 2 (*d2* in Fig. 2), which migrates slightly slower than albumin. Fraction *G* (Fig. 1*G*) shows no bands that stain with lipid dyes.

Preparative Agarose–Starch Gel Electrophoresis of Rat Plasma Proteins

The recovery of total lipoprotein phosphorus was 80 ± 2% (mean ± SEM). To test for linearity, two different amounts of the same plasma sample, 0.33 and 0.44 ml, were run simultaneously. Agreement between the two different amounts of serum applied was better than 90% (Table 1, column 2). The prestaining of the VLDL band with Sudan black reduced its recovery by 9%, and the recovery of the combined LDL–HDL bands was reduced by 5% after prestaining.

As indicated in Table 1, in rat plasma the HDL fractions contain most of the lipid phosphorus, HDL-Band 2 containing more than HDL-Band 1, with the balance being distributed between LDL and VLDL. The chylomicron fraction is relatively large (Table 1) in this instance because the plasma samples used were drawn from rats that had not been fasted during the night preceding the experiment.

TABLE 1 LIPID PHOSPHORUS OF RAT PLASMA LIPOPROTEINS

Fraction	nmoles P/ml Serum*	Percentage Difference between Different Sample Loads*
Chylomicrons	136 ± 10	4.0 ± 2.0
LDL	127 ± 10	4.5 ± 1.6
VLDL	167 ± 10	8.1 ± 3.7
HDL-Band 2	646 ± 22	3.9 ± 1.1
HDL-Band 1	365 ± 25	5.9 ± 1.8

Two electrophoretic separations were performed on each of four samples of prestained rat plasma, using 0.33 and 0.44 ml of plasma. Therefore, each of the values in column 1 represents the mean of eight determinations.

* Mean ± SEM

DISCUSSION

At the outset of this study, the system which had been used for the electrophoresis of human serum lipoproteins (4) was applied to the electrophoresis of rat serum. Although a discrete band corresponding to HDL could be demonstrated, both the LDL and VLDL bands were broad and diffuse. The bands were therefore sharpened by utilizing a discontinuous system. The discontinuous system used in this study was modified from the previously used system (1) to provide a better resolution of the LDL and VLDL bands from each other and from the HDL bands. The inclusion of starch (10) in the gels is not essential but usually produces a better resolution of the bands.

In the present system, a partial resolution of at least two major HDL bands is achieved, but attempts to obtain a better resolution of these bands from each other and from albumin have so far been unsuccessful.

The main advantage of the present system is that it is a simple one. Its main use is in the rapid separation of the major classes of lipoproteins, i.e., LDL, VLDL, and HDL. If resolution of individual HDL is desired, it may be achieved by using acrylamide disc electrophoresis (1) or starch gel electrophoresis (11).

The preparative agarose gel electrophoresis is useful in cases in which the lipid moieties of the lipoprotein bands are being studied and in which the nonlipid material, including the non-lipid-containing plasma proteins, can be eliminated by solvent–water partition. Instead of the 3–4 days required for the recovery of the different lipoprotein classes when preparative ultracentrifugation is used, agarose gel electrophoresis allows the preparation of lipoprotein lipids within 24 hr. It is also useful in separating chylomicrons from VLDL, which may be difficult by differential centrifugation.

The recoveries from the gels consistently averaged about 80% of total plasma phospholipids. The reason for this is not clear. The extent of recovery was similar in duplicate runs of the same sample, regardless of the sample load. It is possible that some lipoproteins, like the chylomicrons, because of their large size do not enter the gel and so are left layered on top of the gel. During or after electrophoresis, because of the handling of the gels, the lipoprotein molecules that have not migrated into the gel may be lost in the upper reservoir buffer. Another possibility which involves basically the same mechanism is an aggregation of some lipoprotein species in the presence of propylene glycol, rendering them incapable of migration into the agarose gel.

As mentioned under Results, prestaining of the lipoprotein bands decreases their recovery by 5–9%, and this limitation must be kept in mind when using this technique. Another limitation that must be considered is the inaccuracy of determination of the chylomicron fraction. The reproducibility of results on the same plasma samples was not determined on successive days, although the agreement between duplicate aliquots tested on the same day was reasonably good (Table 1). However, since the recovery of lipoprotein lipid phosphorus is reproducible, the technique should nevertheless be useful when comparing groups of rats subjected to different experimental conditions.

The technical assistance of E. Rudnicki and A. Gillespie is appreciated. I am grateful to Dr. S. H. Jackson for his review of the manuscript. This work was supported by Grant No. MT-1635 from The Medical Research Council of Canada.

Manuscript received 1 June 1970; accepted 14 December 1970.

References

1. Narayan, K. A., H. L. Creinin, and F. A. Kummerow. 1966. Disc electrophoresis of rat plasma lipoproteins. *J. Lipid Res.* **7:** 150–157.
2. Camejo, G. 1967. Structural studies of rat plasma lipoproteins. *Biochemistry.* **6:** 3228–3241.
3. Papadopoulos, N. M., C. M. Bloor, and J. C. Standefer. 1969. Effects of exercise and training on plasma lipids and lipoproteins in the rat. *J. Appl. Physiol.* **26:** 760–763.
4. Chalvardjian, A. 1969. An apparatus for vertical gel electrophoresis on microscope slides. *Clin. Chim. Acta.* **26:** 174–176.
5. 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.
6. McDonald, H. J., and L. P. Ribeiro. 1959. Ethylene and propylene glycol in the prestaining of lipoproteins for electrophoresis. *Clin. Chim. Acta.* **4:** 458–459.
7. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.
8. Chalvardjian, A., and E. Rudnicki. 1970. Determination of lipid phosphorus in the nanomolar range. *Anal. Biochem.* **36:** 225–226.
9. Narayan, K. A., W. E. Dudacek, and F. A. Kummerow. 1966. Disc electrophoresis of isolated rat-serum lipoproteins. *Biochim. Biophys. Acta.* **125:** 581–590.
10. Karjala, S. A. 1958. Electrophoresis of serum proteins in mixed agar gels. *Makromol. Chem.* **28:** 103–113.
11. Cohen, L., and J. Djordjevich. 1968. Human serum α-1-lipoprotein patterns revealed by starch gel electrophoresis. *Lipids.* **3:** 420–424.