Proteins

Part 2
Introduction

- The total protein test is a rough measure of all of the proteins in the plasma.

- Total protein measurements can reflect:
  - nutritional status,
  - kidney disease,
  - liver disease, and many other conditions.

- If total protein is abnormal, further tests must be performed to identify which protein fraction is abnormal, so that a specific diagnosis can be made.
Principles of Analysis

- The proteins that are frequently analyzed in a clinical laboratory are those in serum and plasma.
- Proteins in other body fluids such as urine and CSF may be also tested.
- Method of analysis can be qualitative, semiquantitative or quantitative.
- Some methods measure all proteins whereas others measure groups of proteins or specific individual proteins.
Lab methods for Total Protein

1. Total nitrogen
2. Kjeldahl
3. UV
4. Refractometry
5. Biuret
6. Dye-binding
1- Total Nitrogen

- It measures all chemically bound nitrogen in the sample, both protein and NPN.
- It is useful in assessing nitrogen balance and monitoring the nitrogen nutritional status in patients receiving parenteral nutrition.
- It uses chemiluminescence.
1- Total Nitrogen

**Principle**

- The sample is heated in presence of oxygen, 1100°C.
- N is oxidized into nitric oxide.
- Nitric oxide is mixed with ozone (O₃) to form an excited No₂*.
- When No₂* returns to the ground state, it emits light.
- The amount of light is proportional to the concentration of N.
- A standard is run for comparison.
2- Kjeldahl Method

- Classic, a reference method (precise & accurate)
- Difficult to perform
- Protein is subjected to heat and strong acid to break it down
- Steps involves protein precipitation (NPN remains in the supernatant) digestion with \( \text{H}_2\text{SO}_4 \) at 340°C in presence of catalyst, \( \text{CuSO}_4 \).
- N is converted into \( \text{NH}_4\text{HSO}_4 \).
- Alkali is added, then ammonia is distilled into standard boric acid solution.
- \( \text{NH}_4\text{H}_2\text{BO}_3 \) "ammonium borate" is titrated with standard solution of HCL to determine the amount of N in the original sample.
3- UV Absorption of Proteins

- Direct methods of total protein estimation which are based on physical properties include ultraviolet.
- Protein solutions show strong absorption in the 280 nm region and in the 210 nm region.
- Virtually all the ultraviolet absorption in serum is attributable to protein.
- The absorption at the higher wavelength (280) is attributable to the aromatic rings of tyrosine, tryptophan, and phenylalanine.
- The absorption at the lower wavelength (210) is mostly attributable to the peptide bond.
3- UV Absorption of Proteins

- Free tyrosine and tryptophan, uric acid, and bilirubin which also absorb light near 280 nm will interfere.

- Determination of total protein by ultraviolet absorption is not routinely used, because of the requirement of expensive cuvets with high transmission at 210 nm.

- Used at research laboratory
4- Refractometry

- The method of refractometry is based on the refraction of incident light by total dissolved solids.
- The velocity of light is changed as it passes the boundary between two transparent layers (air and water).
- The refractive index of water at 20° C is 1.330.
- The addition of solute to water increases the refractive index linearly and the increase in a dilute solution is proportional to the solute concentration.
4- Refractometry

- For serum this reflects the mass of protein present, with the assumption that:
  - the concentrations of inorganic electrolytes and nonprotein organic compounds do not vary appreciably from sample to sample,
  - and that differences in the refractive index reflect primarily differences in protein concentration.

- In practice, the refractometer should be specifically calibrated with serum of a known protein concentration.
5- Biuret reaction

- Indirect methods of total protein determination rely upon the formation of colored complexes which are monitored colorimetrically.
- One of these methods is the biuret reaction.
- It is recommended by the international federation of clinical chemistry.
- In this reaction, cupric ion complexes with the peptide linkages of protein through coordinate bonds to the carbonyl oxygen and amide nitrogen.
- The complexes are violet colored in alkaline solution.
6- Dye Binding Methods

- The ability of proteins to bind dyes such as Coomassie Brilliant Blue has also been utilized in spectrophotometric methods for total protein determination.

- Coomassie Brilliant Blue binds to protonated amine groups of amino acid residues in the polypeptide chain,

- A shift occurs for the absorbance maximum for the dye from 465 nm to 595 nm.

- This method, however, is mainly applied to the assay of total protein in CSF, urine and breast milk, or in the staining of protein bands after electrophoresis.
Albumin/Globulin (A/G) Ratio

- Useful diagnostic information can be obtained by determining the albumin fraction and the globulins
- A significant change in the ratio can point to specific diseases
- Total Protein – Albumin = Globulin
- Albumin levels determined by dye
  - Bromcresol Green (BCG)
    - Sensitive & Most commonly used dye in labs
Salt Fractionation

- Fractionation of proteins is done using precipitation.
- Globulins are separated from albumin by salting out, using sodium salt to cause precipitation of the globulins.
- The albumin that remains in solution in the supernatant is then measured by any of the routine total protein methods.
- Salting out is not used today because direct methods are available that react specifically with albumin in a mixture of proteins.
Albumin measurement

- The most widely used methods for determining albumin are dye-binding procedures.
- The pH of the solution is adjusted so that albumin is positively charged.
- The albumin is attracted to and binds to an anionic dye by electrostatic forces.
- When bound to albumin, the dye has a different absorption maximum than the free dye.
Total Globulins

- Another approach to fractionation of proteins is the measurement of total globulins.
- Albumin can then be calculated by subtraction of the globulin from total protein.
- The total globulin level in serum is determined by a direct colorimetric method using glyoxylic acid.
- Glyoxylic acid condenses with tryptophan found in globulins to produce a purple color.
Total Globulins

- Albumin has approximately 0.2% tryptophan, compared with 2%-3% for the serum globulins.

- When calibrated using a serum of known albumin and globulin concentrations, the total globulins can be determined.

- The measurement of globulins based on their tryptophan content has never come into common use because of the ease and simplicity of the dye-binding methods for albumin.
Abnormal Total Protein

- Serum protein concentrations and the proportions of the individual protein fractions change during a variety of diseases.
- When abnormality is found in the total protein, other techniques can be used to determine the fractions of each protein group.
- An electrophoretic analysis is usually performed.
- If an abnormality is seen on the electrophoretic pattern, analysis of individual proteins within the area is made.
- Abnormalities may be further identified and evaluated by one of the immunological techniques.
Abnormal Total Protein

- Quantitation of total serum protein and its individual fractions is of value in the diagnosis of certain acute and chronic disorders.

- Plasma proteins are often still classified into groups according to their electrophoretic mobility.

- Electrophoresis is usually performed on serum rather than plasma since the fibrinogen present in plasma produces a band in the $\beta$ region that might be mistaken for a paraprotein.
Serum protein electrophoresis

- Serum protein electrophoresis (SPE) is a simple technique for separating serum proteins.
- Cellulose acetate or agarose gel, separates the proteins into distinct bands: albumin, \( \alpha_1 \), \( \alpha_2 \), \( \beta \) and \( \gamma \)-globulins.
- When an electric field is applied to a medium containing charged particles,
  - the negatively charged species migrate toward the positive electrode (anode)
  - while the positively charged particles migrate toward the negative electrode (cathode).
Serum Protein Electrophoresis

- Traditionally alkaline buffer (pH 8.6) allows movement toward anode due to net negative charge of all serum proteins

- Migration order
  - Fastest Albumin
  - Then α1, α2, β, and γ
  - Acid fixed then stained by dyes to visualize on support media cellulose acetate
Serum protein electrophoresis

- The most important diagnostic use of SPE is for the recognition of paraproteins as are usually found in benign or malignant gammopathies.
- Such disorders must be distinguished with additional studies including immunoelectrophoresis.
- It is also used for other serum protein disorders, inflammatory conditions, autoimmune disease, infection, or protein-losing conditions.
- It can also be used to monitor disease progress and response to treatment.
Serum protein electrophoresis

Albumin $\alpha_1$ $\alpha_2$ $\beta$ $\gamma$

Many scanning densitometers compute the area under the absorbance curve for each band. The concentration is then calculated as a percentage of the total protein that was determined by one of the protein methods.
# Nephrosis

<table>
<thead>
<tr>
<th>Condition</th>
<th>Albumin</th>
<th>Globulins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephrosis</td>
<td>↓↓</td>
<td>1 ↓ 2 ↑ β</td>
</tr>
</tbody>
</table>

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

Decreased gamma globulins

<table>
<thead>
<tr>
<th>Condition</th>
<th>Albumin</th>
<th>Globulins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypogammaglobulinemia</td>
<td>N</td>
<td>N N N N</td>
</tr>
</tbody>
</table>
Hepatic cirrhosis

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

<table>
<thead>
<tr>
<th>Condition</th>
<th>Albumin</th>
<th>Globulins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cirrhosis</td>
<td>↓↓</td>
<td>α1 N α2 N β↑ γ↑↑</td>
</tr>
</tbody>
</table>

“β-γ bridging”
Monoclonal gammopathy

Albumin decreased
Sharp peak in gamma region

<table>
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<tr>
<th>Condition</th>
<th>Albumin</th>
<th>Globulins</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. Myeloma</td>
<td>↓</td>
<td>α1 N α2 N β N γ ↑↑</td>
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Isoelectric Focusing

- IEF is a high resolution technique that separates proteins on the basis of their isoelectric point pI.
- Ampholytes of varying pI are added which make a pH gradient.
- The protein when applied to the isogel migrates in the electric field until reach the area of the gel where the pH equals to the pI of the protein.
- Migration stops and the proteins are focused in narrow bands.
Isoelectric Focusing

An ampholyte solution is incorporated into a gel.

A stable pH gradient is established in the gel after application of an electric field.

Protein solution is added and electric field is reapplied.

After staining, proteins are shown to be distributed along pH gradient according to their pI values.
Immunochemical Methods

Specific proteins may be identified by immunochemical assays in which the reaction of the protein (antigen) and its antibody is measured.

Methods using various modifications of this principle include

- radial immunodiffusion (RID),
- immunoelectrophoresis (IEP),
- immunofixation electrophoresis (IFE)
- electroimmunodiffusion,
- and immunonephelometry.
Urine Proteins

- Majority of proteins found in urine arise from:
  - the blood,
  - however some can originate from the kidney and urinary tract
- Proteins appear in urine because they have passed through the renal glomerulus and have not been reabsorbed
- Routine screening in urinalysis
  - qualitative tests for proteinuria are commonly performed using a reagent test strip
- Acid precipitation methods
  - Trichloroacetic acid
Proteins in CSF

- CSF is formed in the choroids plexus of the ventricles of the brain by ultrafiltration of the blood plasma.
- Protein measurement is usually requested on CSF.
- Abnormally increased total CSF proteins may be found in conditions in which there is increased permeability where ultra-filtration occur.
- This can be due to bacterial, viral and fungal meningitis.
Total CSF protein

- The most frequently used procedures are turbidimetric using TCA, sulfosalicylic acid with sodium sulfate.
- Also available are dye-binding methods (e.g., Coomassie brilliant blue)