Diagnostic Protein Electrophoresis & Immunofixation

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Proteins and Electrophoresis: An Overview
Protein Trivia

- The most abundant organic molecule in cells (50% by weight)
- 30-50K structural genes code for proteins
- Each cell contains 3-5K distinct proteins
- About 300 proteins have been identified in plasma
Functional diversity of proteins

- **Structural**
  - Keratin, collagen, actin, myosin
- **Transport**
  - Hemoglobin, transferrin, ceruloplasmin
- **Hormonal**
  - Insulin, TSH, ACTH, PTH, GH
- **Regulatory**
  - Enzymes
- **What else?**
The composition of proteins

• Amino acids (*simple* proteins)
  – 20 common (*standard*) amino acids
• Conjugated proteins contain a *prosthetic group*:
  – Metalloproteins
  – Glycoproteins
  – Phosphoproteins
  – Lipoproteins
  – Nucleoproteins
The size of proteins

• An arbitrary lower limit is a MW of 5,000
• Proteins can have MW greater than 1 million, although most proteins fall in the range of 12-36K
  – 100-300 amino acids
  – Albumin (the most abundant protein in humans) is 66K and contains 550 amino acids (residues)
Protein structure

• Primary structure
  – Amino acid sequence
• Secondary structure
  – $\alpha$-helix or random coil
• Tertiary structure
  – 3-D conformation (globular, fibrous)
• Quaternary structure
  – Multi-protein assemblies
Amino acids (1º structure)

- The amino acid sequence is the only genetically-stored information about a protein
- Each amino acid is specified by a combination of 3 nucleic acids (codon) in mRNA:
  - e.g., CGU=Arg; GGA=Gly; UUU=Phe
Properties of amino acids

- The –R group determines, for the most part, the properties of the amino acid
- Substances that can either donate or accept a proton are called zwitterions.
Acid-base properties of amino acids

\[
\begin{align*}
\text{pK}_1 & \quad \text{pK}_2 \\
\text{pH} & \quad \text{equivalents OH}^{-} \\
+\text{H}_3\text{N} & \quad \text{R} \\
\text{O} & \quad \text{O} \\
\text{H}_3\text{N} & \quad \text{CH} \quad \text{C} \quad \text{OH}^{-} \\
\text{R} & \quad \text{H}_3\text{N} & \quad \text{CH} \quad \text{C} \quad \text{O}^{-} \\
\text{H}_2\text{N} & \quad \text{CH} \quad \text{C} \quad \text{O}^{-} \\
\end{align*}
\]
Acidic and basic amino acids

- **Acidic**
  - Asp \( R=\text{CH}_2\text{COO}^- \)
  - Glu \( R=(\text{CH}_2)_2\text{COO}^- \)

- **Basic**
  - Lys \( R=(\text{CH}_2)_4\text{NH}_3^+ \)
  - Arg \( R= (\text{CH}_2)_3\text{NHC(\text{NH}_2)_2}^+ \)
  - His \( R: \)

**Diagram:**

![Chemical structure of histidine (His)](image-url)
Uncharged amino acids

- Non-polar (hydrophobic) amino acids
  - Ala, Val, Leu, Ile, Pro, Phe, Trp, Met
- Polar (hydrophilic) amino acids
  - Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Stereochemistry of amino acids

- All naturally-occurring amino acids found in proteins have the “L” configuration
Essential amino acids

• Humans ordinarily cannot synthesize:
  – Leu, Ile, Val, Met, Phe, Trp, Thr, Lys, His (Arg)

• Dietary protein is the principal source of essential amino acids
The peptide bond

$$\begin{align*}
\text{H}_2\text{N} & \quad \text{CH} & \quad \text{C} & \quad \text{OH} & \quad \text{H}_2\text{N} \\
\text{R} & & & & \text{R}
\end{align*}$$

$$\text{H}_2\text{O}$$
The peptide bond

Dipeptide
Amino acid composition and protein properties

• The \(-R\) groups determine, for the most part, the properties of the protein
• Proteins rich in Asp, Glu are acidic (albumin is an example)
• Post-translational modifications of proteins have significant effects on their properties, as well.
Coiling (2° structure)

- Linus Pauling described the $\alpha$-helical structure of proteins
- Pro and OH-Pro break the $\alpha$-helix
- Ser, Ile, Thr, Glu, Asp, Lys, Arg, and Gly destabilize the $\alpha$-helix
Folding (3° structure)

- J. C. Kendrew deduced the structure of myoglobin from X-ray crystallographic data.
- Globular proteins have stable 3-dimensional conformations at physiological pH, temperature (Why?)
Myoglobin

- Protein 3° structure is influenced by α and β regions
- Proteins fold in order to expose hydrophilic regions, and sequester hydrophobic regions
4° structure

- Hemoglobin has 4 subunits
  - Two $\alpha$ chains
  - Two $\beta$ chains
- Many enzymes have quaternary structures
Technical Electrophoresis

The Separation
Human Proteins

- Only ~200 of the vast array of human proteins have been characterized.
- Clinical knowledge is technically limited to 25 – 30 relatively high concentration components of blood plasma, CSF (cerebrospinal fluid), urine and other fluids.
- Of these, 15 or so can be visualized by high resolution agarose electrophoresis.
Normal Control

Pre-albumin
\( \alpha_1 \)-Acid Glycoprotein
\( \alpha \)-Lipoprotein
\( \alpha_1 \)-Antichymotrypsin
Haptoglobin
C-3
IgA
IgG
Albumin
\( \alpha_1 \)-Antitrypsin
\( \alpha_2 \)-Macroglobulin
Transferrin
\( \beta \)-Lipoprotein
Fibrinogen
IgM
Measuring proteins

- By reactivity
  - Biuret reaction, Lowry method
- By chemical properties
  - Absorption at $\lambda=260$ nm (Phe) or 280 nm (Tyr, Trp)
- By activity
  - Enzymes, immunoglobulins (antibodies)
- By immunogenicity
Separating plasma proteins

• Chromatography
  – Gel (size exclusion), HPLC, ion exchange, immunoaffinity

• Electrophoresis
  – Starch gel, agarose gel, cellulose acetate, PAGE (polyacrylamide gel electrophoresis)
Electrophoresis: Theoretical aspects

Electromotive force (emf)

$F_{emf} = EQ = \frac{V \cdot Q}{d}$

$F_{drag} = 6\pi r \eta v$

when $F_{emf} = F_{drag}$, velocity is constant
Endosmosis

- Large, highly charged proteins may actually migrate toward the *like-charged* electrode.
Optimizing electrophoresis

- Optimal electrophoretic separations must balance speed and resolution

  - Higher voltage increases speed, but heat causes evaporation of the buffer and may denature proteins or even cause gel molting

  - Higher ionic strength (buffer) increases conductivity, but enhances endosmotic effects
Serum Protein Electrophoresis (SPE)

Albumin  α₁  α₂  β  γ
Albumin

• Most abundant protein in plasma (approximately half of total protein)
  – Synthesized in liver
  – $t_{1/2}$=15-19 days

• Principal functions
  – Maintaining fluid balance
  – Carrier
  – Anti-oxidant activity
  – Buffer
Clinical significance of albumin

• Hyperalbuminemia is rare and of no clinical significance

• Hypoalbuminemia
  – Increased loss (nephrotic syndrome)
  – Decreased production (nutritional deficit, liver failure)

• Analbuminemia

• Bisalbuminemia, dimeric albumin
Pre-albumin

- Thyroxine-binding protein (not an incipient form of albumin), also called transthyretin, or TBPA
  - Also complexes with retinol-binding protein (RBP)
- Only protein that migrates anodal to albumin
- Sensitive marker of nutritional status, since its $t_{1/2}$ is only 2 days
$\alpha_1$-Antitrypsin (AAT)

- Protease inhibitor that binds to, and inactivates, trypsin
- Deficiency is associated with
  - Pulmonary emphysema
  - Cirrhosis
- SPE is only a screening test for AAT deficiency
Other $\alpha_1$ proteins

- $\alpha_1$-Acid glycoprotein (orosomucoid)
  - Biological function is unknown
- $\alpha_1$-Fetoprotein (AFP)
  - Principal fetal protein, used to screen for fetal abnormalities (neural tube defects)
\( \alpha_2 \)-Macroglobulin

- Largest non-immunoglobulin in plasma
- Protease inhibitor
- Increased in nephrotic syndrome (size)
- Complete genetic deficiency is unknown
$\alpha_2$-Ceruloplasmin

- Copper transport protein
- Participates in plasma redox reactions
- Cp levels fluctuate with a variety of physiological states, but measurement is usually to screen for Wilson’s disease (a B cell disorder)
  - Plasma Cp is decreased due to inhibition of synthesis
\(\alpha_2\)-Haptoglobin

- Binds to, and preserves, hemoglobin but not myoglobin
  - Complex also has peroxidase activity, and may be involved in inflammatory response
- Hemolytic diseases can deplete Hp levels
β-Transferrin

- Iron transport protein, and also binds copper
- Transferrin is increased in iron deficiency anemia, as well as pregnancy and estrogen therapy
- Decreased in inflammation, malignancy, or liver disease
$\beta_2$-Microglobulin

- Small protein (MW=11.8K)
- BMG is filtered in the glomerulus, but is reabsorbed in the renal tubules.
  - Urinary BMG levels are a sensitive measure of renal tubular function
- Increased in renal failure
β-Compliment proteins

• C3 and C4 migrate in the β region
• Compliment proteins are decreased in genetic deficiencies, and increased in inflammation.
γ-Region

- Includes immunoglobulins (IgG, IgA, IgM) and C-reactive protein (CRP), an inflammatory index
- Single sharp peak is indicates a paraprotein associated with a monoclonal gammopathy (multiple myeloma)
- CRP is the most sensitive indicator of Acute Phase Reaction
  - Inflammation, trauma, infection, etc.
Protein and Immunofixation Electrophoresis

The Test
Diagnostic Immunofixation Electrophoresis (DIFE) at a Glance

• **Why Get Tested?**
  – To help diagnose and monitor multiple myeloma and a variety of other conditions that affect protein absorption, production and loss as seen in severe organ disease and altered nutritional states.

• **When to Get Tested?**
  – If there is an abnormal total protein or albumin level or if there is a condition that affects protein concentrations in the blood and/or causes protein loss through the urine.

• **Sample Required?**
  – A blood sample drawn from a vein in the arm; sometimes a random or 24-hour urine sample can be acquired.

• **Test Preparation Needed?**
  – None
How is the DIFE test used?

• Electrophoresis is normally used to identify the presence or absence of abnormal proteins, and to identify when different groups of proteins are increased or decreased in serum or urine.

• It is frequently used to detect and identify monoclonal proteins (an excessive production of one specific immunoglobulin).

• Protein and immunofixation electrophoresis are used to help detect, diagnose and monitor the course and treatment of conditions associated with these abnormal proteins, including multiple myeloma and a few related diseases.
Protein is usually normally excreted in the urine in very small amounts. When it is present in moderate to large amounts, it may indicate a problem with the kidneys (glomerulonephritis) or multiple myeloma.

The primary reason protein and immunofixation electrophoresis are requested on urine is to look for monoclonal protein production.

This protein may show up in both the serum and urine, or it may only be seen in the urine.
What is Multiple Myeloma?

- 5-10 per 100,000
- 2x African Americans vs. white
- Malignant neoplasm
- 1% of all malignancies
- Marrow plasma cells > 10%

Multiple Myeloma

- Bone pain
- Fatigue
- Pathological fracture
- Anemia – normochromic, normocytic
- Hypercalcemia
- ESR (erythrocyte sedimentation rate) elevated

Multiple Myeloma

- Osteolytic lesions - ribs, vertebrae, skull, long bones
- Damage to kidneys with free light chain deposition

Multiple Myeloma

Treatment

• Autologous peripheral blood stem cell transplantation
• Chemotherapy – melphalan, prednisone
• Thalidomide
• Immunomodulatory agents

Asymptomatic Myeloma

- ~15%
- Smoldering myeloma
- Indolent myeloma

Non-Secretory Myeloma

- No monoclonal protein detected
- Immunohistochemical analysis
- Symptoms similar to secretory myeloma
- Can detect free light chains in urine

How is DIFE used? Cont’d

- An example is Bence-Jones protein, which is the free light chain component of antibodies (normally, antibodies are composed of four parts, two identical heavy (H) chains and two identical light (L) chains.)

- Sometimes, in multiple myeloma, only one or the other is produced, or it may be produced in excess.

- The small size of Bence-Jones protein allows it to pass through the kidneys by filtration and enter the urine.
Urine protein electrophoresis may also be used to help diagnose the cause and estimate the severity of protein excretion due to kidney damage or disease.

This damage or disease may be due to diabetes, chronic inflammation, an autoimmune condition, or a malignancy (cancerous).

Electrophoresis is not usually necessary to assess the loss of small to moderate amounts of protein due to temporary conditions, such as a urinary tract infection or an acute inflammation.
When is DIFE requested?

- Protein electrophoresis may be requested when a doctor or laboratory technologist is investigating symptoms that suggest multiple myeloma, symptoms such as bone pain, anemia, tiredness, unexplained fractures and recurrent infections.

- It may also be used as a follow-up to other laboratory tests, such as an i) abnormal total protein and/or albumin level, ii) elevated urine protein levels, iii) elevated calcium levels, and iv) low white or red blood cell counts (CBC).
When is DIFE requested? Cont’d

- Immunofixation electrophoresis is usually ordered when the protein electrophoresis test shows the presence of an abnormal protein band that may be an immunoglobulin.
When is DIFE requested? Cont’d

• Electrophoresis tests are most frequently requested when there is a disease or condition that causes a monoclonal protein to be produced.

• Once a disease or condition has been diagnosed, electrophoresis may be used at regular intervals to monitor the course of the disease and the effectiveness of treatment (if any).

• As disease progresses, the amount of protein goes up; with treatment, it usually goes down.
When is DIFE requested? Cont’d

• Monoclonal protein production may be due to a malignant disease, such as multiple myeloma, but it may also be due to a monoclonal gammopathy of undetermined significance (MGUS).

• Most patients with MGUS have no symptoms but they must continue to be monitored regularly as some may develop or progress into multiple myeloma after a number of years. The reasons for this relapse are obscure.
MGUS - Monoclonal Gammopathy of Undetermined Significance

- 1% Healthy persons > 50 yrs old
- 3% > 70 yrs old
- M-Protein < 2.5 g/dL
- Marrow plasma cells < 10%
- No bone lesions
- **No Bence-Jones proteins** (A Bence-Jones protein is a monoclonal globulin protein found in the blood or urine, with a molecular weight of 22-24 kDa. This protein is often suggestive of multiple myeloma or Waldenstrom's macroglobulinemia.)
- **Long term follow up with no treatment**
- 1.5% progress to more serious disease

When is DIFE requested? Cont’d

• Serum protein electrophoresis may also be used when symptoms suggest an inflammatory condition, an autoimmune disease, an acute or chronic infection, a kidney or liver disorder, or a protein-losing condition, even if the total protein and/or albumin concentrations are apparently normal.

• Urine protein electrophoresis may be used when there is protein detected in the urine or when the doctor suspects a monoclonal protein may be present.
What does the DIFE test result mean?

- Protein and immunofixation electrophoresis tests give a rough estimate of how much of each protein is present.

- The value of protein electrophoresis lies in the proportions of proteins and in the patterns they create on the electrophoresis graph (gel).

- The value of immunofixation electrophoresis is in the identification of the presence of a particular type of immunoglobulin or fragment of an immunoglobulin.
What does the DIFE test result mean?

• For example, diagnostically certain conditions or diseases may be associated with decreases or increases in various serum proteins.

• This is reflected according to the following illustrations:
DIFE Clinical Significance

Albumin

- ↓ *Decreased* with malnutrition and malabsorption, pregnancy, kidney disease (especially nephrotic syndrome), liver disease, inflammatory conditions and protein-losing syndromes

- ↑ *Increased* with dehydration (e.g., diarrhea)
DIFE Clinical Significance

Alpha1 globulin (α1 globulin)

- ↓ *Decreased* in congenital emphysema (α1-antitrypsin deficiency, a rare genetic disease), or severe liver disease

- ↑ *Increased* in acute or chronic inflammatory diseases
DIFE Clinical Significance

Alpha2 globulin (α2 globulin)

- **↓ Decreased** with hyperthyroidism or severe liver disease, hemolysis (red blood cell fragility and breakage)

- **↑ Increased** with kidney disease (nephrotic syndrome), acute or chronic inflammatory disease
Beta globulin (β globin)

- **↓ Decreased** with malnutrition, cirrhosis (e.g., hepatitis)

- **↑ Increased** with hypercholesterolemia, iron deficiency anemia, some cases of multiple myeloma or monoclonal gammopathy of undetermined significance (MGUS)
DIFE Clinical Significance

Gamma globulin (γ globulin)

- \(\downarrow\) Decreased in a variety of genetic immune disorders, and in secondary immune deficiency

- \(\uparrow\) Increased
  - Polyclonal: chronic inflammatory disease, rheumatoid arthritis, systemic lupus erythematosus (SLE), cirrhosis, chronic liver disease, acute and chronic infection, recent immunization
  - Monoclonal: Waldenstrom’s macroglobulinaemia, multiple myeloma, monoclonal gammopathies of undetermined significance (MGUS)
DIFE Clinical Significance

• Immunizations within the previous six months can increase immunoglobulins, as can drugs such as phenytoin (Dilantin), procainamide, oral contraceptives, methadone and therapeutic gamma globulin.

• Aspirin, bicarbonates, chlorpromazine (Thorazine), corticosteroids and neomycin can affect protein electrophoresis results.
The Test Sample

• As indicated, protein electrophoresis is a method for separating the proteins found in blood (serum) or urine.

• During the test, an electric current is used to move the proteins across a thin layer of agarose/polyacrylamide gel.

• The distances that individual proteins travel depend on their size, shape and electrical charge.
The Test Sample

- These separated proteins may be detected by the use of a dye that binds to (stains) all of the proteins and reveals a characteristic pattern of bands.

- Each band indicates the presence of a particular protein, while the size of the band is a rough indication of the quantity.

- This pattern of bands is converted into a visual graph, showing vertical spikes or peaks where there is a lot of protein and smaller peaks or valleys where there is less.
The Test Sample

• A newer method called capillary zone electrophoresis (CZE) separates proteins by passing them through a long, thin column, producing a graph that is very similar to the one made by running the protein through a gel.
Specific proteins of interest can be identified by first mixing them into the gel with monoclonal or polyclonal antibodies, then washing away all the other proteins prior to staining. This procedure is called immunofixation electrophoresis (IFE).

A slightly different method, immuno-electrophoresis, was used in the past to identify specific proteins. However this technique has been largely superceded by IFE because IFE is easier to perform and interpret.
The Test Sample

- Normally, serum proteins are separated into five or six major groupings by protein electrophoresis. These fractions are called albumin, α₁, α₂, β and γ (the β fraction is sometimes divided into β₁ and β₂).

- Albumin, which is produced in the liver, forms its own group and accounts for about 60% of proteins in the blood.

- 'Globulins' is a collective term used to refer to proteins other than albumin. With the exception of the immunoglobulins (produced by activated B lymphocytes) and some complement proteins, most of the globulins are produced in the liver.
The Immunoglobin Molecule Migration Spectrum

- In the 1930’s, Elvin Kabat and others showed that proteins in a fraction of serum called \( \gamma \)-globulin had activities of antitoxins, precipitins and agglutinins that had previously been thought to be separate activities.

- The active molecules in the \( \gamma \)-globulin fraction were given the name, antibodies.

- It had been determined by several means that the proteins with antibody activity (immunoglobulins) had a molecular weight of approximately 150 kDa.
The Immunoglobin Molecule Migration Spectrum

![Diagram showing the migration spectrum of immunoglobin molecules with peaks at different migration distances for albumin and globulins, labeled α, β, and γ.

Absorbance vs. Migration distance graph with positive and negative charge markers (+) and (-).

- Albumin peak is prominent at lower migration distances.
- Globulins range from α to γ with β in between.

The diagram illustrates the varying migration distances of different globulin types based on their charge and size.
The Test Sample

• The bands seen on protein electrophoresis form characteristic patterns. Alterations to these patterns are associated with a variety of different diseases and conditions.

• For example in multiple myeloma (a cancer of certain types of white blood cells called plasma cells), the uncontrolled growth and division of a malignant plasma cell leads to the production of large amounts of a single type of immunoglobulin (antibody).
The Test Sample

• In contrast to other proteins in serum, which are typically of a single type, antibodies (immunoglobulins) must differ from each other to be able to recognize bacteria, viruses and other 'foreign' substances. Each time the body is exposed to a virus, for example, one plasma cell replicates and makes a group (or clone) of plasma cells to produce antibody to eliminate it.

• Since our total immunoglobulin represents antibody made by many clones, we refer to it as a polyclonal pattern. When there is a cancer of plasma cells, only one type of antibody is produced, termed a monoclonal pattern. This abnormal protein can be seen as a characteristic band on the electrophoresis gel.
The Immunoglobulin Molecule Migration Spectrum

Agarose gel immunofixation electrophoresis of normal serum.
Example

• An agarose gel electrophoresis first separates the proteins in a serum sample. Antiserum against the protein of interest is spread directly on the gel. The protein of interest precipitates in the gel matrix. After a wash step to remove other proteins, the precipitated protein is stained. This method is qualitative and is used to identify proteins found in multiple myeloma.

• Below is the immunofixation electrophoresis gel from a serum sample analyzed. After electrophoresis, the precipitated proteins are stained with Acid Violet, a stain.
Example

- Blood is drawn from a vein (venipuncture), usually from the inside of the elbow or the back of the hand. A needle is inserted into the vein, and the blood is collected in an air-tight vial or a syringe. Preparation may vary depending on the specific test.
Example

- The SP lane represents a routine serum protein electrophoresis of this specimen. On the next three protein separations, antiserum against IgG, IgA, and IgM were applied to the G, A, M lanes respectively. Antiserum to kappa light chain was added to the next protein separation and antiserum to lambda light chain to the last protein separation.
Acute Phase Reactants

• Other ACPs include $\alpha_1$-acid glycoprotein, haptoglobin, and ceruloplasmin
Normal SPE

Albumin $\alpha_1$ $\alpha_2$ $\beta$ $\gamma$
Immediate response pattern

Decrease in albumin
Increase in APR haptoglobin
Delayed response pattern

Albumin decreased
Haptoglobin increased
Gamma globulins increased
Hypogammaglobulinemia

Decreased gamma globulins
Nephrotic Syndrome

- Decreased albumin
- Increased $\alpha_2$-macroglobulin
- Decreased gamma globulins
Hepatic cirrhosis

Decreased albumin (synthesis)
Increased gamma globulins (polyclonal gammopathy)

“β-γ bridging”
Monoclonal gammopathy

Albumin decreased
Sharp peak in gamma region
Protein-losing enteropathy

Decreased albumin
Decreased gamma globulins
Increased $\alpha_2$-macroglobulin