Electrophoretic separation of plasma lipoproteins in agarose gel

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ABSTRACT A method has been developed for the separation of serum or plasma lipoproteins by electrophoresis in an agarose–agar gel mixture. The gel is applied to the surface of a thin polyester photographic film strip. With minor alterations in technique either single samples on individual strips or many samples on one large sheet may be processed. After fixation and dehydration the transparent film is stained with Sudan Black B and washed with water. The finished electrophoretogram can be obtained in 5 hr and consists of widely separated bands of lipoprotein fractions on a colorless transparent background, ideally suited for scanning with a densitometer.

Plasma samples from different subjects show pre-β lipoproteins of different mobilities. An effect of gel concentration on the extent of lipoprotein migration is demonstrated. The clear-cut separation of lipoproteins by this method will facilitate the classification of hyperlipoproteinemias and improve quantitative estimates of lipoprotein distribution.

SUPPLEMENTARY KEY WORDS rapid · plastic film strip · hyperlipoproteinemias · pre-β lipoproteins · mobility · Clofibrate

DURING RECENT YEARS the interest of investigators and clinicians in characterizing the plasma lipoprotein patterns of patients with hyperlipidemia has increased. Much of this interest has been stimulated by improved procedures for the paper electrophoresis of plasma lipoproteins (1), which permitted Fredrickson and his associates to classify the hyperlipoproteinemias into five distinct types (2). However, the interpretation of the paper electrophoretogram remained difficult for the inexperienced. The method of lipoprotein electrophoresis to be described employs as the supporting medium agarose or an agarose–agar mixture which is applied to the surface of a transparent polyester film strip. The resulting stained electrophoretogram shows widely separated lipoprotein bands on a clear colorless background, from which excellent densitometric scans can be made.

Agar and agarose have been widely employed as supporting media for electrophoresis (3). The use of the plastic film strip was first described by Cawley (4) and by Giri (5) for the separation of serum proteins in agar. During the past several years a modification of Cawley's procedure in our laboratory produced wide separation of the five serum protein fractions. The densitometer scan revealed that the optical density fell to zero between the albumin and the several globulin peaks. Accordingly, it seemed appropriate to apply this technique to the electrophoretic separation of plasma lipoproteins. The method described here permits the processing of either a single sample on an individual strip (procedure A), or several samples applied to the gel on one large strip (procedure B).

EXPERIMENTAL PROCEDURES

Samples

Blood samples were collected in the morning from subjects who had usually eaten a light, fat-free breakfast. Serum or EDTA–plasma (1 mg of EDTA per ml of blood) was used. For serum samples blood was allowed to clot in a test tube at room temperature for 2 hr. The clot was gently freed from the wall of the test tube. The red cells were sedimented from plasma or serum by centrifugation at 800 *g* for 10 min at 10°C. The serum or plasma was subsequently stored at 4°C.

Electrophoretic Equipment and Materials

The Plexiglas electrophoretic cell used in this study was made in our laboratory and is larger than those commercially available. (Source of supply or specifications obtainable from the author.) It is a horizontal type, 38 cm long and 28 cm wide, and accommodates eight individual gel strips or one large strip for 14 samples. There is a compartment for cooling water on the underside of the horizontal platform, spanning the 13 cm between the buffer wells. The electrophoretic cell manufactured by

E. C. Apparatus Corp., University City, Pa. (Cat. No. EC 401, research model, 20 × 30 cm), produces excellent separations on five individual strips (procedure A) or on seven samples applied to one large strip (procedure B). In both cells the electrical contact with the agarose gel is made with Whatman No. 3 filter paper, chromatographic grade.

Any regulated DC power supply of at least 250 v, 50–100 ma output will suffice. Current required is 10 ma per strip. The Heathkit regulated voltage DC power supply, model I.P. 32, Heath Company, Benton Harbor, Mich., is inexpensive and has proven to be satisfactory. The Spinco Duostat DC power supply (Beckman Instruments, Inc.) with both voltage and current regulation, 50 ma output, is adequate for use with the E.C. cell.

Cronar polyester film, order code 41, thickness 0.004 inch, clear base, subbed one side, in a roll 12 inches × 100 ft, was obtained from E.I. du Pont de Nemours & Co., Inc., Wilmington, Del. From this roll strips 35 × 150 mm, or large sheets 13 × 35 cm are cut with a paper cutter. The cut strips are flattened by placing them on a glass plate in a drying oven at 80–85°C.

Agarose–Agar Solution

Agarose powder, Cat. No. 83901, was purchased from Bio-Rad Laboratories, Richmond, Calif.; Ionagar powder No. 2, Cat. No. L12, from Consolidated Laboratories Inc., Chicago Heights, Ill.

Barbital buffer, 0.05 M, pH 8.6 was prepared by dissolving 2.76 g of diethyl barbituric acid and 15.40 g of sodium diethyl barbiturate in 1500 ml of distilled water. EDTA is not added to the buffer solution.

Bovine Albumin Powder, Fraction V, was obtained from Armour Pharmaceutical Co., Kankakee, Ill. 25 g of the powder is dissolved in approximately 75 ml of normal saline, and the pH is adjusted to 8.6 with 1 M Tris buffer before the solution is made up to 100 ml.

The agarose–agar solution required in procedures A and B is prepared as follows. 0.25 g of agarose is added to 50 ml of 0.05 M barbital buffer and brought to a gentle boil in a small Erlenmeyer flask lightly stoppered with a glass marble. In a second flask 0.3 g of agar in 50 ml of the buffer solution is also prepared. Magnetic stirring is employed to prevent scorching. When these solutions are clear and colorless they are removed from the hot plate and allowed to cool to 45–50°C. To a beaker containing 40 ml of the solution of agarose is added 10 ml of the agar solution. After mixing, the beaker is placed in the water bath at 45°C. To this mixture 1 ml of the solution of bovine albumin is added with stirring.

Gel and Sample Application

Procedure A. 10 Cronar film strips are placed on a section of plate glass which has been warmed under hot tap water and placed on a *level* surface. A few drops of water under each strip hold them in place and prevent warping during the addition of the hot agarose mixture. A template drawn on white paper and placed under the glass plate defines the boundaries within which the agarose mixture will be spread on the strips and indicates the line of origin along which the chrome steel rods, 2 mm in diameter and 20 mm long, forming the sample troughs, will be placed.

4 ml of the agarose mixture is drawn into a preheated (about 60°C) pipette and delivered onto the central two-thirds of the first strip. The tip of the pipette is used to spread the solution quickly from edge to edge and to within 1 cm of each end of the strip as indicated by the template. Immediately after the gel has been spread a chrome steel rod is placed in the liquid agarose mixture about 35 mm from one end of the strip on the origin line drawn on the template. All 10 strips are coated within 10 min. About 15 min after the last strip has been coated the agarose mixture will have gelled sufficiently on the first strip to allow the metal rod to be removed. It is gently moved to free it from the gel and is picked up with a magnet.

While the agarose mixture is cooling on the film strips, the serum samples are prepared with the aid of a micropipette and pipette control. Disposable pipettes were obtained from Scientific Products, Flushing, L.I., N.Y. They are recalibrated to deliver 50 μl between marks. The hand-operated micropipette control, Cat. No. k-76361, was obtained from Kontes of California, Berkeley, Calif. 50 μl of serum is transferred by means of the micropipette, with its control, into a small test tube in a water bath at 45°C. 25 μl of the warm agarose solution is added. The tube is mixed briefly and replaced in the water bath. 50 μl of the sample–agarose solution (containing 33 μl of serum) is applied carefully but quickly to the trough in the gel. The solution is spread evenly as the pipette is emptied. One of the extra strips is used if an application is unsatisfactory. Several minutes are allowed for the last sample to gel in the trough before the strips are placed in the electrophoretic cell, the origins being aligned along the cathodal side.

Double thickness filter paper wicks (Whatman No. 3, chromatographic grade) are applied to complete the electrical circuit between the wicks in the buffer well and the gel. These wicks must be immersed in the buffer for at least 24 hr before use and stored in a tray of buffer between uses. Two wicks, one placed on top of the other, are employed to decrease the resistance to the flow of current. The wicks overlap the gel by approximately 1 cm. To 3 ml of the bovine albumin solution a few crystals of bromophenol blue are added. A drop of this dyed albumin solution applied to the gel surface of the first and eighth strip at the origin serves to mark the extent of the

migration of albumin during electrophoresis. The cell is closed. Water from the circulating bath at 25°C is passed through the cooling compartment of the cell. A constant current of 10 ma per strip is applied for approximately 2 hr, or until the dye marker has migrated 70 mm. In order to maintain constant current, if a constant voltage power supply is used, the operator should reduce the voltage after 15 min and perhaps again after 30 min, after which the current should remain constant at 10 ma per strip.

Procedure B. In procedure B a large sheet of Cronar film, 15 × 35 cm, is coated with the agarose–agar mixture as follows. The 13 × 34 cm area to be covered by the gel is outlined on the film strip with a waterproof glass marker (Sanfords). The lines drawn by the marker form a barrier beyond which the agarose mixture will not spread. A glass plate is heated as before under hot tap water and placed on a *level* surface. The corners of the Cronar sheet are taped to the plate.

The agarose–agar mixture containing bovine albumin is prepared in the manner described above. Onto the central two-thirds of the film strip 50 ml of this solution is poured from the beaker and spread rapidly over the area outlined with the tip of a pipette. The template, Fig. 1, which forms the wells is placed in position about 15 mm inside and parallel to one of the long edges of the liquid gel. The template is constructed by soldering brass rods 2 × 15 mm, spaced 8 mm apart, to a 35 cm length of rectangular brass stock, $^1/_8$ × $^3/_8$ inches. The transverse plates at each end of the template are fitted with brass machine screws, No. 4–40, and lock nuts. The screws are adjusted so that the brass rods make an indentation on the surface of the gel. These wells should be deep enough to contain 20 μl of plasma but should not extend below one half the thickness of the gel.

After 15–20 min the template is removed. The film strip is placed in the electrophoretic cell; 20 μl of undiluted serum or plasma is delivered directly into the wells from a Hamilton microsyringe. The buffer-saturated wicks and dye marker are applied and the cell is closed. 15 min are allowed for the serum sample to diffuse into the gel; a current of 80 ma (approximately 200 v) is then applied. Under these conditions the dye marker will migrate approximately 50 mm in 2 hr.

Fixing and Staining

In procedure A, after electrophoresis has been completed, the individual strips are placed in a staining rack and are immersed for 30 min in a tray of fixing solution consisting of 5% glacial acetic acid in 75% ethanol. The fixing solution appears to partially dehydrate the gel and to remove the buffer salts. Upon removal from the fixing solution, the under sides of the strips are dried before they are placed on a glass plate in the drying oven at 80–85°C for 20 min. The surface should now appear completely transparent. If a few buffer crystals remain on the surface, they will not interfere with scanning. Their presence, however, indicates that the fixing solution should be discarded.

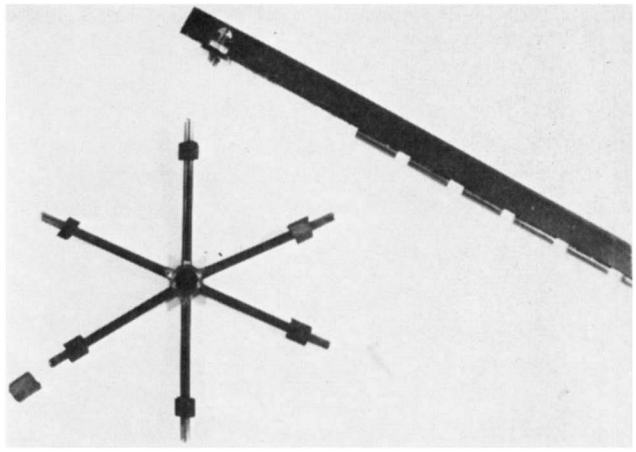

FIG. 1. In the upper portion is shown the template used in procedure B for making the sample wells in the agarose gel. The short brass rods are 15 mm long and are spaced 8 mm apart. The depth of the wells is made one half the thickness of the gel by adjustment of the screws on the cross plate on each end of the template. The wells should accept 20 μl of sample.

The hub and spoke assembly shown in the lower part of the figure is used for rotating the gel strips in the dye solution. A motor mounted on the top of the plexiglass cover plate of the dye container rotates the assembly at 1–2 rpm. Rotation of the strips in the dye solution reduces the staining time.

If the procedure of rotating the strips in the dye is followed, holes are punched in the anodal end of the film strip and the strips are hung singly or in pairs from the spokes of the rotating assembly, Fig. 1. This apparatus is rotated at 1–2 rpm by a motor mounted on the top of a plexiglass plate covering the 2 liter beaker used as the staining cylinder. Small sections of rubber tubing are fitted on the spokes to secure the strips in place.

Sudan Black B, Cat. No. 629 and Oil Red O, Cat. No. 689, were purchased from the National Aniline Div., Allied Chemical Corp., New York. A stock solution of Sudan Black B is made with 200 mg of dye per 100 ml of 60% ethanol. After mechanical agitation for 60 min at room temperature the stock solution is allowed to stand overnight. The undissolved dye will settle out and filtration is unnecessary. A 1/100 dilution of the stock solution will have an optical density (OD) of 0.28–0.30 when read in the Beckman DU spectrophotometer at 590 mμ, 1 cm light path. A working solution is made by diluting the stock solution with an approximately equal volume of 60% ethanol to a final concentration of 14.0–15.0 units of OD, i.e. a 1/100 dilution of the working standard has OD 0.14–0.15 at 590 mμ. The working solution will remain at this concentration for several weeks at room temperature regardless of the number of strips stained.

We discard a working solution when its OD falls below 13.0.

The dye solution of Oil Red O is made by saturating 60% ethanol with the dye according to the description of Jencks and Durrum (6). This solution is used directly as the working solution. It requires storage at 37–40°C to maintain maximum concentration during the 3–4 wk before it is discarded.

The strips are rotated for 1 hr in Sudan Black B or for 2 hr in Oil Red O. Otherwise the strips are replaced in the staining racks and immersed in either of the dye solutions for 6 hr. After removal from the dye, the strips are washed under running tap water, while the soft gel surface is rubbed gently with the finger tip. The strips are then placed, gel side down, on blotting or filter paper as the under surface of each is cleansed with a cloth moistened with 95% ethanol.

In procedure B the fixing and staining procedures for the large strip are essentially the same as described for procedure A. If three holes are punched in the long side across from the sample wells, the sheet can be hung from and rotated by the hub and spoke assembly for 30 min in the fixing solution. After removal from the drying oven, the sheet is resuspended and rotated in the Sudan Black B dye solution for 1 hr.

Densitometry

The Spinco model RB Analytrol, Spinco Div., Beckman Instruments, Inc. Palo Alto, Calif., provides for densitometric scanning of stained electrophoretograms with automatic integration of the peaks of optical density. The B-2 cam is employed. For Sudan Black B a 600 mμ interference filter is placed in the front filter holder; none in the rear. A simple, custom-made film strip drive is mounted on the side of the instrument and reduces the scanning speed from 8 to 4 cm/min. Alternatively, the Microzone scanning attachment, Spinco part No. R102, may be employed after the aperture slot in the film strip carrier has been lengthened by 0.75 cm at each end.

Control Strips

Control strips containing a fixed concentration of human serum are made as follows. 0.25 g of agarose is brought to a boil in 50 ml of distilled water. When this solution has cooled to 45–50°C, 5 ml of control serum or serum from a subject with normal serum lipid levels and 1 ml of the Fraction V solution are added. 2 ml of this mixture is applied to the central 70 mm of 25 Cronar film strips. A level surface for this application is imperative. The gel is allowed to dehydrate at room temperature. One control strip is stained and scanned each day the dye is used. This serves as a check on the dye uptake by lipoproteins of known concentration.

Miscellaneous

Additional materials include several rectangular pieces of 1/4 inch plate glass, 14 × 9 inches; a constant temperature circulating water bath at 25°C for cooling the electrophoresis cell; a constant temperature water bath at 45°C to be used in the preparation of serum samples in procedure A; an electric hot plate with magnetic stirrer for preparing the agarose and the agarose–agar gels; and Spinco staining racks and plastic staining trays, parts No. 300-827 and 300-835.

RESULTS

The results of lipoprotein electrophoresis in agarose–agar gel are shown in Figs. 2–4. In Fig. 2 aliquots of serum from a normal subject and from patients with Type II–V hyperlipoproteinemia (2) were separated into lipoprotein fractions in agarose gel (*a*) and on filter paper (*p*). In the normal subject whose plasma sample is shown at the left, there was a faint pre-β band visible on the paper strip, which does not appear in this photographic reproduction. In agarose, however, the pre-β lipoprotein in this normal subject is clearly visible. A pre-β band has been clearly visible in all agarose gel electrophoretograms carried out on plasma samples from more than 500 subjects, including 200 teenagers with generally low serum lipid values. In Fig. 2, the second pair of electrophoretograms from the left show an unusually prominent β band, and demonstrate the pattern characteristic of Type II abnormality. The clearly defined β and pre-β bands are seen in the agarose strip.

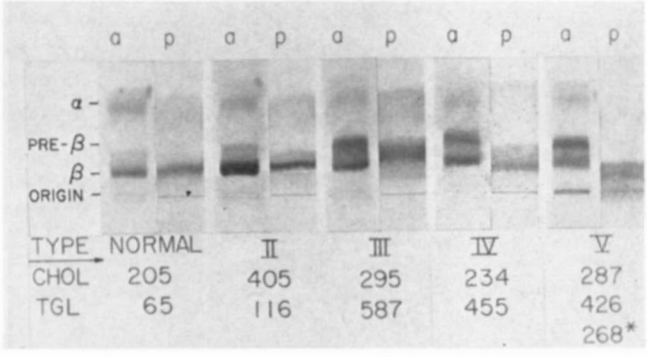

FIG. 2. Serum electrophoretic patterns, stained with Sudan Black B, from a normal young male subject and from subjects with Types II, III, IV, and V hyperlipidemia (2). In each case the pattern on the left, *a*, was obtained with the use of the agarose mixture as the supporting medium, whereas on the right, *p*, filter paper was used. The corresponding values for total serum cholesterol (CHOL) and triglyceride (TGL) are shown. For Type V the value is also given (asterisked) for the triglyceride content in the chylomicron fraction (d < 1.006, 10,000 *g* for 10 min).

Although these photographic reproductions of the lipoprotein bands are less than optimal, particularly for the paper technique, the wide separation of the pre-β from the β lipoprotein band in agarose is apparent. When chylomicronemia is mild to moderate, as in Type V, the alimentary particles remain at the origin.

In our series we had four patients with Type III hyperlipoproteinemia. In two of these the β and pre-β regions stained more intensely than the portion between them, and the densitometer scan revealed a peak at each end of the plateau. An example of this type of pattern is seen in Fig. 2, Type III. In contrast, in Fig. 4, sample *1–2* the broad band of uniform density that is more generally characteristic of this lipoprotein pattern is demonstrated. In both kinds of Type III patterns, however, the lipoproteins with both β and pre-β mobility had densities <1.006 in the ultracentrifuge (2).

In Fig. 2, the fourth agarose strip shows the lipoprotein pattern characteristic of Type IV patients, in which the pre-β band is broader and stains more densely than the β.

Our series of patients contained only two with the Type V abnormality. The example shown in Fig. 2 was obtained from one of these patients 36 hr after admission to the hospital for one of his many bouts of abdominal pain. At this time the chylomicronemia was moderate in extent. The chylomicrons in the serum sample remained at the origin.

Fig. 3 illustrates the variation in mobility of the pre-β lipoprotein. The first example represents the type of pattern often seen in subjects with familial Type II hyperlipoproteinemia, in which the slow pre-β band does not separate widely from the β. In example *2* the pre-β band has intermediate mobility whereas in example *3* a fast pre-β fraction is demonstrated. Fast-moving fractions have been seen after fat feeding in normal subjects and in subjects treated with Clofibrate (a hypolipoproteinemic drug). The fourth example, showing two pre-β bands, occurs frequently but not invariably in Clofibrate-treated subjects, as well as in some normal subjects. The variation in pre-β mobility is also seen in Fig. 4.

The results of the electrophoretic separation of several samples on one large sheet of Cronar film as described in procedure B is demonstrated in Fig. 4. The excellent reproducibility between duplicate samples may be assessed visually. Since the agreement between duplicate densitometric scans has also been good, we no longer carry out electrophoretic analyses in duplicate. It should be noted that for densitometry this large sheet may be cut into individual electrophoretograms with a paper cutter.

The data presented in Table 1 shows the effect of varying the gel concentrations upon the migration of lipoproteins. Portions of a serum sample from a normal subject and of another sample from a patient with Type IV hyperlipoproteinemia were used. The data show that the pre-β lipoproteins migrate further from the origin with decreasing concentrations of agarose. Although the migration of the β-lipoprotein increases somewhat under these conditions the net effect of decreasing the gel concentration is to increase the distance between the β and pre-β peaks. In Table 1, column 5 (β migration) demonstrates that in 0.6% agarose and in the agarose–agar

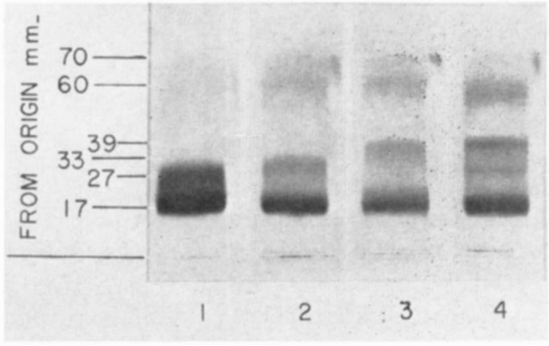

FIG. 3. Variation in mobility of the pre-β bands. Slow, intermediate, and fast-moving fractions are seen in samples *1*, *2*, and *3*, respectively. In example *4* the fast-moving pre-β bands appeared after Clofibrate therapy in a subject with Type IV hyperlipoproteinemia (2). The slow-moving pre-β lipoprotein in example *1* has been frequently seen in subjects with familial Type II hyperbetalipoproteinemia.

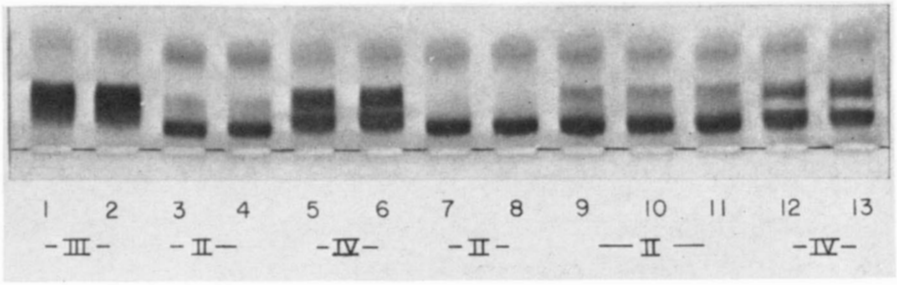

FIG. 4. Electrophoresis in agarose gel performed on a large sheet of the plastic film Cronar. The patterns were obtained from hyperlipoproteinemic subjects. The plasma samples were applied in duplicate (in triplicate in channels *9*, *10*, and *11*). Example *1–2* demonstrates the lipoprotein pattern seen in Type III subjects; *3–4* that in Type II; *5–6*, Type IV; *7–8*, familial Type II; *9–10–11*, Type II patient during treatment with Clofibrate; *12–13*, Type IV. The variation in mobility of the pre-β-lipoprotein bands is evident. The fast-moving pre-β-lipoprotein seen in *9*, *10*, and *11* is a characteristic feature seen during Clofibrate therapy.

TABLE 1 RELATION OF THE CONCENTRATION OF AGAROSE AND OF THE AGAROSE–AGAR MIXTURE TO THE MIGRATION DISTANCE OF LIPOPROTEINS IN 0.05 M BARBITAL BUFFER

Lipoprotein Pattern Type	Gel Type and Concn	Distance from Origin to Peak of			Separation between β and Pre-β Peaks
		α	Pre-β	β	
		mm			
Normal subject	Agarose				
	1.2%	124	52	32	20
	1.0%	117	58	37	21
	0.8%	125	65	35	30
	0.6%	123	72	34	38
	Agarose–agar 8:2	122	73	35	38
Type IV	Agarose				
	0.8%	129	50	27	23
	0.6%	125	74	41	33
	Agarose–agar 8:2	128	75	42	33

The distance of migration of the individual peaks was measured in eight strips for each variable. Average values are shown.

mixture the Type IV β fraction migrated somewhat faster than the normal β. The faster-moving β band may also be seen for the Type IV subjects in Fig. 4, examples *5*, *6*, *12*, and *13*.

DISCUSSION

It has been shown that the results of serum lipoprotein electrophoresis in agarose–agar gel are superior to those obtained by paper electrophoresis. The principal advantages of the gel medium are the clear-cut separations of the pre-β lipoproteins from the β fraction, and the appearance of the stained bands on a transparent, durable plastic strip. This preparation also provides for well-separated peaks with the densitometer, Fig. 5.

The preparation of the agarose–agar mixture, the spreading of the gel on the plastic strip, and the application of the serum samples is more time-consuming than the paper technique. In procedure B on the large sheet, 60 min is required to set up 14 samples. Of these 60 min, however, 20 min are spent waiting for the gel to solidify. During this time another Cronar sheet for 14 samples may be prepared. Thus 28 samples can be set up in approximately 75 min. With the filter paper method it takes 60 min to prepare four Durrum type cells and to apply 32 samples, or 16 samples if duplicate strips are used. Although the use of a fixing solution is an additional step in the gel procedure it does not add appreciably to the time involved. The time required for staining may be reduced from 6 to 1 hr if the strips are rotated in the more concentrated solution of Sudan Black B. It therefore becomes possible to complete the electrophoretic separations and their staining within 1 working day. With regard to the cost and the care required to operate the equipment we have found that there is no appreciable difference between the two techniques.

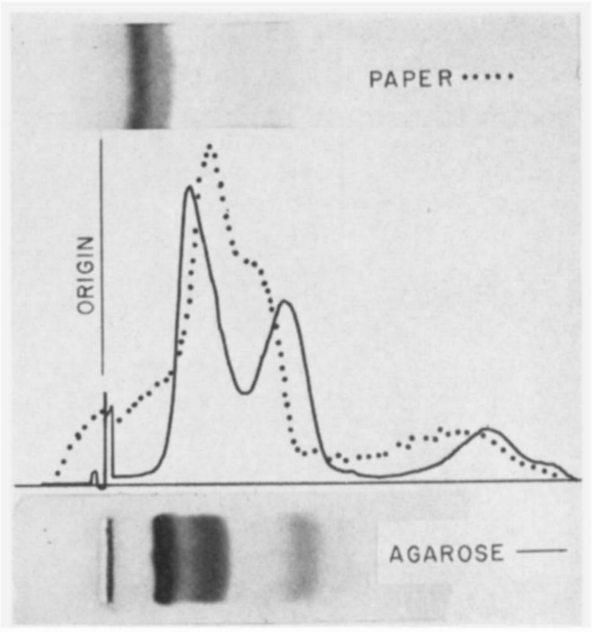

FIG. 5. Densitometer scan of an agarose gel electrophoretogram (solid curve) compared with the scan of a paper strip (dotted curve). Note that the drive speed for the electrophoretogram relative to chart speed has been reduced by one-half, to 4 cm/min. The plasma sample was collected from a Type IV subject 4 hr after fat loading, 2 g/kg body weight. Although this photograph does not show a chylomicron band at the origin in the paper strip, its presence is evident from the densitometer scan. Note the dense chylomicron band, with negligible trailing, in the agarose strip. Correlation of this band with the triglyceride content of the isolated chylomicron fraction has not been successful.

Hatch et al. (7) have reported on the good correlation found between the densitometer scan of paper electrophoretograms and the ultracentrifugal schlieren patterns. A similar comparison has been made with agarose gel as the electrophoretic medium. Because of the clear definition of the pre-β band in this technique there was significant improvement in the correlation between the levels of pre-β lipoproteins and S_f classes 12–250 (8).

Table 1 suggests that the use of agarose at concentrations lower than those employed in this study might result in further resolution of the β from the pre-β fraction, but it is not practicable to employ dilute solutions of agarose. At a concentration of 0.5%, agarose forms a very delicate film at room temperature and must be solidified at 4°C. For this reason it was found helpful to harden the agarose gel by the addition of 0.6% agar as described. This is particularly important in procedure B where the notched bar is employed to produce the sample wells in the large gel sheet. Agarose alone at a concentration of 0.6% does not form a gel rigid enough to prevent the edges of the wells from collapsing inward. For use with the individual strips, however, 0.6% agarose without added agar has proven satisfactory.

The molarity of the buffer solution influences the extent of migration and the degree of separation between the lipoprotein bands. In his initial description of the agar technique for the electrophoretic separation of serum proteins, Cawley (4) compared the use of 0.1, 0.075, and 0.05 M barbital buffers and observed that wider separations of the protein fractions were obtained in 0.05 M buffer. We have repeated these experiments and have likewise found that the use of the less concentrated buffer increases the extent of the migration and of the separation of plasma lipoproteins.

The addition of serum albumin to the gel broadens the lipoprotein bands and eliminates the irregularities in their shape. This produces a smooth densitometric scan. The mechanism of action of serum albumin in this role is unknown. Lees and Hatch have suggested that the adsorptive sites in the paper may be saturated by the excess of albumin present and that this permits uniform flow of lipoproteins (1). This may be the case with agarose. Agar contains a large number of charged groups which result in the absorption of lipoproteins to the agar gel. Pure agarose is an uncharged linear galactose polymer. During its purification, however, a number of sulfate and other charged groups may persist. In the presence of excess albumin these charged sites may be saturated (3). Additionally, the metal-bind capacity of albumin may prevent the oxidation of lipoproteins by cupric ion (9).

The decision whether to perform electrophoresis of lipoproteins on individual strips or on the large Cronar sheet depends upon the degree of definition required and the number of samples to be analyzed. If the densitometric scan of the electrophoretogram is to be used as a means of comparing this technique with another, the procedure of mixing serum with agar before application of the sample produces sharper resolution of the lipoprotein fractions. For this reason the results of lipoprotein separation performed by procedure A was used in establishing the correlation with the results of analytical ultracentrifugation. However, as discussed above, considerably less time is required to set up a large sheet and to apply unmodified serum samples directly in the well. The large sheet may be cut into individual electrophoretograms and each scanned with the densitometer. The scan obtained by this procedure occasionally differs by 10% from the scan derived from the individual strip. This difference appears to be the result of a broadening of the pre-β lipoprotein band and an increase in its distance of migration. For determining the phenotype in an epidemiological study this variation will not be a handicap.

Although we prefer the use of the more concentrated solution of Sudan Black B because it shortens the staining time, current studies in our laboratory indicate that there is no major difference between the uptake of Sudan Black B and of Oil Red O by each lipoprotein fraction.

In normal subjects and in patients with Type II, III, and IV hyperlipoproteinemias, chylomicrons that are found in plasma after a fatty meal or after fat loading remain at the origin as they do on paper. When a small amount of trailing occurs the densitometer trace does not reach the base line between the chylomicron peak and the β peak, as shown in Fig. 5.

Under certain conditions involving dense chylomicronemia, trailing of lipoproteins from the pre-β region to the origin will occur in the gel as well as on paper. In normal subjects, or subjects with the Type IV abnormality, after a fat meal of 2 g/kg body weight, the level of chylomicrons in blood rarely exceeds 300 mg/100 ml as chylomicron triglycerides. At this concentration the chylomicrons will remain at the origin and little or no trailing will be seen in the electrophoretogram. In our experience plasma samples containing 1500 mg/100 ml or more of triglycerides will show no trailing provided the triglyceride is confined to the pre-β fraction. There are two unusual conditions, fortunately both rare, in which extensive trailing has been observed. In the Type I abnormality and in patients with Type V disease before or during an acute episode of abdominal pain, chylomicronemia is very dense. Electrophoretograms made from these samples containing 1000 mg/100 ml or more of chylomicron triglycerides show considerable trailing. Attempts to circumvent the trailing by diluting the plasma sample with normal saline or with an albumin solution have been unrewarding primarily because the β, pre-β, and α lipoproteins become equally diluted and appear as faint bands in the stained electrophoretogram.

Another complication encountered during the gel electrophoresis of samples containing excessive quantities of chylomicrons is the observation that the gelled sample at the point of origin becomes reliquified 1 or 2 hr after electrophoresis has been started. To avoid this difficulty most of the chylomicrons should be removed by gentle centrifugation (10,000 *g* for 30 min) or by allowing them to form a layer at the top of the test tube. Electrophoresis of the subnatant plasma proceeds normally despite some trailing.

Rapp and Kahlke (10) have recently reported a method for the separation of lipoproteins in 0.8% agarose gel which is very similar in principle to the technique presented here. There are, however, several points of difference. Instead of using the Cronar film strip, the authors spread the agarose and performed the electrophoresis of 48 samples on a large glass plate. The densitometric trace or a photograph of the lipoprotein bands constitute the permanent record. Albumin is not added to the agarose, with the result that the lipoprotein bands are narrow and take on a crescent shape (3). For calculation of the relative mobilities of the lipoproteins

vitamin B_{12} is added to the dyed albumin mixture. The vitamin is carried from the origin by electroendosmosis in the direction of the cathode (opposite to the electrical migration of the lipoproteins) and provides a marker from which migration distances may be calculated.

Note Added in Proof. In procedure A, if 1.0% agarose is used to dilute the serum or plasma sample, chylomicrons, if present, remain evenly distributed in the sample well and are not concentrated at the anodal lip of the well as shown in Fig. 5. The increased concentration of agarose used for this purpose also minimizes trailing, but does not change the migration rate of the other lipoprotein bands. We now use this modification routinely.

A major contribution to the perfection of this technique was made by Dr. Frederick T. Hatch, Lawrence Radiation Laboratory, Livermore, Calif., who noted improved resolution of the lipoprotein bands after serum albumin was added to the agarose gel mixture.

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