Agarose Gel Electrophoresis

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Introduction

A system for plasma protein fractionation and for nomenclature according to charge has been inherited from Tiselius’ technique of moving boundary electrophoresis. The nomenclature was maintained on transition to zone electrophoresis with filter paper as supporting medium. The clinical use of electrophoresis in plasma-protein analysis is still based on the simple electrophoretic separation of plasma proteins, according to their relative mobility, into albumin, α₁, α₂, β, and γ-globulins in spite of the knowledge that each of the classical electrophoretic zones contains two or more major plasma proteins that often are subject to independent metabolic regulation. Specific analysis of individual proteins is increasingly used as a supplement to electrophoretic analysis.

Thirteen plasma proteins together comprise more than 95% of the serum protein mass. Their distribution on agarose electrophoresis and their average relative concentrations are given in Figure 1. Each classical electrophoretic zone contains between two and six of these 13 components. Some proteins are spread between more than two of the electrophoretic zones. Therefore, scanning the zone pattern gives good information about only two of the predominant proteins, albumin and IgG, which represent about 90% of the protein in the albumin and γ zones, respectively. With the naked eye it is easier to detect minor mobility and concentration changes (two-dimensional scanning) from comparative electrophoresis on agarose gel with a parallel series of plasma samples than from the conventional one-dimension scanning diagrams used to calculate proteins in the various zones. With increased complexity in the composition of an electrophoretic fraction there is an increasing risk that the abnormality of a separate component will go undetected by scanning the fraction.

Clinical use of electrophoretic results depends on the resolution achieved for the proteins and on the experience of the interpreter. Resolution of the proteins was improved when cellulose acetate, which had lower protein absorption, was substituted for filter paper as the supporting medium. Techniques such as electrophoresis on starch gel and polyacrylamide, which add a molecular sieving effect, have been of great importance in biochemistry. Nevertheless, identification of components is often complicated if the analyst does not have access to information of clinical diagnostic value.

Wieme (1) introduced cooled agar as a support for clinical electrophoresis. The negatively charged polysaccharide unit cause disturbing electroosmotic water flow, and they interact with some proteins.

Agar is extracted from certain red seaweeds. It can be fractionated into two fractions, one with a high content of sulfate and carboxyl groups (called agarpectin), the other an almost neutral fraction (agarose). The medium-endosmosis type of agarose is suitable for protein separation from most biological fluids. The low-endosmosis type may be valuable for separating certain proteins such as lactoferrin, lysozyme, and cationic proteins from leukocytes.

Electrophoresis on agarose gel with one of two buffer systems, barbituric acid–sodium barbiturate (2) or Tris–barbiturate (3), is a valuable screening method for detection of abnormal distribution of proteins within and between various zones. The agarose electrophoresis can easily be combined with immunofixation (4) for identification of M-components where the separation system has higher resolution than is the case for ordinary immunoelectrophoresis. Grossly abnormal concentrations of proteins such as prealbumin, albumin, α₁-antitrypsin, α₂-macroglobulin, α and β-lipoproteins, transferrin, C3-complement, and IgG are detected by visual inspection.

Principle

The galactopyranose network of a 10 g/L agarose gel allows relatively free electrophoretic mobility, according to charge, of proteins with sizes up to more than 10⁶ daltons. For larger, fibrillar molecules, an increasing resistance to mobility is observed up to five to ten million daltons, where migration is completely inhibited. Thus the gel allows almost free migration of all plasma proteins except chylomicrons, very-low-density lipoproteins, immune complexes, and fibrinogen polymers. The plasma proteins are separated by zone electrophoresis at pH 8.6, so that all major proteins (except some IgG) migrate toward the anode if the gel is free from endosmotic water flow.

The plasma protein separation achieved at a given volts per centimeter value depends mainly on buffer composition and gel temperature. Increasing the concentration of the buffer from 0.04 to 0.10 mol/L decreases the resolution in the β–γ
zone and increases it in the albumin–α2 zone. If Ca²⁺ ions are added to the buffer, resolution of the major β-globulins (transferrin, β₁-lipoproteins, C₃, and IgA) is increased. A cooling system aids in balancing the heat production in the gel during the run. After electrophoresis is finished the proteins are denatured and made visible by staining. Series of samples are run in parallel to facilitate a comparative evaluation (Figure 2).

Materials and Methods

Reagents

Arnse Seakem™ (ME), manufactured by FMC Corp., Marine Colloids Division, Rockland, ME 04841. Several batches with electroendosmosis (mₑ) 0.13–0.19 were suitable.

Electrophoresis buffer: Barbiturate–sodium barbiturate buffer, pH 8.6, 75 mmol/L, with calcium lactate 2 mmol/L. Prepare the buffer with diethylbarbiturate, 10.35 g in approximately 1 L of distilled water heated to boiling; add 2.88 g of calcium lactate and 65.7 g of sodium diethylbarbiturate when the barbitial has dissolved, and dilute to 5 L.

Fixing solution: Add 15 g of picric acid (Merck) to 1 L of water. After storage overnight at room temperature, filter the solution and add 200 mL of concentrated acetic acid.

Destaining solution: Mix 200 mL of glacial acetic acid, 900 mL of methanol, and 900 mL of distilled water. This solution can be reused repeatedly after passage through a column with activated charcoal to remove the dye.

Staining solution: Dissolve 5 g of Amido Black 10B (Merck) in 2 L of destaining solution by heating to 60 °C. Filter the solution after cooling to room temperature and before use.

Indicator solution: Dissolve 2 g of bromphenol blue in 100 mL of ethanol/water (95/5 by vol).

Apparatus

Electrophoresis chamber with a water-thermostated cell (5). Available from Behring Diagnostics; Bio-Rad Laboratories; LKB, Rockville, MD; or MRA Corp., Clearwater, FL. Power supply, minimum 300 V and capacity of 150 mA per electrophoresis cell.

Circulating cooler with flow rate of 1–2 L/min and adjustable between 0 and 20 °C.

Flexible polyester film, Gelbond™, 7 mm thick, available in rolls from FMC Corp., Marine Colloids Division, Rockland, ME.

Sample application foil, available from LKB, Rockville, MD, or Pharmacia Fine Chemicals, Piscataway, NJ. A similar application foil is also included in the precast and prebuffered agarose gel system Panelgel™, from Worthington Diagnostics, Freehold, NJ 07728.

Whatman No 1 filter paper, 1 cm width, in rolls.

U-frame, for casting gels, 205 × 110 × 1 mm, available from Bio-Rad Laboratories or Behring Diagnostics. Glass plate is 205 × 110 × 1.5 mm from Bio-Rad Laboratories or Behring Diagnostics.

Sample-application syringe, 1–5 μL, with stoppers, obtainable from Hamilton or SMI.

Electrode wicks, seven layers of Whatman no. 1 papers, "Wetex" cloths, or "Ultra wicks," from Bio-Rad Laboratories; alternatively, vertical agarose wicks can be used (5).

Collection and Handling of Specimens

Venous blood is drawn into Vacutainer Tubes containing disodium ethylenediaminetetraacetate (Becton-Dickinson). After centrifugation, the supernatant fluid is stored at 5 °C if analysis is to be performed within the next two to three days. Storage at −20 to −26 °C will disturb some of the patterns, but at −70 °C all the native electrophoretic pattern is preserved. Serum may be used if analyzed on the day of blood sampling.

Procedures

Casting the gel. Agarose solution is prepared as a 8 g/L suspension of agarose in barbital buffer. Heat to boiling to dissolve, using an electric heater equipped with a magnetic stirrer. Cool the clear solution to about 50 °C and pipet it into a mold consisting of two thin glass plates enclosing a Gelbond™ polyester sheet. Spray 0.5 mL of ethanol/water (95/5 by vol) on one glass plate and place the polyester sheet of similar size with its hydrophobic side down. (This is tested by seeing the behavior of a drop of water on both sides; the water will "spread" on the hydrophilic surface, whereas it will "bead" on the hydrophobic one.) Make sure that no air bubbles remain between the plastic sheet and the glass plate. Place the U-frame and then the second glass plate, and clamp them together with strong paper clamps. Pour 20 mL of warm agarose solution between the plates. Allow the agarose to solidify, cover the open upper edge with tape, and store in the refrigerator until use. Plates can be stored for at least a week, but resolution is better if the agarose plates are prepared only one day before use.

Sample application. Apply a slip of Whatman No 1 filter paper about 2.5 cm from one long side of the gel where the samples will be applied. Remove the strip immediately after...
Fig. 11. gel slowly detergent syringe, tween the touching 20 in Fig. 6. 10. Increased a-lipoprotein. 9. Decreased Hp and C3. Increased a2M and strongly increased polyclonal IgG (systemic lupus erythematosis). 8. Normal pattern. 7. Increased a-lipoprotein and IgA (alcohol abuse). 6. Decreased Hp, strongly increased polyclonal anodal IgG (IgG3). 5. Decreased Hp, strongly increased polyclonal IgG (IgG3). 4. Increased acute-phase reactants and fast albumin (penicillin). 3. Increased a-lipoprotein. 2. Decreased a-lipoprotein and strongly increased /3-lipoprotein. 1. Slightly increased a-lipoprotein, Hp 2–2, and increased CRP.

Fig. 12. Examples of patterns

1. Slightly increased a-lipoprotein, Hp 2–2, and increased CRP.
2. Decreased a-lipoprotein and strongly increased /3-lipoprotein.
3. Increased a-lipoprotein.
4. Increased acute-phase reactants and fast albumin (penicillin).
5. Hypoalbuminemia, decreased a-lipoprotein and acute phase reactants, hypotransferrinemia, and increased polyclonal IgG (liver cirrhosis).
6. Increased acute-phase reactants with strongly increased polyclonal IgG.
7. Normal pattern.
8. Hypoalbuminemia, increased proteins of high relative molecular mass (glomerular protein losses).
9. Lipolysis, increased acute-phase reactants.
10. Genetic variant, double C3.
11. Decreased Hp and C3. Increased a2M and strongly increased polyclonal IgG (systemic lupus erythematosis).
12. Normal pattern.

Cool water should be circulated through the cell and adjusted to 10–14 °C, to keep the gel temperature during running near room temperature. Electrophoresis is done at 20 V/cm in the gel for 45 (40–60) min, when the blue-stained albumin band has migrated 5.5 cm.

Fixing, drying, and staining. Immediately transfer the plate to the picric acid–acetic acid solution for 15 min. Cover the gel with wet Whatman no. 1 filter paper, taking care to avoid air bubbles between paper and gel. The drying time can be shortened by applying a 1-cm-thick layer of paper towels, upon which is placed a glass plate and a weight of 1–2 kg for about 10 min. Remove the soft papers and dry the gel in a stream of hot air.

Then place the dried gel in the staining solution for 10 min. Rinse the gel plate in tap water and soak it in two baths of destaining solution during 10 min until the background is colorless. Finally, dry the plate again in a stream of hot air.

Technical Discussion

Electrophoresis of plasma proteins does not require a highly purified agarose. Most important is reproducible quality from batch to batch. We therefore recommend that a one- to two-year supply be stocked. Most agarose qualities are not as reproducible as that of Seakem ME. If other batches of agarose are used it is advisable to perform test runs with agarose concentrations ranging from about 7 to 12 g/L, to ascertain optimum separation conditions and efficient tensile strength for easy handling of the gel. A good indicator of resolution is the distinctness of the group-specific globulin bands in front of the a3-macroglobulin fraction (Figure 1). The mobility of lipoproteins and C-reactive protein is related to the agarose quality. Use of the Gelbond polyester film for gel support facilitates handling and storing the finished product.

The sample-application system we recommend is that suggested by Cawley (6) and simplifies the procedure as compared to the well-forming system (5). The application foil is rinsed in isotonic saline and can be reused 100–200 times. Effective cooling by good contact between the gel and the cooled surface is important. A potential gradient in the gel of 15–20 V/cm may be used in most apparatuses. This corresponds to a total field strength of 250–300 V and a current of...
Interpretation of the Agarose Gel Electropherogram in Terms of Variation of Individual Proteins

The ability of the agarose gel electrophoresis to reveal different proteins varies with protein concentration, dye-binding capacity, and degree of electrophoretic homogeneity. The dye-binding capacity generally decreases with increasing oligosaccharide and lipid content. Figure 1 gives a schematic presentation of the relative concentration and the electrophoretic heterogeneity of the major plasma proteins in a healthy subject having the most frequent phenotypes. The individual concentration--mobility profiles are drawn to indicate the degree of electrophoretic heterogeneity and to show the discrepancy between the demarcation of electrophoretic fractions and the range of individual proteins.

Table 1 lists the individual proteins that may be present in such a concentration that they influence the electrophoretic pattern. Those given in parentheses normally are present in such low concentration that they do not influence the pattern significantly. Examples of abnormal electrophoretic protein patterns are given in Figure 2.

100–140 mA, depending on the conductivity of the electrode wicks.

Resolution can be slightly improved if gel thickness is decreased to 0.5 mm and the potential gradient in the gel increased to 30–35 V/cm. However, most commercially available equipment has too low a heat conductivity to give good results at voltages higher than those recommended here.

The temperature of the cooling water must be adjusted to exclude overheating of heat, which is detected by the condensation of water on the underside of the glass plate that covers the electrophoresis chamber. Overcooling, however, gives rise to fluid drops on the gel surface, resulting in a blurred band pattern. The buffer can be reused several times, but the polarity of the current has to be changed every run to avoid pH shifts in the buffer vessels. Albumin focuses in a relatively narrow band, and the high local protein concentration makes it important to have an effective precipitating agent such as picric acid, to avoid blurring of the pattern. Amido Black 10B is used because it gives distinct zones and facilitates the visualization of α- and β-lipoprotein. Coomassie Brilliant Blue is two to three times more sensitive and is of great value when one is analyzing a more dilute protein solution.

The Prealbumin Zone

The proteins in this zone are barely discernible, diffusely demarcated, and vary slightly in mobility. It is difficult to detect any proteins if the pattern is not inspected against a white background under reflected light. Normally the thyroxine- and α2-microglobulin-binding prealbumin is the only protein discerned in this zone. Prealbumin shows a variation of migration and band spread, depending on the degree of complex formation and probably on genetic polymorphism. So far, only the total relative concentration of prealbumin is of clinical interest. In disorders of intermediary triglyceride metabolism or in secondary lipolysis, α-lipoproteins may migrate in the prealbumin zone. Albumin molecules to which acid substances (e.g., drug metabolites) are firmly linked may also appear here.

Pathology. An increased prealbumin fraction may be observed in alcohols. A remarkable concentration decrease within one day is a regular finding as part of the acute inflammatory response (cause unknown). The concentration remains low in active chronic inflammation. Decreased prealbumin is a characteristic finding early in liver cirrhosis. The principle clinical use of prealbumin estimation is in the early detection of decrease in functioning liver-cell mass.

The Albumin Zone

This fraction is broad because the concentration of albumin is higher than that of other proteins, and also because of its electrophoretic heterogeneity, both acquired and of genetic origin. In healthy subjects the albumin fraction has the same width except in the relatively uncommon heterozygotes (prevalence <1:100) for fast or slow albumins. The most common genetic variant is characterized by a cathodal broadening of the albumin fraction. A series of albumin variants with anodal or cathodal widening of the fraction has been observed. Heterozygotes with clear separation of the albumin
into two bands are said to have "bisalbuminemia" (prevalence <1:10 000).

Pathology. Decreased albumin concentration is a common finding caused by changes in intra- to extracellular distribution, water retention, or decreased synthesis. Examples are conditions of acute or chronic inflammatory response, cardiac or renal insufficiency, abnormal intestinal or other external losses, advanced cirrhosis, and advanced stages of malignant disease.

Hypoalbuminemia may also be a sequel to the infusion of dextran or of such high production of immunoglobulins that they contribute to the colloidal osmotic pressure. Hyperalbuminemia is a sign of dehydration.

The electrophoretic mobility and spread of albumin may be influenced by bound exogenous and endogenous charged organic substances. Bromphenol blue, which is often used as a marker during electrophoresis, is recognized in the albumin front. The most common endogenous causes of increased albumin mobility are hyperbilirubinemia, the presence of nonesterified fatty acids, or high intake of aspirin. Penicillic acids are covalently bound to albumin, and a fast albumin fraction with a normal biological half-life is often recognized weeks after parenteral treatment with high doses of penicillin.

Decreased mobility of part of the albumin fraction is usually hereditary, but contamination with heavy metals such as copper may give a similar pattern.

The Albumin–α1 Interzone

This area is usually evenly stained, owing to the presence of the heterogeneous α-lipoproteins. In exceptional cases, α-fetoprotein may appear as a faint band in the middle of the zone. Rapid-migrating α1-antitrypsin variants may appear from the middle of the cathodal limit of the zone. In the latter the color may be abnormally intense because of atypical α-lipoproteins or very high concentrations of orosomucoid.

α1-Lipoproteins comprise one of the most heterogeneous plasma protein groups electrophoretically and chemically. The usual position is from the albumin zone into the α1 band. The mean mobility increases with increasing concentration of non-esterified fatty acids. Normally, the color intensity of the zone between albumin and the α1 band mainly reflects the α-lipoprotein concentration. This intensity is high in women during puberty, after the intake of estrogens, and during pregnancy.

Pathology. α-Lipoproteins on the anodal side of albumin indicate abnormal lipolysis. A high α-lipoprotein fraction with normal or slow mobility is a common finding in chronic alcoholics. Moderate decrease in concentration of the α-lipoprotein fraction is part of the inflammatory response.

A marked decrease of the α-lipoproteins with decreased mean mobility and altered immunoreactivity is a regular finding in acute hepatitis and precedes hyperbilirubinemia during a relapse. Similarly, the α-lipoprotein zone is "faded" in patterns from cases of liver cirrhosis, particularly during a relapse. The α-lipoprotein zone also is less intense during biliary occlusion, in which the concentration of the β-lipoprotein band is increased and the presence of lipoprotein-X may be demonstrated.

Decreased mobility of the anodal α-lipoprotein fraction results in a blurred α1 band. This may be seen in severe malnutrition and in alcoholics, particularly after drinking bouts. A high α-lipoprotein fraction with normal or slow mobility is a common finding in chronic alcoholics. Moderate decrease of the α-lipoprotein fraction belongs to the inflammatory response.

Decrease of the α-lipoproteins accompanies several inherited lipoprotein disorders, such as lecithin:cholesterol acyltransferase (EC 2.3.1.43) deficiency and Tangier disease.

α-Fetoprotein is a normal trace component migrating in the zone between albumin and the α1 band.

Pathology. α-Fetoprotein may cause a faint band when present in 100-fold excess of normal, in sera from cases of primary cancer of the liver or embryonic tumors.

Orosomucoid is slightly heterogeneous and spreads on the anodal side of the α1-antitrypsin band. Because of its low dye-binding capacity (high sialic acid content) it is usually not recognized.

Pathology. The α1 band may appear broadened and slightly fuzzy on the anodal side if the orosomucoid concentration exceeds 2 g/L. It is impossible to judge by appearance whether an abnormally fuzzy α1 band is a result of increased α1-lipoproteins or of increased orosomucoid. An increase of the orosomucoid fraction is a regular finding within one to two days of onset of an inflammation. Increased values are also seen during glomerular filtration insufficiency and often during treatment with prednisolone and other corticosteroid analogs. One of its catabolic routes is disappearance through glomerular filtration because of its low relative molecular mass (45 000). Anodally fuzzy α1 bands are common in uremia.

The α1 Zone

α1-Antitrypsin is responsible for the normal α1 band, which has a minor adjacent cathodal satellite band. This is an expression of the microheterogeneity of α1-antitrypsin. Inherited and acquired mobility variants are common and may cause broadening, slight displacement, or doubling of the α1 band.

About 15% of Caucasians have genetic α1-antitrypsin (protease inhibitor, Pi) variants detectable on agarose gel electrophoresis. Most of these individuals are heterozygotes; half of their α1-antitrypsin appears in the normal Pi M band, half in a second band on its cathodal or anodal side. These variants are of no clinical significance. The most frequent cathodal variants are S with slightly and Z with markedly decreased concentration (60 and 15% of the average, respectively). Homozygotes for S and Z have no band in the normal α1-antitrypsin position. Subjects with the classic α1-antitrypsin deficiency have a scarcely discernible band with slightly decreased mobility. α1-Antitrypsin has a reactive SH-group, and for this reason thiol compounds may be bound and sometimes alter the mobility.

Pathology. α1-Antitrypsin belongs to the acute-phase reactants, increasing one to two days after tissue injuries. Selective or dominating α1-antitrypsin increase is a remarkably valid sign of liver injury and a sensitive sign of increased estrogen, and it doubles, e.g., during pregnancy. A decreased α1 band is an indication for specific measurement of α1-antitrypsin and genetic typing (Pi-typing) (θ).

The α1-α2 Interzone

On the anodal side of the usually well-demarcated α2 band, one to three adjacent faint bands are recognized. They represent primarily the group-specific component, inter-α-trypsin inhibitor, and pregnancy zone protein in pregnant women. When present, haptoglobin of type 1:1 overlaps these minor bands on the anodal side of α2-macroglobulins. In such cases the cathodal part of the α2 zone appears empty.

Pathology. During acute inflammatory response a new band may appear on the cathodal side of α1-antitrypsin. The protein responsible has not been identified.

The bands in this zone are more darkly stained during an acute inflammatory response and during pregnancy. Inspection is not sufficient to ascertain which of the proteins has increased. Complexes between α-chains and α1-antitrypsin

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or albumin may be found in myeloma patients with \( \kappa \)-chain Bence Jones proteinuria. They are linked though an SS-bridge and are cleaved on mild reduction. Free light chains of \( \lambda \)-type may also appear in this zone.

The Broad \( \alpha_2 \) Band

The complex protein composition of the broad \( \alpha_2 \) band zone cannot be settled by inspection, although two proteins dominate, \( \alpha_2 \)-macroglobulins and haptoglobins. Both show such an individual variation and varying zone spread that a quantitative analysis of one of them is necessary for an interpretation of the composition of the fraction. Haptoglobins also show a wider normal range than most other plasma proteins. A distinct front-sharpening of the \( \alpha_2 \) band, however, indicates a major \( \alpha_2 \)-macroglobulin fraction. This front demarcation is usually quite apparent in children and often in the aged. The \( \alpha_2 \)-macroglobulin concentration is also greater during childhood.

\( \alpha_2 \)-Macroglobulin is so dominant in the \( \alpha_2 \) zone during early infancy that variation of other \( \alpha_2 \)-globulins is not recognizable.

Pathology. Increase of the \( \alpha_2 \)-macroglobulin fraction has a low diagnostic significance. It occurs regularly in cases of selective glomerular protein loss, but not in intestinal protein losses.

A slight increase is common in liver cirrhosis, in diabetes, in malignancy, and in older people. Low \( \alpha_2 \)-macroglobulin values also have low diagnostic significance in spite of the key position of the protein as the major acceptor of proteases released into the intercellular space and the short biological half-life of these complexes. No apparent decline of the concentration in plasma is observed in acute pancreatitis, in spite of the increased intra-abdominal \( \alpha_2 \)-macroglobulin catabolism. Low values are common during the final days of life.

\( \alpha_2 \)-Antiplasmin decreases earlier and more markedly than does \( \alpha_2 \)-macroglobulin in pancreatitis.

The great increase of plasma haptoglobin during inflammatory response and the decrease during increased intravascular hemoglobin release (e.g., slight hemolysis, B12 and folic acid deficiency, cancer metastases in liver and bone marrow, sepsis, hepatitis, liver cirrhosis, splenomegaly) make the haptoglobin contribution to the \( \alpha_2 \) zone most variable and a quantitative analysis advisable. Treatment with 17-hydroxylated androgens augments the inflammatory increase of plasma haptoglobin. Malignant plasma cell proliferation may cause an M-component of the IgA class to migrate in the \( \alpha_2 \) zone.

Several other \( \alpha_2 \)-globulins show a disease-related variation, but their concentration is too low to influence the appearance of the \( \alpha_2 \) zone appreciably.

The intensity of the \( \alpha_2 \) band may be increased and extended cathodally when hemoglobin–haptoglobin complexes have been formed. In this case the sample is usually red.

The \( \alpha_2-\beta_1 \) Intergzone

The background stain of this zone has low intensity. The cold-insoluble globulin (fibrinoligin) of fibroblast origin causes a weak, narrow band in the middle of the zone. Its concentration declines when plasma clots, because of its linkage to the fibrin. It also constitutes the bulk of the cryoprecipitate in heparin-treated plasma at 4 °C.

Fuzzy bands and abnormally high mobility may appear when concentrations of pre-\( \beta_1 \) and \( \beta_1 \)-lipoproteins are low, especially after storage of samples.

On electrophoresis of sera with apparent hemoglobin contamination (>1 g/L), the free hemoglobin migrates in the cathodal \( \alpha_2-\beta_1 \) interzone, overlapping the anodal part of the \( \beta_1 \) band.

Pathology. The concentration of cold-insoluble globulin is slightly influenced by disease, but tends to decrease during the inflammatory response. Increased concentration is regularly found in cholestasis of pregnancy. M-components (narrow and broad) of all Ig classes and of light-chain type may appear in this zone.

The \( \beta_1 \) Zone

The color intensity of this band varies roughly proportionally to the transferrin concentration. Splitting of the fraction into one slower or one faster band is the expression of heterozygosity for a transferrin variant. In such cases the two bands have similar and half-normal intensity. These variants are of no clinical significance.

After cleavage of complement component C3, the C3c fragment migrates overlapping or adjacent to the anodal side of the \( \beta_1 \) band. This is usually a sampling and storage artifact, which is avoided by collecting blood directly into a tube with ethylenediaminetetraacetate. C3 cleavage regularly occurs on storage of serum. The cleavage rate is faster in sera from subjects with inflammatory response.

Pathology. Intensely or weakly stained \( \beta_1 \) bands indicate high and low transferrin concentrations, respectively. The transferrin increase during pregnancy, after estrogens, and in iron deficiency is apparent to the naked eye. The decrease during inflammatory response usually follows the hypoalbuminemia but the transferrin decrease is a slightly more sensitive marker of malnutrition than hypoalbuminemia, especially in alcoholics.

Broad M-components, predominantly of the IgA class, appear in this zone. Their molecular size heterogeneity and complex formation are the cause of their extension in the anodal and cathodal direction. The other M-components usually have a sharp demarcation.

The \( \beta_1-\beta_2 \) Intergzone

A slightly wavy \( \beta_1 \)-lipoprotein band with a sharper cathodal than anodal demarcation dominates this zone, where the background stain is mainly caused by IgA. The mobility of the \( \beta_1 \)-lipoproteins increases with the amount of nonesterified fatty acids bound and decreases with increasing concentration of the fraction. This concentration-dependence of the mobility is caused by interaction with an agrose contaminant and therefore varies from batch to batch. The mobility of the \( \beta_1 \)-lipoproteins decreases with increasing concentration of Ca\(^{2+}\) in the buffer.

Pathology. Comparative electrophoresis of fresh sera renders it possible to judge whether \( \beta_1 \)-lipoprotein concentrations are high or low. A cathodally displaced band of high intensity indicates an abnormally high \( \beta_1 \)-lipoprotein concentration; a low amount results in faster mobility.

High color intensity over this zone, extending into the anodal \( \gamma \)-zone, is a characteristic finding with increased IgA.

M-components of all classes may be found in this zone.

The \( \beta_2 \) Zone

This zone is mainly caused by the third complement factor (C3). A series of genetic variants occurs with slightly higher or lower mobility. In the heterozygotes the amount of C3 is equally divided between the two bands. Deficiency variants exist but are extremely rare. Normally the C3 band has a small bump on the anodal side. C3 is cleaved during storage of serum and the predominant conversion product is C3c, which has a fast \( \beta_1 \) mobility. During the early conversion a weak band appears on the cathodal side of C3. This product is very labile.

Pathology. C3 is one of the acute-phase reactants, increasing in concentration with a lag phase of some five to seven
days. It also increases during biliary obstruction. Normal or low C3 values after the first week of inflammatory response suggest an abnormal C3 activation. Conversion products of complement activation in vivo are rarely found in blood samples because of the short biological half-life of the cleavage products. A weak C3 band in combination with the occurrence of conversion products suggests in vitro activation. Low values owing to increased complement activation in vivo may be seen early in acute nephritis, membranoproliferative glomerulonephritis, systemic lupus erythematosus, and similar conditions with intravascular immune complexes. The concomitant signs of inflammatory response may be slight. Complement activation following the classical pathway in antigen–antibody reactions—e.g., in hemolytic anemia—is often followed by no recognizable decrease in C3 in spite of a marked decrease in C4.

M-components are common in the C3 zone.

The γ Zone

On the cathodal side of the C3 band follows the extended γ zone. On analysis of serum, the intensity and distribution of color mainly depend on the concentration of the various subclasses of IgG. Gross abnormalities are observed with the naked eye. Not even faint bands are accepted as a normal finding in the γ zone. High IgM concentration has some influence on the color intensity in the anodal part of the γ zone where fibrinogen migrates if plasma is being analyzed.

Fibrinogen causes a sharp band with an adjacent fraction of 10 to 20% on its anodal front. The intensity of the band reflects adequately the fibrinogen concentration. The large size and slow mobility of the molecules probably preclude detection of genetic variants with single amino acid substitutions. Fuzzy demarcation of the fibrinogen band without trailing is sometimes found in subacute pancreatitis.

Pathology. Faint single bands may appear in the γ zone secondary to the proliferation of selected Ig-producing cell clones as a result of the appearance of certain other proteins.

A labile conversion product of C3 in the anodal γ zone has been mentioned earlier. C-reactive protein migrates on the cathodal side of the bulk of IgG and often reaches a concentration such that a visible band appears in cases of acute and chronic inflammatory response. Interaction of C-reactive protein with agaee gel contaminants causes variations in band intensity from batch to batch of agaee.

Another cause of a single band, or more often two or three faint bands (<2 g/L), in the γ zone is oligoclonal proliferation of some Ig cell clones. This is often seen after viral infections and in malignancy.

Dark, narrow bands (M-components) are seen in myeloma, macroglobulinemia, and in other conditions with more or less malignant monoclonal immunocyte proliferation. Immunologic classification of the M-component is necessary to determine its class. Monoclonal Ig increase is usually but not regularly characterized by a sharply demarcated band. A series of narrow bands with stepwise increasing or decreasing IgG concentration may be found in the middle or the cathodal γ zones in monoclonal Ig cell proliferation. It is seldom difficult to decide if poly- or monoclonal plasma cell increase has caused an atypical protein partition in the γ zone, because limited polyclonal origin always causes a much fuzzier demarcation than does monoclonal origin. If any doubt remains, the problem is solved by light-chain typing, which will give only one type if the origin is monoclonal cell proliferation.

The concentration of the M-component roughly indicates the size of the proliferating immunocyte clone. Immunoglobulins of the classes A, M, D, and E have half or shorter survival times in plasma than do the predominant immuno-

globulins of IgG subclasses 1, 2, and 4. This must be considered when one is estimating the cell mass from the concentration of the M-component. Increasing M-component concentration indicates increasing cell mass. It is important to recognize a concomitant decrease in concentration of immunoglobulins deriving from the other immunocyte populations, as a generally depressed Ig value suggests malignant growth of the clone producing the M-component. The concentration of Bence Jones protein in urine is correlated to malignancy of the cell clone.

M-components in a concentration at or below 10 g/L in old people are usually a sign of comparatively innocent immunocyte proliferations. M-components of class IgM in this low concentration interval arouse suspicion of malignant lymphoma or reticulosis if there are concomitant signs of inflammatory response with no apparent cause.

Polyclonal Ig cell stimulation, apparent as increased staining of part or all of the γ zone, regularly indicates increased IgG synthesis. Grossly increased concentrations (>25 g/L) primarily suggest hyperimmune states. The most common causes are systemic lupus erythematosus, chronic active hepatitis, abscess formation of microorganism origin, or malignancy with bone marrow involvement. It may be a rare accidental finding in subjectively healthy individuals.

Moderate hypergamaglobulinemia develops in response to various antigenic stimuli, but never reaches abnormal concentrations that are recognizable in electrophoretic patterns earlier than three weeks.

Hypogammaglobulinemia is easily recognized on comparative electrophoresis and usually indicates serious diseases (e.g., diabetes, myelosclerosis, malignant plasma- or reticular-cell proliferation, widespread metastases) if losses (intestinal or renal) and disturbances of the glucosteroid metabolism can be excluded. Hypogammaglobulinemia in adults without apparent cause is an indication to search for Bence Jones proteinuria.

M-components of IgM or IgG classes may appear on the cathodal side of the normal γ zone in rare cases. Lysozyme normally migrates here, but seldom increases to a concentration causing a visible band except in myeloproliferative disease.

Some examples of common electrophoretic patterns are given in Figure 2. A more detailed collection is given in ref. 7.

A summarized scheme for analysis of electrophoretic patterns is given in Table 2.

Comparison of the Electrophoretic Pattern of Proteins in Plasma and Cerebrospinal Fluid

Normally, some 80% of the proteins of cerebrospinal fluid (CSF) originate from plasma, and the rest have been synthesized locally. The biological half-life for the plasma proteins entering the subarachnoid space is about one week, and therefore the composition of CSF proteins follows that of plasma, but with some delay. The filtration resistance for plasma proteins rapidly increases with molecular size and therefore the ratio of large proteins (IgM, β-lipoproteins, and α2-macroglobulins) to albumin normally is much lower in CSF than in plasma. This molecular size discrimination diminishes with increasing CSF protein concentration.

The locally synthesized share of the proteins normally diminishes with increasing concentration of CSF proteins. The CSF has to be concentrated at least 150-fold before electrophoresis.

The electrophoretic pattern of concentrated CSF is characterized by a strong prealbumin fraction, which migrates slightly faster than in plasma. The albumin and α1 zone are similar to those in plasma, but the share of α-lipoproteins is
Table 2. Analysis of Electrophoretic Patterns

<table>
<thead>
<tr>
<th>Electrophoretic zones</th>
<th>Look for</th>
<th>Possible causes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prealbumin zone</td>
<td>Decrease</td>
<td>Low liver cell-mass, inflammatory response, malnutrition</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin zone</td>
<td>Decrease</td>
<td>Inflammatory response, malnutrition, losses, malignancy, increased extracellular volume.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fast anodal front</td>
<td></td>
<td>Drugs (penicillin), jaundice</td>
</tr>
<tr>
<td>Interzone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α1-Lipoproteins</td>
<td>Decreased</td>
<td>Liver cell lesions</td>
</tr>
<tr>
<td></td>
<td>Increased</td>
<td>Malnutrition, alcohol abuse, postmenopausal, high estrogen level</td>
</tr>
<tr>
<td>α1 zone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α1-Antitrypsin</td>
<td>Polymorphism</td>
<td>Genetic variants, inflammatory response, liver cell injury, genetic deficiency</td>
</tr>
<tr>
<td></td>
<td>Increase</td>
<td></td>
</tr>
<tr>
<td>Relative decrease</td>
<td></td>
<td>Losses</td>
</tr>
<tr>
<td>α2 zone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α2-Macro-globulin</td>
<td>Increase</td>
<td>Age dependent in children and elderly (&gt;70), selective proteinuria, inflammatory response</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>Increase</td>
<td>In vivo hemolysis, hepatosplenomegaly, increased ineffective erythropoiesis</td>
</tr>
<tr>
<td>Relative decrease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β1 zone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
<td>Polymorphism</td>
<td>Genetic variants, (Bence Jones protein, M-component, hemoglobin)</td>
</tr>
<tr>
<td></td>
<td>Increase</td>
<td>Iron deficiency, estrogens</td>
</tr>
<tr>
<td></td>
<td>Decrease</td>
<td>Malnutrition, losses, inflammatory response</td>
</tr>
<tr>
<td>β-Lipoprotein</td>
<td>Increase</td>
<td>Hypercholesterolemia, decreased mobility with an increased intensity suggests biliary obstruction</td>
</tr>
</tbody>
</table>

Table 2. Continued

<table>
<thead>
<tr>
<th>Electrophoretic zones</th>
<th>Look for</th>
<th>Possible causes</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2 zone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td>Selective</td>
<td>Malignancies, mucosal</td>
</tr>
<tr>
<td></td>
<td>Increase</td>
<td>infection, rheumatoid arthritis, alcohol abuse</td>
</tr>
<tr>
<td>C3</td>
<td>Polymorphism</td>
<td>Genetic variants, inflammatory response, biliary obstruction</td>
</tr>
<tr>
<td></td>
<td>Position</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increase</td>
<td></td>
</tr>
<tr>
<td>γ zone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Increase</td>
<td>Inflammatory response</td>
</tr>
<tr>
<td></td>
<td>Relative</td>
<td>Fibrinoysis, inadequate sampling procedure</td>
</tr>
<tr>
<td></td>
<td>Decrease</td>
<td></td>
</tr>
<tr>
<td>Immunglobulins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sharp bands</td>
<td></td>
<td>M-component</td>
</tr>
<tr>
<td>Diffuse increase</td>
<td></td>
<td>Polyclonal increase</td>
</tr>
<tr>
<td>Banded appearance</td>
<td></td>
<td>Oligoclonal increase, viral infection, malignancy</td>
</tr>
</tbody>
</table>

decreased and the α1 band is more diffuse. The α2 zone appears faint and fuzzy (owing to its very low relative α2M content), with more proteins in the α2-β1 zone. The β1 band is similar to that for plasma, while the β2 band is doubled because of C3 and of a locally produced carbohydrate-deficient transferrin. A tendency to band sharpening is often recognized in the middle of the γ zone. It is IgG in nature, but no corresponding concentration gradient is observed in plasma.

At the cathodal end of the γ zone a faint band is located, which is caused by the small (10,000 daltons), labile "γ-trace" fraction.

Clinical interest in electrophoretic analysis of CSF is limited to the immunoglobulin pattern, in search of signs of local immunoglobulin production in or around the central nervous system. Mono- and oligoclonal pattern in the γ zone of CSF when the plasma pattern is polyclonal is a reliable indicator of local immunocyte response to microorganisms, viruses, or antigen–antibody complexes. This typical, usually oligoclonal, band pattern is blurred when concomitant inflammatory reaction results in increased protein leakage of plasma proteins into CSF, i.e., in an increased protein concentration in CSF.

Electrophoretic comparative CSF and plasma protein analysis is of no interest when the CSF protein concentration exceeds 2 g/L, because even high local IgG production can no longer be distinguished from the electrophoretic pattern due to presence of plasma proteins.

Comparison of the Electrophoretic Pattern of Proteins in Urine and Plasma

Primarily determinant for the urinary protein pattern are the composition and amount of proteins permeating the glo-
merular filter in relation to the tubular reabsorptive capacity. Normally, this is limited to some 200 mg/day.

On urinary loss of larger amounts of protein, the comparative electrophoretic analysis distinguishes the three major possibilities: nonselective glomerular proteinuria, selective glomerular proteinuria, and Bence Jones proteinuria.

In nonselective glomerular proteinuria, the lesions of the glomerular membranes are so severe that there is no discrimination in filtration rate between molecules in the size range of albumin and IgG (70 000 to 150 000 daltons).

The amount of protein (albumin) in the urine tells us more about the lesion than does a urinary pattern that mimics the serum pattern.

Albumin normally passes the glomerular filter five to 10 times more readily than does IgG. The degree of glomerular selectivity is better reflected in the albumin/IgG ratio than by a comparative electrophoretic analysis.

In selective glomerular proteinuria the electrophoretic urinary protein patterns diverge more from the plasma pattern.

In severe selective glomerular proteinuria the plasma is depleted of the proteins of low relative molecular mass, which are enriched in the urine. The urine pattern is dominated by albumin, the α1 band (α1-antitrypsin), and the β1 band (transferrin), while the cathodal part of the pattern is empty. Simultaneously, the plasma pattern shows the pattern typical of glomerular protein loss with decreased albumin and increased concentration of all large proteins (α2-macroglobulins, cold-insoluble globulin, β-lipoproteins, IgM, and fibrinogen).

Mild, selective glomerular proteinuria (<1 g/L) is a common finding in inflammatory conditions and in circulatory insufficiency and is therefore of marginal clinical interest. The inflammatory response gives increased osmollytic excretion, causing the α1 band to blur in the anodal direction. The inter-α zone is partly occupied by Zn–α2-glycoprotein and other small proteins. The small loss of proteins in urine has no feedback effect on the plasma protein pattern. Reasons for running electrophoretic analysis on urine in cases showing mild proteinuria are limited to the suspicion of Bence Jones proteinuria or of proximal tubular proteinuria.

Bence Jones Proteinuria

Urinary excretion of κ or λ immunoglobulin light chains of monoclonal origin (Bence Jones proteinuria) results in the appearance of a more or less dark single (or usually double) band somewhere in the α1 to the slow γ zone. Its nature may be demonstrated by immunoelectrophoresis but preferably is shown by immunofixation of an agarose electrophoresis with anti-κ and anti-λ antisera. The result of conventional immunoelectrophoretic analysis is more difficult to interpret than the immunofixation result if the concentration of the monoclonal light chain is low, because polyclonal light chains always occur in the urine.

The excretion of increased amounts of light chains through the glomeruli causes competition with other proteins of low relative molecular mass for the limited reabsorptive capacity in the proximal tubular section of the nephron. Therefore, the protein pattern in Bence Jones proteinuria often shows bands suggesting insufficient tubular reabsorption.

Pure tubular proteinuria is a mild proteinuria of less than 0.5 g/day, characterized by excretion of the smaller (<50 000 daltons) proteins that normally permeate the glomerular filter and that are normally reabsorbed by the proximal tubular cells.

The predominant proteins of low molecular mass giving rise to electrophoretic bands in concentrated urine are α2-microglobulin (retinol binding protein), β-microglobulin (constant piece of the HLA-antigen), and "γ-trace." Equally abundant, but electrophoretically heterogeneous, are protein HC (9) and polyclonal light chains.

The proportions of these proteins vary in disease. The pure tubular lesions are rare, but a protein pattern similar to "tubular proteinuria" is common in advanced glomerular filtration insufficiency. This is explained by the increasing concentration in plasma, when filtration is insufficient, of all molecules that normally are eliminated by filtration. The load of small proteins increases in the few remaining functioning nephrons and thus surpasses the reabsorptive capacity of the proximal tubular cells. Primary proximal tubular insufficiency is combined with normal glomerular filtration (normal plasma creatinine) in contrast to secondary tubular insufficiency with primary glomerular filtration insufficiency (uremia). In cases of normal plasma creatinine values and slight proteinuria electrophoretic analysis of concentrated urine may be of supplementary interest if other laboratory signs of proximal tubular insufficiency exist.

In conclusion, urine electrophoresis is of great clinical value where Bence Jones proteinuria is suspected, but otherwise is of marginal interest.

Contributed Comments (from Evaluators E.S.T. and L.K.)

We evaluated the described technique with use of an LKB electrophoresis chamber 2117 Multiphor. The power supply was a Scarie Model 3-1155, current and voltage regulated. Temperature was maintained at 10–14 °C by use of a recirculating pump connected to an ice bath reservoir.

We have the following comments regarding specific aspects of the method.

Reagents: Picric acid is difficult to obtain because it is no longer manufactured by many companies. It is available from MCB Chemicals (cat. no. PX1165-01).

Apparatus: The cooling device for the electrophoretic chamber does not have to be a cryostat temperature-regulated device. An acceptable substitute is an ice bath with a recirculating pump such as the one used for an aquarium or decorative fountain. One can be obtained at a local hardware store for about $30.00 to $40.00. This performs well when connected to a recirculating water bath in a large Styrofoam container. Crushed ice can be added as needed to maintain the temperature in the range of 10–14 °C.

Fixing, drying, and staining: Problems developed in removing the wet Whatman no. 1 filter paper after the drying and removal of picric acid. The gel had a tendency to pull away from the polyester sheet. A helpful hint is to spray the filter paper with distilled water before attempting to remove it.

Accuracy: Because the method is largely qualitative and subjectively semiquantitative, accuracy was estimated by comparing the electrophoretic pattern with immunochromatographic quantitation of specific proteins. In general, we observed that abnormalities appearing in the electrophoretic pattern were reflected by abnormalities in concentrations of the major serum proteins.

Precision: Subjective evaluation of precision as determined by different observers appeared to be excellent. The reproducibility of patterns within a given run, as well as between runs, appeared quite good.

Reliability: The method is reliable when the materials and directions specified are followed. It is important that a power supply of good quality and an electrophoretic chamber of good construction be used.

Normal ranges and abnormal ranges in disease: The method relies on visual inspection of the stained electrophoro-
retic pattern. As such, it is a qualitative procedure; assessment of normal vs. abnormal patterns is subjective. In evaluating this aspect of the method, electrophoretic patterns we judged to be normal were compared with quantitative values for major serum proteins in the patients' samples under study. Similar comparison was made with quantitative protein values when there were abnormal electrophoretic patterns. In most instances, it was possible to determine an abnormality in pattern by visual comparison with a selected normal. The abnormalities appeared either as an increase or decrease in staining intensity of individual bands in the electrophoretic strip. Based on the relative intensity of staining of a band, a prediction was then made as to the underlying protein abnormality. This was then correlated with the quantitative data and in most instances good correlation was noted, similar to the correlations discussed by the Submitters.

Conclusion: The method exhibits good characteristics of workability, accuracy, precision, and reliability. It can be recommended as a qualitative screening procedure for detecting abnormalities of the major proteins and as an important adjunct to specific quantitation of serum proteins.

References

Editor's note: The reader is reminded that Selected Methods do not bear the official imprimatur of the Association. They are methods that seem durable and generally useful, and that have been checked by several evaluators. As detailed elsewhere [Clin. Chem. 19, 1207 (1973)], these methods are offered here for criticism by the world community of users, and will be revised appropriately before being collected into a bound volume, Selected Methods of Clinical Chemistry. The last such volume was published by the Association in 1977.

No reprints of these papers will be available, because they are not regarded as necessarily being final versions.