Agar Gel Lipoprotein Electrophoresis: A Correlated Study with Ultracentrifugation

R. M. Iammarino, M. Humphrey, and Paul Antolik

Interest in lipoprotein electrophoresis has been stimulated by a reappraisal of hyperlipoproteinemic syndromes and definition of five basic types according to Fredrickson (Types I, II, III, IV, V). The basic analytic reference for lipoprotein studies remains ultracentrifugal analysis. We have adapted an agar gel lipoprotein electrophoretic system employing a photometric scan and quantification of lipoprotein fractions based on post-electrophoresis Sudan Black B staining. This method of lipoprotein partitioning has been compared with lipoprotein analysis by ultracentrifugation. Statistically significant correlation between the methods is demonstrated.

Lipoprotein electrophoresis forms the basis of a new classification of hyperlipoproteinemic syndromes (1, 2). While some interpretations can be made from cholesterol and triglyceride estimations alone, a unique feature of this classification rests with an accurate, reproducible lipoprotein electrophoresis. Five basic groups are defined (Types I, II, III, IV, V) based on patterns of paper lipoprotein electrophoresis and ancillary data. Since specific diet and drug treatment exists for the clinical management of the “Types,” laboratory analyses are most important.

In an attempt to establish paper electrophoretic separation of lipoproteins in our laboratory, technical difficulties were encountered: a high background stain with oil red O, occasional difficulty in interpretation of the pre-β region, and poor definition of the α-lipoprotein band. Scanning the strips by various devices was without success. The dye fades in time and this process is hastened by exposure to light. We could not interpret the electrophoretogram in all instances, and a recent
report indicates our experience is not unique. Pries et al (3) reported discrepant results by independent observers in 20–40% of the patients studied. To compound these difficulties the separation of lipoproteins on paper requires an 18-hr electrophoresis run and a 6-hr staining period. These difficulties prompted investigation of other methods. A method of agar gel electrophoresis was developed from the methods proposed by Ressler (4), Cawley (5), and Bell (6). Advantages included a shorter electrophoresis run, use of a stable dye—Sudan Black B—with low background staining, and a preparation which can be scanned and quantified.

Ultracentrifugal analysis remains the primary method of lipoprotein separation. Since our aim was to provide a quantitative method of lipoprotein electrophoresis, we performed a series of analyses comparing lipoprotein electrophoresis and ultracentrifugal analysis. Analysis of 33 consecutive patients by these methods forms the basis of this report.

**Materials and Methods**

**Electrophoresis Reagents**

1. **Agar-Panagar®**—(Colab-Chicago Heights, Ill)
2. **Barbital buffer**—2.76 g diethylbarbituric acid; 15.40 g sodium diethylbarbiturate dissolved in 1.5 liters of distilled water. This will prepare a solution with a pH of 8.6 and an ionic strength of 0.050. (Scientific Products—E4950)
3. **Acetic acid**—10% (v/v) in absolute ethanol
4. **Absolute ethanol**—also 50% and 60% (v/v)
5. **Stain**—stock solution: 1 g Sudan Black B (Fisher Scientific No. S-668) in 1000 ml 60% ethanol
6. **Sodium hydroxide**—20% (w/v)

Panagar®, supplied as a preweighed, prepackaged agar tablet, is dissolved in 25 ml of barbital buffer pH 8.6 and 25 ml of distilled water. Following dissolution at room temperature with gentle stirring, the agar-buffer-water combination is heated on a stirring hot plate to 95–100°. (Albumin added to the agar or buffer was not found necessary in this system.) Exactly 2.5 ml of the solubilized agar solution is transferred to clean, dry standard microscope slides (1 × 3 in.).

A transverse cut is made in the agar by the use of a plastic template and stainless steel cutting bar.* The dimensions of the trough are 15 × 15.5 mm, and the cut is made at a point 2.0 cm from the edge of the microscope slides. The agar trough is gently removed with a split applicator stick. When such a cutter is used, the trough holds 20 μl of

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*These can be purchased from A. Panichielli, 3836 Everlawn St, West Mifflin, Pa 15122.
sample. Sample application is made by the use of a microcapillary pipette (Drummond Microcaps®). The slides on a plastic tray are placed in a Beckman Durrum cell which contains barbital buffer, pH 8.6, 0.050 ionic strength. The polarity is adjusted so that the anodal migration moves toward the center of the slide. The electrophoresis is carried out for 1.5 hr at 100 v constant current mode using the Beckman Duostat power supply. Occasional difficulty was encountered in some runs with the appearance of an irregular migration during which the center part of the protein migrates at a slower rate than the lateral portions producing a "bat wing spread." The most likely cause for this difficulty is time lag following removal of the agar trough with drying of the sample application site. The protein bands migrate as a uniform front with the α1 lipoprotein migrating with the albumin band. Following electrophoresis the slides are fixed in 10% acetic acid and absolute ethanol for 10 min and for an additional 30 min in a solution of absolute ethanol. The slides are dried after covering with moistened filter paper. When the slides are completely dried they are transferred to a working stain solution containing 50 ml of stock stain solution which has been freshly filtered. The working stain solution contains 0.1 ml of 20% NaOH added immediately prior to staining. The slides remain in this solution for 1 hr at room temperature. The background stain is removed by three rinses of 50% ethanol, each rinse lasting approximately 1-2 min. This is essentially the staining method of Uriel (8). The slides are air dried. The stained agar surface dries to a thin tough coat bound firmly to the glass slide. The slide is scanned in a Photovolt® densitometer. Use of a 545-nm filter is employed with a narrow slit and a slow scan speed. In this instrument various settings are provided in an attempt to give a logarithmic response. In our system "response 10" provided a fairly uniform and reproducible result. When coupled with the integrator, appropriate cuts are made in the lipoprotein electrophoretic peaks and the calculations made to express the fractions in terms of percentage. Figure 1 illustrates the essential features of the method. When indicated (study 8 and 10) high lipid samples were diluted with normal saline to give better electrophoretic definition of the lipoprotein bands. The slides are labelled and stored in a regular slide file for reference and rescanning if necessary. On repeat scanning over a 6-month period no dye instability has been observed.

Ultracentrifugation

Analysis of lipoproteins by ultracentrifugation was performed by one of us (P.A.). The technics employed are modified from the method
as reported by Lewis, Green, and Page (9). A Beckman Spinco Model L centrifuge was employed and in this method two lipoprotein fractions are obtained. In the initial spin, the specific gravity of the serum is adjusted with salts (NaCl and KBr) so that lipoproteins with the density of 1.063 or less are obtained. This is the low density lipoprotein (LDL) fraction. Following this the specific gravity is adjusted to separate lipoproteins within the range of 1.063–1.21. This is the high density lipoprotein (HDL) fraction. These are removed following the second spin. Analysis of the three fractions, LDL, HDL, and native serum is performed for cholesterol as adapted by the method of Sperry and Webb (10) and triglycerides adapted from the method of Van Handel and Zilversmit (11).

**Clinical Study**

Blood was drawn following an overnight fast. After clotting, the blood was centrifuged at room temperature and the serum was removed. No preservative was used. The samples were stored in the
refrigerator for periods up to 1 week prior to analysis. We avoided freezing of specimens due to the disruptive effect on lipoproteins. Patients included abnormals followed for some time because of hyperlipoproteinemic syndromes, and a normal group undergoing a longitudinal health profile survey.

Results

Our basic question was the validity of lipoprotein partitioning following agar gel electrophoresis as compared with ultracentrifugal partitioning. The values of total serum cholesterol and triglycerides were combined for this study as the approximate total lipid value. The percentage of low density lipoprotein (< 1.063) was calculated from the amount of cholesterol and triglycerides in the low density fraction compared to the totals in native serum. High density lipoproteins (1.063–1.21) were similarly calculated. The sum of low density and high density lipids equaled the total in native serum (analyzed separately) ±5%. For this study the pre-β and β fractions electrophoretically were added and identified as total electrophoretic β. The standard deviation of the lipoprotein electrophoresis on replicate analyses of the same serum was ±3% by this method. These data are shown in Table 1. This table shows the result of analysis of 27 serum samples. Sera with wide ranges of lipid were used in an attempt to see if the method correlated over these ranges. Table 2 shows the analysis from 3 patients performed in duplicate over a 3-month period. In general, it is noted that the electrophoretic β (pre-β and β) is lower than the percentage LDL obtained by ultracentrifugation. A possible explanation for this constant observation may be offered in that phospholipids were not analyzed. Since a higher percentage of phospholipids is noted in the HDL lipids, this phospholipid would account for a greater proportion of electrophoretic stainable α lipid and a correspondingly lower percentage of electrophoretic β lipid. Of course, other causes may exist for the constant observed difference, and perhaps as analytic techniques improve for electrophoretic separation better correlation may be demonstrated. A somewhat better agreement at higher values is noted, and this may be due to the lower relative proportion of phospholipid in these sera. Repeat analysis in these studies (Table 2) shows the analytic variation for both separation methods is small.

Although no patients with hyperlipoproteinemia of Fredrickson Type I was analyzed during this study, one patient (study No. 8) with Type V hyperlipoproteinemia was seen (Fig 2). It will be noted that the chylomicon fraction remains at the origin or more properly slightly to the anodal side of the origin.
LIPOPROTEIN ELECTROPHORESIS

### Table 1. COMPARATIVE LIPOPROTEIN STUDIES

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Ranges: 104–3,157 mg/100 ml, 37–150 mg/100 ml

### Table 2. VARIATIONS IN LIPOPROTEIN SEPARATIONS

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<th>Difference</th>
<th>% Total % LDL electrophoretic</th>
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Analysis from 3 patients was performed Jan.–March 1968.
Aliquots of the two fractions obtained by the ultracentrifugation analysis were subjected to electrophoresis after dialysis. The separations are shown in Fig 3. The HDL fraction shows some β lipid. Carry-over contamination with incomplete removal of the LDL fraction was thought to be the most likely explanation for this, although there is a possibility of high density particles with a β migration which extends much further under the same conditions of electrophoresis than the full serum pattern. Changes due to salt interaction during the ultracentrifugal separation are most likely responsible for this difference in electrophoretic mobility.

Statistical analysis of the combined β fractions of the electrophoretic separations and the LDL (<1.063) fraction of the ultracentrifugal separation was performed. Regression analysis of these data give the following best fit equation: \( Y = 1.09X - 8.9 \), where \( Y \) is the percent β estimated by electrophoretic separation and \( X \) is the LDL ultracentrif-

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**Fig 2.** A lipoprotein scan from Study 8 of elevated lipid. The serum was diluted 1:4 prior to electrophoresis. The zones are labelled and the figures at the bottom of the graph are data from the integrator used to calculate percentage.
u gal value (as percentage). This is expressed in graphic form in Fig 4. The mean squared deviation of these data from this equation does not differ by F test from the mean squared deviation calculated from the theoretical perfect regression equation: $Y = X$.

**Discussion**

While agar gel lipoprotein electrophoresis was not new, we felt a certain hesitancy in completely accepting this method without some attempt to compare ultracentrifugal separations with electrophoresis as had been done for paper electrophoresis by Fredrickson et al (2).

**Fig 3.** Electrophoresis of the native serum and separated fraction obtained following ultracentrifugation.

**Fig 4.** Correlation is shown between ultracentrifugal $\beta$ and electrophoretic $\beta$. The solid line represents a perfect correlation; dashed line represents the mean and range of the lines derived from regression analysis.
We believe these data show that the agar gel method of lipoprotein electrophoresis is an accurate method for the lipoprotein partitioning of human serum. The well known influence of diet as a modifying factor in lipoproteins as well as the use of newer drugs favorably influencing blood lipid and lipoprotein patterns makes intelligent management of these disorders a process of continuing monitoring. The cost and difficulties of ultracentrifugal analysis mitigate against this as a practical solution. Fredrickson has advocated the use of paper lipoprotein electrophoresis. Others have advocated systems employing cellulose acetate (12) or gelatinized cellulose acetate (13) and cellulose acetate filtration (14). We have not tested these systems and cannot comment upon them, but the methods of agar gel lipoprotein electrophoresis herein reported provide in our opinion an easy, quantitative, reproducible technic which compares favorably with ultracentrifugal monitoring at a fraction of the cost and which offers certain advantages over paper lipoprotein electrophoresis.

While recognizing our difficulties with paper lipoprotein electrophoresis, we would like to indicate that agar gel lipoprotein electrophoresis requires the development of certain technics which require time and patience to acquire. We feel the type of agar is extremely important, and several were tested. The agars include: (1) Special Agar, Noble® (Difco), (2) Pure Agar, Beringwerke, and (3) Agarose, electrophoretic grade (General Biochemicals, Corp.). Panagar® provided the most reproducible system and was easiest to work with. The agar must be well solubilized before transfer to the slides. Several systems of sample application were attempted and some prestaining methods employing Sudan Black B were used. Of importance is that a narrow band of serum be applied. The system employed by Cawley (5) whereby glass rods are imbedded in the agar while moist and removed did not work in our hands, since we could not obtain a smooth enough agar front surface at the point of origin. The actual electrophoresis was used in a system designed primarily for immuno-electrophoresis and will be reported (15). The agar gel system of Weime (7) employing petroleum ether overlay was not tested for this method. The influence of pH and ionic strength were tested with pH ranges of 8.4–9.0 and ionic strength of 0.025–0.075. The conditions described are optimal in our experience for the type of agar employed. The greatest variable in the system was the type of agar used. When establishing this method in our laboratory, we determined protein stains on the same electrophoretic runs (Fig 5) and were able to demonstrate, as others have, that the α, lipoprotein of paper electrophoresis migrates with the albumin band in the agar system. The β band of the lipoprotein migrates
with the $\beta$ protein band; however, the pre-$\beta$ band is a position of minimal protein staining between the $\beta$ and $\alpha_2$ globulin bands. A study (16) on the nature of the pre-$\beta$ lipid provides an explanation of the failure of the pre-$\beta$ band to stain deeply with protein stain. This finding is quite consistent with the well-known characteristics that this zone represents the endogenous very low density lipoprotein rich in triglycerides and low in protein. Analysis of this zone on the electrophoretogram is of increasing importance since recognition of certain forms of carbohydrate-induced hyperlipoproteinemia are dependent upon it. Any new method of lipoprotein electrophoresis will in large measure be judged on how well the pre-$\beta$ is demonstrated. The report of Bresterman (17) indicates that the pre-$\beta$ was found in a high proportion (198 of 200) of individuals presenting with myocardial infarction. We feel that this method provides a practical approach to a more accurate quantification of pre-$\beta$ lipoprotein than is possible with paper

![Diagram of lipoprotein electrophoresis](image)

**Fig 5.** A slide showing the zones stained employing the protein stain, right, and the lipid stain, left.
electrophoresis. During the study, a report appeared (18) employing a modified system of agar gel electrophoresis which demonstrates up to nine bands in pathologic sera. This paper demonstrates a system of photometric scanning not unlike the one herein reported.

Quantitation of the $\alpha$ band is of some significance in the clinical monitoring of patients with lipoprotein disorders. Levy et al (16) have shown that $\alpha$ lipoprotein varies inversely with pre-$\beta$ lipoprotein. A recent report by Cohen and Djordjevich (19) employing starch gel lipoprotein electrophoresis demonstrates three patterns of $\alpha$ lipoprotein. They also showed that these patterns differed in men and women.

The chylomicron band is also of practical importance. This band is identified in agar gel lipoprotein electrophoresis and this separation stands in contrast to lipoprotein electrophoresis employing cellulose acetate as a support. On cellulose acetate the chylomicron band has no specific zone of migration and can be found anywhere on the electrophoretogram according to Kohn (20) or as $\alpha_2$ globulin as reported in the system of Colfs and Verheyden (23). A recent report (21) concerning a comparison of agar gel lipoprotein electrophoresis with ultracentrifugal studies indicates that lipoproteins with an Sf greater than 400 do not enter the gel and therefore cannot be analyzed. We cannot comment on this since most of our sera were devoid of significant chylomicron components.

We are currently employing a total lipid analysis and quantitative agar gel lipoprotein electrophoresis in patients with suspected or proven lipoprotein abnormalities. Normal values, variations, and clinical studies using this system of lipoprotein analysis will be the subject of another report (22).

Addendum

Since submission of this paper, a report has appeared (23) which describes a similar method of lipoprotein electrophoresis in agarose-agar employing a similar method of scanning. Studies were carried out correlating this agar gel lipoprotein electrophoresis with that obtained by employing the paper electrophoresis system as modified by Fredrickson.

An excellent summary (24) of the clinical and laboratory classification of hyperlipoproteinemia has also appeared since this article was submitted. This article contains much clinically useful material and summarizes current management by diet and drugs.

References

6. BELL, H. E., and IRWIN, W. C., Personal communication.
20. KORN, J., Personal communication.
22. IMMARDINO, R. M., Unpublished data.