Rapid Large-Scale Screening of Blood Proteins by Use of Cellulose Acetate Electrophoresis on a Solid Support

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We describe a new principle of cellulose acetate electrophoresis (solid-support electrophoresis), in which cellulose acetate membranes are given a solid support, instead of being suspended as heretofore. The new technique is particularly well suited for screening procedures, because many samples can be processed economically on a single run. Also described for the first time, in conjunction with solid-support electrophoresis, is a method of (a) simultaneously using continuous and discontinuous buffer systems with specially (series) oriented membranes, or (b) a single-sample application for fractionating different constituents simultaneously (proteins, lipoproteins, isoenzymes, etc.).

Additional Keyphrases screening procedure · multiple-constituent determination in a single run

Analysis of serum proteins by electrophoresis has become routine in most clinical laboratories since cellulose acetate was introduced as a supporting medium. Little progress has been made during the past decade, however, in improving the design of the equipment used with these membranes or the techniques associated with their use. Thus, commercial equipment such as the "Microzone Cell" (Beckman Instruments, Fullerton, Calif. 92634), which enjoys widespread use, suffers from a number of disadvantages: it cannot be used to screen large numbers of specimens, cost of equipment is relatively high, and an inordinate amount of technologists' time is required. Furthermore, over half of the membrane, potentially useful for specimens, is wasted, owing to the perforations used to suspend them.

We describe here:

1. A new method of cellulose acetate electrophoresis, in which a solid support is used ("solid-support cellulose acetate electrophoresis") instead of suspending the membranes.

2. The construction of an electrophoretic apparatus for "solid-support cellulose acetate electrophoresis" that requires only minutes to assemble from readily available materials at a cost of less than $5.

3. A method of analyzing a large number of samples rapidly with the new procedure.

4. A method for using cellulose acetate membranes more economically by applying the specimens across the largest dimension.

5. A method of using cellulose acetate membranes one on top of the other, so that a single sample application can be used to simultaneously determine multiple constituents such as isoenzymes, proteins, haptoglobins, and glycoproteins.

Materials and Methods

Electrophoretic Unit

The assembled unit (Figure 1) consists of three interlocking kitchen plastic containers ("Rubbermaid Instant Drawer Organizer," Rubbermaid, Inc., Wooster, Ohio) measuring 9 × 3 × 2 cm (cat. No. 2915) or 30 × 3 × 2 cm (cat. No. 2917), the outer two containers serving as buffer reservoirs and the inner container serving as an "ice box" for cooling the membrane; a 10 × 20 cm, 20 × 20 cm, or 10 × 30 cm glass plate to serve as a bridge between the buffer reservoirs; and two removable electrodes made from 28-gauge platinum wire wound about plastic rods 9 cm long and 0.5 cm in diameter. The smaller chambers (9 × 3 × 2...
cm) were housed in a plastic sweater-storage box measuring 34 × 27 × 8 cm, whereas the larger containers were covered by a plastic tray (both available from Sears, Roebuck & Co., Chicago, Ill.).

“Sepaphore III” 5.7 × 14.6 cm (punched and unpunched; Gelman Instrument Co., Ann Arbor, Mich. 48106) and cellulose acetate strips 2.5 × 30 and 5 × 20 cm (Schleicher and Schuell, Inc., Keene, N.H. 03431), respectively, were used as the supporting medium. For quantitation, a densitometer (cat. No. 552) with automatic printout (Photovolt Corp., 1115 Broadway, New York, N.Y. 10010) was used. The data were fed into a desk computer (Smith-Corona Marchant, 299 Park St., New York, N.Y. 10017), programmed for calculating percentages and grams of individual fractions per 100 ml.

Multisample Applicators and Specimen Holders

Two types of multiple sample applicators were used (Figure 2):

1. Multisample applicators made from stainless steel, which were essentially similar to those described by Kohn (1).

2. Modified Cordis applicators (Cordis Laboratories, P.O. Box 428, Miami, Fla. 35127). These plastic applicators were modified by removing segments of plastic so that spacing of the “teeth” in the applicators was similar to that in those just described.

Sample holders were made from acrylic plastic. Grooves were cut on both surfaces of each plate so that five rows of platforms (labeled A through E) were created on each surface, i.e., 20 platforms per row on one surface (numbered consecutively) and 10 platforms per row on the opposite surface. Individual platforms (for each specimen) were spaced so they meshed with one of the teeth of the applicators (Figure 2). The sample holders were stored in an airtight plastic box with shelves, to minimize evaporation of specimens.

Methods

Preparation of cellulose acetate membranes for electrophoresis. All membranes were wetted from below in barbital buffer (pH 8.6, 0.5 mol/liter) except those used for separation of hemoglobin variants and of serum haptoglobins; the latter were wetted with TBES buffer¹ (60 mmol/liter, pH 9.1) before an electrophoretic run.

Membrane orientation on bridge. Series-oriented membranes: When the Sepaphore III membranes were used, three were positioned separately on a 20 × 20 cm glass plate, parallel with one another and 1 cm apart, so that the migration path measured 5.7 cm for each membrane. The membranes were connected in series (intermembrane wicks) by a double thickness of wetted folded paper toweling (Selffold Towels, Fort Howard Paper Co., Green Bay, Wis.; folded edges were removed with a paper cutter and reused indefinitely) measuring 2 × 7 cm (Figure 3). The plate containing the membranes was used as a bridge between the buffer reservoirs by positioning it over the edges of the middle chamber. The membranes were cooled from below by placing ice in the middle chamber (“icebox”). Reservoir wicks, 16 × 10 cm, also cut from paper towels (“Selffold”), were wetted in the reservoirs and positioned so that about 1 cm of one end of each wick overlapped the edges of the two outside membranes; opposite ends of the wicks were inserted into the buffer reservoirs, which contained about 200 ml of barbital buffer (pH 8.6, 60 mmol/liter). A four-inch photographic roller (available at most photographic shops) was used to squeegee several thicknesses of dry paper towel

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¹ Nonstandard abbreviations used: TBES, tris(hydroxymethyl)aminomethane-sodium ethylenediaminetetraacetate-borate; TLC, thin-layer chromatography (i.e); PAS, periodic acid-Schiff reagent; LDH, lactate dehydrogenase.
over the cellulose acetate membranes, including the edges of the wicks, so as to force out all excess fluid between the membranes, wicks, and glass plate, while simultaneously causing the membranes to become flattened and intimately attached to the TLC plate. The paper towels, which soaked up the excess buffer, were then discarded. The maneuver was repeated if any surface fluid remained. Electrodes were then placed in the anodic and cathodic reservoirs. On occasion, two electrophoretic units were run in parallel; when this was the case, the first unit was allowed to equilibrate (with current flowing) during the time the second unit was being readied. After equilibration (5 min) the current was turned off and the samples applied to all membranes as described below. When few specimens (14 or less) were to be analyzed, the single membrane to be used was positioned in the center of a 10 × 20 cm glass plate.

For a larger number of specimens (as many as 84), six cellulose acetate membranes were subjected to electrophoresis simultaneously on a single 20 × 20 cm TLC plate by use of the following procedure: Two 1 × 15 cm strips were cut from the edges of a paper towel with a paper cutter. Each strip was wetted in buffer solution, and the strips were placed so as to lay flush with the cathodic and anodic ends of the glass plate, respectively (referred to as “cathodic and anodic connecting wicks”).² The towelling was then squeegeed into place so that all excess buffer was removed. For convenience, these wicks were left in position for reuse after an electrophoretic run. Two membranes (cathodic and anodic) were then placed into position so that one edge of each overlapped the cathodic and anodic connecting wicks, respectively. A third membrane was then placed between the two membranes. All three membranes were then connected by wetted intermembrane wicks. The membranes and wicks were then squeegeed onto the TLC plate so as to remove all excess buffer, and fourteen specimens were applied to each of the three membranes as described. The plate was inverted and three additional membranes with connecting wicks were positioned and squeegeed onto the opposite surface of the plate as described. Several thicknesses of paper toweling, 21 × 5 cm, were wetted with buffer and draped over the rounded upper edges of the buffer chambers, to serve as anodic and cathodic reservoir wicks, respectively. The edges of the glass plates were then laid over the rounded lips of two buffer reservoirs so that the anodic and cathodic connecting wicks (located on one surface of the TLC plate) came into contact with the reservoir wicks. A second set of reservoir wicks were then positioned over the upper anodic and cathodic membranes as already described. Fourteen specimens were then applied to the upper three membranes (a total of 84 specimens per plate). If desired, two units could be run in parallel (total of 168 specimens).

PARALLEL-ORIENTED MEMBRANES: Three to five wetted membranes were superimposed on one another. The edges of the membranes (longest dimension) were placed between the leaves of two wetted, folded paper towels cut to 15 × 1 cm. All were simultaneously squeegeed onto a 10 × 20 cm TLC plate before an electrophoretic run, then electrophoresed as described above. Occasionally, a second set of membranes was placed on the opposite side of the plate as described above for series-oriented membranes. Runs were carried out at 400 V and 15–20 mA for about 45 min (single set of membranes).

The larger (5 × 20 cm size) membranes were used as described above, except that more specimens, 23, were applied per membrane. The narrow size (2.5 × 30 cm) membranes were used for screening hemoglobin variants, haptoglobins, and hepatitis-associated antigen (Australia antigen) (Figure 4), because the respective components migrate relatively short distances. On occasion the latter membranes were used to screen serum proteins (Figure 5).

Preparation of specimens. To “label” the specimens, 1–2 dry granules of bromphenol blue powder were added to each numerically labeled tube of serum. A drop of each specimen was then placed on the specimen plate in numerical order.

Application of specimens. SERIES-ORIENTED MEMBRANES (SINGLE SURFACE): After the electrophoretic system was equilibrated, the power supply was turned off, a multispecimen applicator

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² Wicks were reused indefinitely.
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to a specimen plate, after which any excess specimen accumulated on the sides of the applicator was removed with a piece of tissue paper, wiped vertically. Specimens were then applied by placing the applicator firmly against the membranes for about 15 s. They were positioned as follows:

Cathodic membranes: on an imaginary line joining the cathodic 1/4 of the strip with anodic 1/4 in the middle
Middle membrane: on an imaginary line joining the cathodic 1/4 of the strip with the anodic 1/4
Anodic membrane: on an imaginary line joining the anodic 1/4 of the strip with the cathodic 1/4

Adequacy of sample application could be easily judged by the bromphenol blue dye “label.” On a few runs, sera were applied to only two of the three membranes, a hemoglobin preparation being applied to the third membrane.

Parallel-oriented membranes: Samples were applied with the modified Cordis applicator. A double application was used (one on top of the other).

Electrophoretic technique. A potential of 250 V was applied for 20–30 min. When two electrophoretic units were run concomitantly, they were connected in parallel and the higher voltage mentioned above was used (no safety “interlock” switch is described with the apparatus, so due caution must be used in operating it). After a run was completed, membranes (either parallel or series-oriented) were removed from the glass plate and stained for proteins, lipoproteins, haptoglobins, or LDH isoenzymes. After staining, the membranes were squeegeed onto individual pieces of “Cronar” film (E. I. du Pont de Nemours & Co., Wilmington, Del. 19898), prepunched along one edge to fit a three-ring notebook. After drying, membranes were cleared in a mixture of acetic acid–methanol (40:60, by vol), dried, and stored in the notebook.

Stains. Ponceau Red S (2 g/liter in a solution of trichloroacetic acid (75 g/liter) and sulfosalicylic acid (75 g/liter)) was used to stain for proteins and PAS for glycoproteins (2), van der Helm’s technique (3) was modified3 for LDH isoenzymes, an ozonation technique was used for lipoproteins (2), and an o-dianisidine method for haptoglobins.4

Results

With the electrophoretic procedures used as described, protein patterns were well resolved on either series- or parallel-oriented membranes or on individual membranes. After staining series-oriented membranes, the point of sample application was found to be in the φ2 area in the anodal strip, the “slow” β-globulin area in the middle strip, and the “slow” α-globulin area in the cathodal strip. Protein fractions migrated about 2.5 cm in 20 min. Pathologic sera and variant-type hemoglobins were readily identified by visual inspection.

With parallel-oriented membranes, two applications of the Cordis applicator (for 30 s) were necessary to supply sufficient sample for three to five membranes. Electropherograms for serum proteins, isoenzymes, and lipoproteins were eminently satisfactory with this procedure. Figure 6 illustrates a representative example of the patterns obtained with LDH isoenzymes. Quantitative data for the different protein fractions obtained for a pooled serum control by the techniques described above were essentially similar to those previously obtained with suspended membranes.

Discussion

That cellulose acetate membranes possess many advantages over filter paper and gels as a supporting medium for the fractionation of serum proteins by electrophoresis is generally recognized, and has led most clinical laboratories to adopt this supporting medium. Use of these membranes has been described for demonstrating isoenzymes (4), lipoproteins (5), glycoproteins (6), hemoglobin

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* Sodium cyanide was eliminated and the quantity of phenazin methosulfate reduced to one-half. Substrate ingredients were combined in 1.5% “Jonagar No. 2,” on which the supporting medium was incubated for 45–60 min at 37° C.

* A hemoglobin C solution (400 mg/100 ml) was used to saturate each specimen before electrophoresis. Specimens were stained with a mixture containing 1 g of o-dianisidine and 6 g of H2O2 per liter (pH 4.7).
variants (7), and haptoglobins (8). Nevertheless, they possess certain objectionable characteristics, such as comparatively great cost, difficulty in handling large sheets of the material, and a relatively large expenditure of technologists' time in applying a large number of samples. Use of a multi-sample applicator to circumvent the last objection has recently been described (1), but this approach is impractical because of difficulty in suspending large, fragile sheets of the material. We have largely eliminated the basis for these criticisms.

As far as we know, all previous apparatuses designed for use with unbacked cellulose acetate membranes have been based on the principle of membrane suspension, a principle necessitating a special means of support, with its attendant complications. The principle described and utilized above contrasts with this—a membrane is intimately attached to a solid glass support so as to eliminate all buffer solution except that present in the membrane, effectively forcing an electric current to flow through the latter. Furthermore, the solid support permits the in situ use of a multi-specimen applicator while simultaneously providing the rigidity needed for optimum, reproducible resolution over relatively short migration distances. Although "Mylar"-backed membranes (Helena Laboratories, 1530 Lindbergh Dr., Beaumont, Texas 77704) may be used without suspension, these are relatively expensive and possess other shortcomings: the cellulose surface tends to peel away from the Mylar backing, there is difficulty in wetting, and the plastic backing acts as an electrical insulator, preventing the membranes from being used on top of each other ("parallel" orientation). We emphasize the fact that the use of "parallel" or "series"-oriented cellulose acetate membranes has not heretofore been described and represents a new approach to the use of this supporting medium. These innovations not only permit samples to be analyzed for various blood constituents on a single run, but also permit the concomitant use of multiple buffer systems—continuous or discontinuous, acid and alkaline.

Finally, a significant decrease in technologists' time and costly materials may be expected in the routine fractionation of serum proteins with the techniques described. With respect to materials, 30-cm lengths of membranes are more economical than shorter lengths, especially when purchased in the narrow (2.5 cm) width. Narrow membranes are particularly economical for screening blood specimens for hemoglobin variants, haptoglobins, hepatitis-associated antigen, and the like, because the respective components move relatively short distances, which makes use of wider strips relatively wasteful. The time required for analysis is substantially decreased when multiple studies are carried out simultaneously (e.g., determination of LDH isoenzymes and variant haptoglobins) with "parallel orientation" of the membranes. The innovations described above seem to provide a significant improvement in cellulose acetate electrophoresis for the clinical laboratory.

References


* i.e., the buffer in the reservoir and in the supporting medium can either be the same or different.