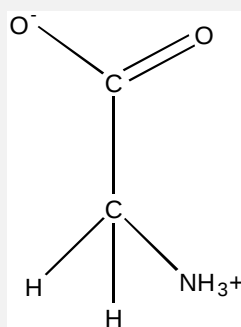


Spectrophotometric Determination of Total Protein-Biuret Method

Background: Proteins are essential to human life. They are macromolecules which are assembled on an as needed basis. They regulate many bodily functions and can function as enzymes.

Biological/Clinical Significance

Proteins are macromolecules with molar masses ranging upwards of 6000 Daltons. They are formed from covalently bonded amino acid units. The simplest amino acid is glycine whose structure is:



Proteins are formed by the linkage of these amino acid groups via an amine group-carboxylic acid group covalent bond which also produces water. Hundreds to thousands of amino acids are present in proteins. There are 20 standard amino acid groups which vary only in what is called the “side chain”. That is, the group which is attached to the central carbon (in glycine it is Hydrogen, in others it may be a methyl group or a phenyl group or something else). It is the combination of these 20 amino acids and how they are ordered which dictates the function of a protein.

Proteins form between 50 and 70 % of a cell's dry weight and are found in all cells, secretions, fluids and excretions of the body. The concentration of proteins in the body ranges from 6.0 g/dL to 8.3 g/dL. The most abundant protein is albumin which can make up 60% of the total protein concentration.

Emphasis and Technique: The experimental procedure teaches the concepts of spectrophotometric analysis. The Lambert-Beer Law relationship is introduced as are rudimentary analytical spectroscopy considerations. Standard (or calibration) curves are generated and the goodness of fit to a linear relationship emphasized.

¹ A Dalton is historical terminology and is equivalent to 1 atomic mass unit.

Chemistry of the Analysis

Spectrophotometric analysis relies on the interaction of electromagnetic radiation (light) with the matter of interest. Strictly speaking, every compound has a distinct absorption spectrum which allows its identification, in many cases, in the presence of other compounds. In addition to the identification of a compound, it is also possible to determine quantitatively the concentration of that compound. The relationship between absorbance and concentration is given by the Lambert-Beer Law and is written mathematically as:

$$A = \epsilon |c$$

where A is the absorbance, a unit-less quantity;

ϵ is the molar absorptivity constant (a constant of the compound having units of $L \cdot mol^{-1} \cdot cm^{-1}$); and

$|$ is the path length over which the light interacts with the sample in cm.

In a more practical sense, the absorbance is defined as the negative logarithm of the transmittance. This is given mathematically as:

$$A = -\log T = -\log \frac{I}{I_0}$$

where I is the intensity of the light beam when the sample is present; and

I_0 is the intensity of the light beam when everything but the sample is present.

(in some cases this is called a background or a blank)

In analytical spectrophotometry, the molar absorptivity of the unknown compound may not be known. It is therefore a common practice to generate a calibration or standard curve. A standard curve is generated by measuring the absorbance of a series of samples for which the concentration is known. Then, since the Lambert-Beer Law shows a linear relationship between absorbance and concentration, a linear least squares fit of the standard curve will yield a mathematical relationship between the absorbance and concentration. This relationship in turn can then be used to calculate the concentration of the unknown sample.

The Biuret Method, which is the most widely used method for total protein determination, relies on the complexation of Cu^{2+} by the function groups involved with the peptide bond. A minimum of two peptide bonds is needed for the complexation to occur. Upon complexation, a violet color is observed. The absorbance of the Cu^{2+} -protein complex is measured at 540 nm and compared to a standard curve.

Reagents

- 1) NaOH, 6.0 M. Dissolve 60g of NaOH in distilled water and dilute to 250 mL. Store in a tightly closed polyethylene bottle at room temperature.
- 2) Biuret reagent. Dissolve 1.50 g of copper (II) sulfate in 250 mL of distilled water. Add 4.5g of sodium potassium tartrate and 2.5 g of potassium iodide. After solids have dissolved, add 50 mL of 6.0 M NaOH and dilute to a total volume of 500 mL with distilled water. Store in a tightly capped polyethylene bottle at room temperature. The reagent is stable for approximately six months.
- 3) Biuret reagent blank. Prepare exactly as in step 2 but omit the copper (II) sulfate.
- 4) Sodium Azide solution 1.5 mM. Add 0.05 g of sodium azide to 250mL of distilled water. Dilute to a total volume of 500 mL.
- 5) Protein standard. Dissolve 1.0 g of 7 mL of sodium azide solution. Dilute to 10 mL total volume with sodium azide solution.
- 6) Standard solutions. Dilute the Protein standard to 20, 40, 60, 80, and 100 g/L by adding the appropriate amount of water. The total volume of each should be 10 mL.

Procedure

- 1) Pipette 5.00mL of the biuret reagent into a each of 7 test tubes.
- 2) Pipette 5.00mL of the biuret blank reagent into a each of 7 test tubes.
- 3) Prepare a reagent-series by adding 100 μ L of each of the protein standards to five separate test tubes filled with the biuret reagent. Prepare a reagent blank by adding 100 μ L of water to a sixth test tube different test tube filled with biuret reagent. Prepare the serum unknown by adding 100 μ L of serum to a seventh test tube filled with biuret reagent. Mix each tube by placing a piece of a parafilm on the top and inverting several times.
- 4) Prepare a blank-series by adding 100 μ L of each of the protein standards to five separate test tubes filled with the biuret blank reagent. Prepare a reagent blank by adding 100 μ L of water to a sixth test tube different test tube filled with biuret blank reagent. Prepare the serum unknown by adding 100 μ L of serum to a seventh test tube filled with biuret blank reagent. Mix each tube by placing a piece of a parafilm on the top and inverting several times.
- 5) Allow the cuvettes to stand at room temperature for 30 minutes.
- 6) Using the reagent-series blank, zero the Spec 20 at 540 nm and measure the absorbance of the reagent series including the serum unknown. Be sure to mix by inversion before making this measurement.
- 7) Using the blank-series blank, re-zero the Spec 20 and measure the absorbance of the blank series including the serum unknown. Be sure to mix by inversion before making this measurement.
- 8) Conduct a blank subtraction by subtracting the absorbance of the blank-series from its reagent series counter part. Plot the new absorbance vs concentration, perform a least squares fit of the standard curve and determine the concentration of the unknown.