

Assays for Total Protein

UNIT 3.4

Total protein assays are used to analyze hundreds of industrial, agricultural, and biotechnology products. They are also basic for research purposes, especially for determining the specific activity (i.e., total activity/total protein) of enzymes, antibodies, and lectins. Clearly, accuracy and precision of specific activity measurements depend as much on accurate measurement of total protein as on determination of total activity.

In performing total protein assays, there are five issues of concern: (1) sensitivity and technique of the method, (2) clear definition of units, (3) interfering compounds, (4) removal of interfering substances before assaying samples, and (5) correlation of information from various techniques. These concerns are discussed at length in the Commentary of this unit.

This unit describes three copper-based assays to quantitate total protein: the biuret method (Basic Protocol 1), a variation of the Lowry method (Hartree-Lowry method; Basic Protocol 2), and the bicinchoninic acid (BCA) assay (Basic Protocol 3). Acid hydrolysis of a protein is coupled with ninhydrin detection to quantitate amino acid content of a sample (Basic Protocol 4). Ultraviolet spectrophotometry is used to measure total protein (Basic Protocol 5) and evaluate samples for the presence of contaminants. The Coomassie dye binding, or Bradford, assay (Basic Protocol 6) is a quite simple assay and frequently is quite sensitive, although it sometimes gives a variable response depending on how well or how poorly the protein binds the dye in acid pH. Finally, dry weight measurement (Basic Protocol 7) is used to quantitate pure protein. Support protocols describe a technique for heat sealing glass tubes (Support Protocol 1) for acid hydrolysis, sample dialysis in polyacrylamide gel wells to remove low-molecular-weight contaminants (Support Protocol 2), and TCA precipitation to precipitate and concentrate proteins and remove low-molecular-weight contaminants (Support Protocol 3).

BIURET ASSAY FOR QUANTITATION OF TOTAL PROTEIN

Biuret assays are the oldest and least sensitive assays for total protein among the colorimetric (spectrophotometric) methods. Nevertheless, the biuret assay is still frequently used because it is easy to perform, reagents are easily prepared, and the assay is markedly less susceptible to chemical interference than other copper-based assays. The assay is based on polypeptide chelation of cupric ion (colored chelate) in strong alkali. Catalog listings for many commercially available proteins and enzymes include their specific activities based on biuret protein ($\text{mg-biuret-protein}^{-1}$). In general, biuret assays are useful for samples containing ~ 1 to 10 mg protein/ml , which is diluted ~ 5 -fold by the added reagent to give a concentration of 0.2 to 2 mg/ml final assay volume (f.a.v.). Most proteins produce a deep purple color, with maximum absorbance (λ_{max}) at 550 nm .

Materials

- Calibration standard: e.g., 10 to 20 mg/ml BSA (see Table 3.4.1)
- Buffer or solvent used to prepare the protein-containing sample
- Sample containing protein at 1 to 10 mg/ml
- Biuret reagent (see recipe)
- Spectrophotometer and 1-cm cuvettes

1. Prepare a dilution series of calibration standard in the buffer or solvent used to prepare the sample.

Use bovine serum albumin (crystallized or lyophilized or one of the Cohn Fraction V preparations which are 96% to 98% protein and 3% to 4% water; Sigma) for the

BASIC PROTOCOL 1

Detection and Assay Methods

3.4.1

Table 3.4.1 Slopes of Calibration Plots for the Biuret Assay^a

Protein	Assay conditions	Slope [A_{650} ($\mu\text{g/ml}$ f.a.v.) ⁻¹ cm ⁻¹]
BSA	—	2.3×10^{-4}
BSA	—	2.6×10^{-4}
BSA	Glucose concentration 0.4 to 400 mM	2.7×10^{-4}
BSA	—	2.7×10^{-4}
β -lactoglobulin	—	2.5×10^{-4}
Cellulase enzymes	—	2.2×10^{-4}
BSA ^b	—	3.2×10^{-4}
BSA ^c	—	1.9 to 2.8×10^{-4}
BSA ^d	—	2.3×10^{-4}

^aAbbreviations: BSA, bovine serum albumin; f.a.v., final assay volume.

^bWatters (1978).

^cGoshev and Nedkov (1979).

^dBeyer (1983).

calibration standard Concentrations of albumin in the final assay volume (f.a.v.) of 5 ml may range from -0.40 to 2.50 mg protein/ml—to produce A_{550} readings from -0.10 to 0.70 in 1-cm. cuvettes—so the dilution series should run from 2 to 12.5 mg/ml.

- In separate test tubes, add 1 ml of protein-containing sample, of each dilution of the calibration standard, or of the buffer or solvent used to prepare the sample (reference standard) to 4 ml biuret reagent. Incubate 20 min at room temperature.

The modest amounts of detergent (e.g., deoxycholate or SDS) used to help solubilize proteins from tissue or insoluble biomaterials that will be added with the protein sample have negligible effects on this assay (see references in Table 3.4.1), although they sometimes have marked effects on the Hartree-Lowry assay (Basic Protocol 2). Mild reducing agents and strong oxidizers adversely affect the assay.

- Measure the net absorbance of the sample, calibration standards, and reference standard at 550 nm (A_{550}) in 1-ml cuvettes. If the spectrophotometer does not automatically give net absorbance readings, subtract the value of the reference standard from those obtained for the sample and calibration standards.

Sample should not be diluted after the addition of biuret reagent. In general, reaction mixtures for spectrophotometric assays should not be diluted after development even if the color (absorbance) is too intense. Dilution will decrease the required excess of cupric ion, thus upsetting chemical equilibrium. If the sample has an $A_{550} > 1$ or 2 (depending on the spectrophotometer), dilute a sample of the original protein and repeat the assay

- Prepare a calibration plot by graphing the net A_{550} values for the standards versus protein concentration ($\mu\text{g protein/ml}$ f.a.v.). Determine the protein concentration of the sample by interpolation from the plot.

Plot the data as shown in Figure 3.4.1 and calculate the slope and its units. (The plots in this unit show net absorbance as the ordinate.) The slope of this plot is directly proportional to the apparent spectrophotometric absorption coefficient (the sensitivity of the assay) in the same units.

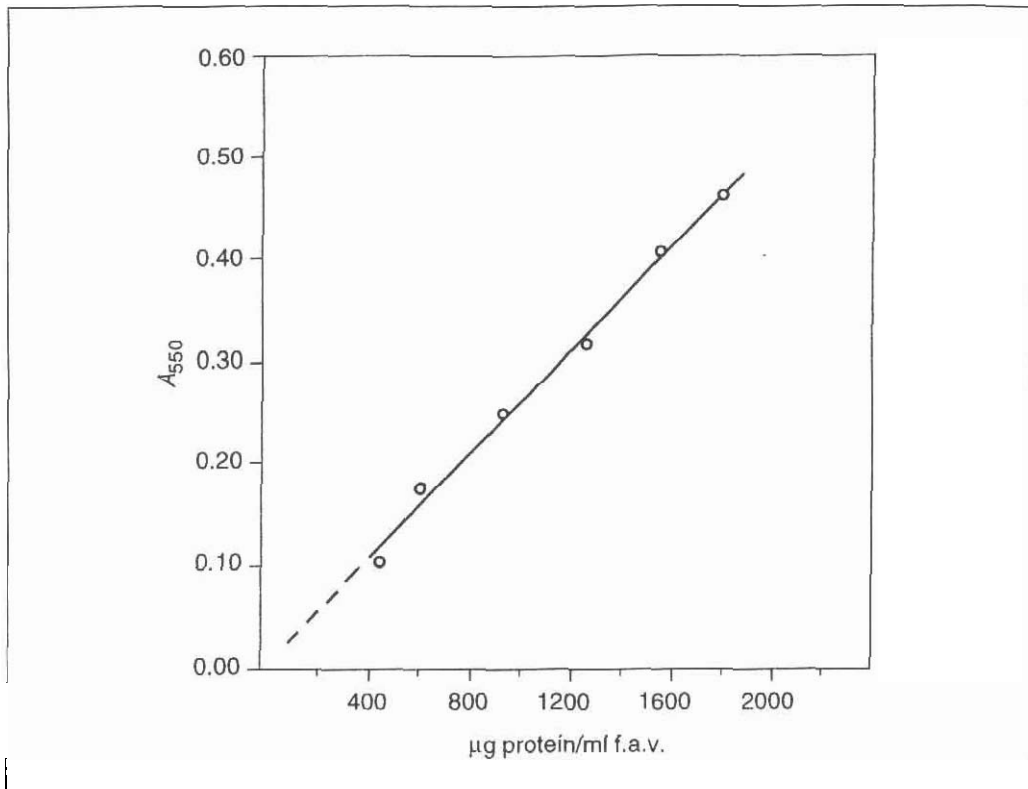


Figure 3.4.1 Biuret assay calibration plot using bovine serum albumin. Slope = $2.26 \times 10^{-4} A_{550} (\mu\text{g protein/ml f.a.v.})^{-1} \text{cm}^{-1}$.

HARTREE-LOWRY ASSAY FOR QUANTITATION OF TOTAL PROTEIN

The original **Lowry** method for total protein analysis was first described in one of the most cited papers in biochemistry (**Lowry et al.**, 1951). The assay is a colorimetric assay based on cupric ions and **Folin-Ciocalteu** reagent for phenolic groups. The assay has been reinvestigated many times and sometimes improved. Most of these studies were designed to discern how interfering compounds distort the assay and how detergents **solubilize** otherwise insoluble proteins. The literature related to this assay was comprehensively reviewed by Peterson (1983). This protocol describes **Hartree's** version of the **Lowry** assay (**Hartree**, 1972). This version uses three reagents instead of five, produces more intense color (increased sensitivity) with some proteins, maintains a linear response over a larger concentration range (30% to 40% greater), is less easy to overload, and overcomes the reagent salt coprecipitation encounter with **Lowry** reagents. **Finally**, the reagents **formulation** offers some advantages in storage stability. It is somewhat less laborious than the original **Lowry** assay, and it maintains the sensitivity of the original.

Materials

- Calibration standard: 300 μg crystalline **BSA** per ml in water
- Buffer or solvent used to prepare the protein-containing sample
- Sample containing protein at 100 to 600 $\mu\text{g/ml}$
- Hartree-Lowry reagents A, B, and C (see recipes)
- 50°C water bath
- Spectrophotometer and 1-cm **cuvettes**

1. Prepare a dilution series of calibration standard in the same buffer or solvent used to prepare the sample to give concentrations of 30 to 150 $\mu\text{g/ml}$.

**BASIC
PROTOCOL 2**

**Detection and
Assay Methods**

3.4.3

Concentrations of albumin in **the final assay volume (f.a.v.)** of 5 ml may range from -30 to 300 $\mu\text{g protein}/5 \text{ ml f.a.v.}$ to produce A_{650} readings from -0.20 to 0.80.

2. Add 1.0 ml of the protein-containing sample, of each dilution of calibration standard, or of the buffer or solvent used to prepare the sample (reference standard) to 0.90 ml of Hartree-Lowry reagent A in separate test tubes. Incubate 10 min in a 50°C water bath.
3. Cool the tubes to room temperature.
4. Add 0.1 ml of Hartree-Lowry reagent B to each tube and mix. Incubate 10 min at room temperature.
5. Rapidly add 3 ml Hartree-Lowry reagent C to each tube and mix thoroughly. Incubate 10 min in a 50°C water bath, then cool to room temperature.

The final assay volume is 5.0 ml.

6. Measure the net absorbance the sample, calibration standards, and reference standard at 650 nm (A_{650}) in 1-cm cuvettes. If the spectrophotometer does not automatically give net absorbance readings, subtract the value for the reference solution from those obtained for the sample and calibration standards.
7. Prepare a calibration plot by graphing the net A_{650} values for the standards versus protein concentration ($\mu\text{g protein}/5 \text{ ml f.a.v.}$). Determine the protein concentration of the sample by interpolation from the plot.

Plot the data as shown in Figure 3.4.2 and calculate the slope and its units. The slope of a calibration plot is proportional to the spectrophotometric absorption coefficient, and is a measure of the sensitivity of assay over the range indicated on the horizontal axis. Comparisons can be made with other measures, such as the weight absorption

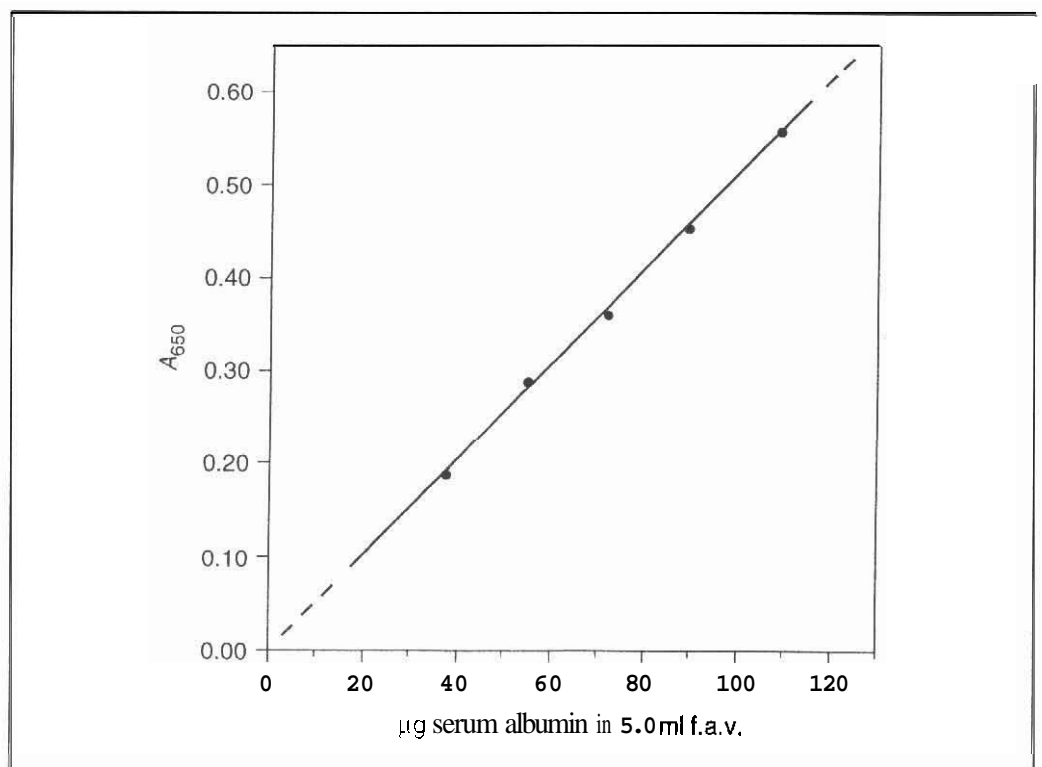


Figure 3.4.2 Hartree-Lowry assay calibration plot using bovine serum albumin. Slope = 2.5×10^{-2} $(\mu\text{g protein}/\text{ml f.a.v.})^{-1} \text{ cm}^{-1}$.

coefficient ($E^{1\%}$) at the same wavelength. Units in the concentration factor in the denominator of $E^{1\%}$ are (dry g)/100 ml of final assay volume.

The units in this protocol, $\mu\text{g protein/ml final assay volume (f.a.v.)}$, are used to permit comparison with values in the literature. It is not always clear whether the milligram or microgram amounts of protein indicated on the axes of these plots refer to the concentration in the input sample or to the final concentration after the addition of reagent(s). Differences in volumes of input versus final developed samples frequently involve dilution factors of 10^1 to 10^2 . As an example, the $E^{1\%}$ values in the original report describing this procedure (see Table 4 in Lowry et al., 1951) are ~ 230 at 750 nm with 3-mm pathlengths. These values can be converted to units presented in this protocol by reconciling the two means for expressing the slopes (see Equation 3.4.1).

$$E_{1\text{ cm}}^{1\%} = \frac{230 A_{\lambda}}{\frac{\text{g}}{100\text{ ml f. a. v.}} \times \frac{10^6 \mu\text{g}}{\text{g}} \times \text{cm}} = \frac{2.3 \times 10^{-2} A_{\lambda} \times \text{cm}^{-1}}{\mu\text{g/ml f. a. v.}}$$

Equation 3.4.1

BICINCHINONIC ACID (BCA) ASSAY FOR QUANTITATION OF TOTAL PROTEIN

BASIC PROTOCOL 3

The bicinchinonic acid (BCA) assay for total protein is a spectrophotometric assay based on the alkaline reduction of the cupric ion to the cuprous ion by the protein, followed by chelation and color development by the BCA reagent. Either a micro or a semimicro procedure, the latter generating a final assay volume of 2 or 3 ml, may be used; the semimicro procedure is most frequently used.

The BCA assay for total protein is somewhat variable: it has differing sensitivities in response to incubation time, incubation temperature, standard protein used for calibration, and other factors (Smith et al., 1985). Certain classes of compounds such as reducing sugars and ammonium ions interfere with the assay, sometimes severely. However, if interfering compounds are eliminated (e.g., by dialysis; see Support Protocol 2), the BCA assay has a good combination of sensitivity and simplicity, and it has some advantages over the Lowry technique (see Background Information).

Materials

Calibration standard: 1 mg BSA/ml
Buffer or solvent used to prepare the protein-containing sample
Sample containing protein
BCA reagent A/reagent B mix (see recipe)
Spectrophotometer and cuvettes

1. Prepare a dilution series of calibration standard in the buffer or solvent used to prepare the sample to cover the range 0.2 to 1.0 mg/ml.
- 2a. For a 2.1-ml final assay volume: Mix 100 μl of protein-containing sample, of calibrating standard, or of buffer used to prepare the sample (reference standard) with 2 ml BCA reagent A/reagent B mix in separate tubes.
- 2b. For a 4.2-ml final assay volume: Mix 200 μl of protein-containing sample, of calibrating standard, or of buffer used to prepare the sample (reference standard) with 4 ml BCA reagent A/reagent B mix in separate test tubes.

The final assay volume depends on the size of the available cuvettes.

Detection and Assay Methods

3.4.5

3. Incubate the samples and standards 30 min at 37°C, then cool to room temperature.
4. Measure the absorbance of the sample, calibration standards, and reference standard at 562 nm (A_{562}). If the spectrophotometer does not automatically give net absorbance readings, subtract the values for the reference standard from those obtained for the sample and calibration standards.
5. Prepare a calibration plot by graphing the net A_{562} values for the standards versus protein concentration ($\mu\text{g protein/ml f.a.v.}$; see Fig. 3.4.3). Determine the protein concentration of the sample by interpolation from the plot.

Figure 3.4.3 shows a calibration plot prepared using BSA as the protein standard and a 2.1-ml final assay volume (f.a.v.). The slope of the plot is a measure of the sensitivity of the assay. The slopes of plots such as Figure 3.4.3 are also related to the absorption coefficients of the colored complexes. The units attached to the slope must take into account the extent of dilution and be consistent with the protocol used to develop the colored complexes. The value of the slope can be used to compare the different methods—e.g., Smith et al. (1985) compared the BCA and Lowry methods using BSA as calibrating protein. For the BCA method, the slope was 1.5×10^{-2} to $2.6 \times 10^{-2} A_{562} (\mu\text{g protein/ml f.a.v.})^{-1} \text{cm}^{-1}$; for the Lowry method, the slope was 1.6 to $2.3 \times 10^{-2} A_{750} (\mu\text{g protein/ml f.a.v.})^{-1} \text{cm}^{-1}$.

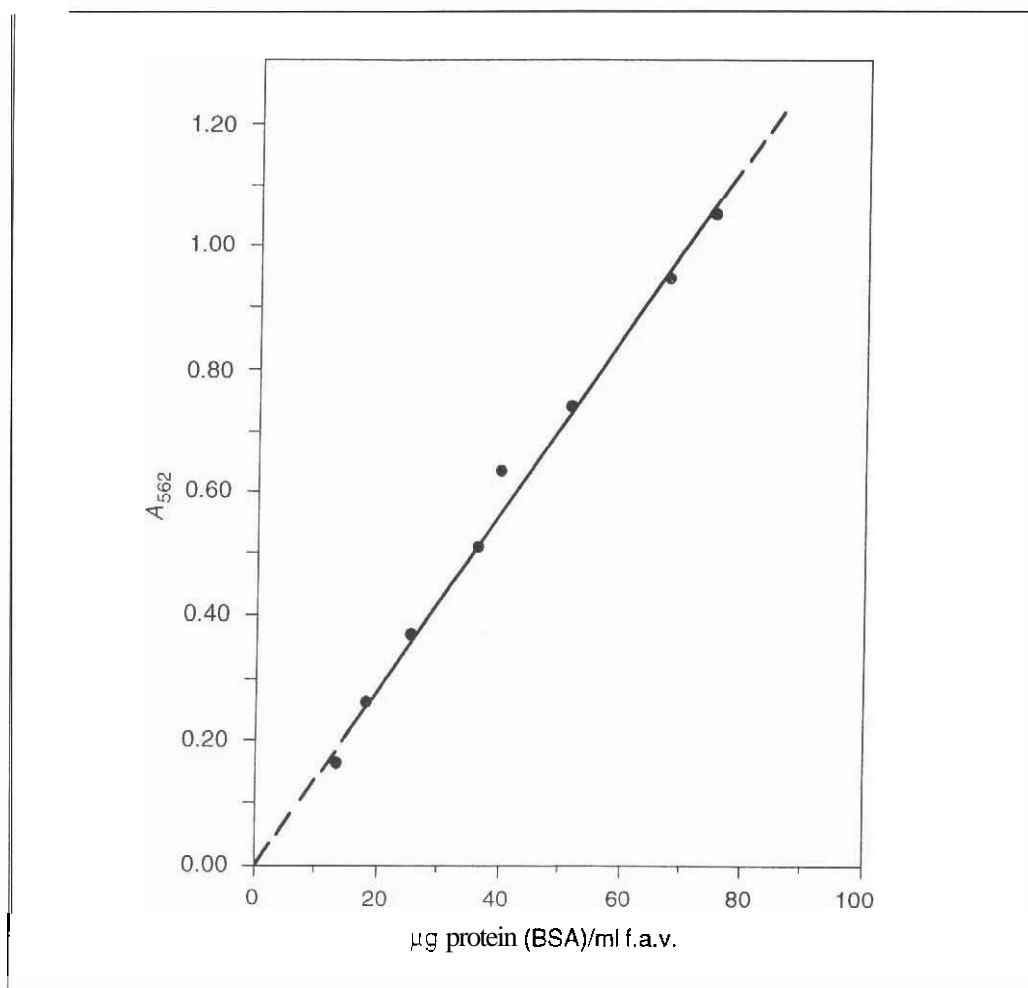


Figure 3.4.3 Bicinchoninic acid assay calibration plot using bovine serum albumin. Slope = $1.5 \times 10^{-2} A_{562} (\mu\text{g protein/ml f.a.v.})^{-1} \text{cm}^{-1}$.

ACID DIGESTION–NINHYDRIN METHOD FOR QUANTITATION OF TOTAL PROTEIN

In this assay, proteins are hydrolyzed to amino acids with 6% sulfuric acid at 100°C. The hydrolysates are neutralized and the amino acids quantitated by ninhydrin **derivatization** and **spectrophotometry** at 570 nm (A_{570}). This method is resistant to interference by phenolic and related compounds that may affect **Folin reaction–dependent** analyses.

Individual amino acids do not produce equal "color yields" upon reaction with ninhydrin. However, most proteins—except those with unusual compositions such as high hydroxyproline and **proline** (collagens), extraordinary sulfur contents (cysteine; e.g., alcohol dehydrogenase and thionein), or densely glycosylated proteins (e.g., **invertase** and **glycophorin**)—give reasonable results using **leucine** as a calibration standard. Ammonium ion generates strong color with ninhydrin, nearly equivalent to that produced by **leucine**. However, TCA precipitation of the proteins prior to acid hydrolysis will remove NH_4^+ , which will be left in the supernate (see Support Protocol 3).

Materials

H_2SO_4

NaOH

Sample containing protein

Calibration standard: 0.2 mg/ml leucine, diluted $\frac{1}{2}$ to $\frac{1}{20}$

Ninhydrin reagent (see recipe)

Isopropanol diluent: 50% (v/v) isopropanol in water

Heat-sealable tubes

100°C water bath

1. Based on the percentage H_2SO_4 (e.g., 6%) to be used in this assay, determine the volume of NaOH required to neutralize the reaction.

For example, 6% H_2SO_4 is $-2.3\text{ N (H}^+)$, so it would take -15.3 ml of 6% (w/v) NaOH (1.5 N) to completely neutralize 10 ml of 6% H_2SO_4 .

In this protocol, % H_2SO_4 refers to a v/v dilution of concentrated H_2SO_4 .

The total volume of the reaction mixture after the addition of acid, base, and ninhydrin reagent to a protein solution containing 10 to 20 mg protein should be close to 3 but no more than 6 ml. If necessary, use more concentrated acid and base solutions to prevent excessive dilution of the sample.

2. In a heat-sealable tube, add H_2SO_4 to an 0.02- to 0.05-mg protein sample to give a final concentration of 3% acid. Seal the tube under a nitrogen blanket (see Support Protocol 1) and incubate 12 to 15 hr (overnight) at 100° to 105°C.

Protein in aqueous solution may be used directly if it does not contain many additional amino acids and oligopeptides. If the sample is dilute or contains contaminants or interfering compounds, the protein can be precipitated and concentrated using TCA (see Support Protocol 3). After the supernatant is removed, 3% H_2SO_4 can be added directly to the TCA precipitate for hydrolysis.

3. Open the tube and add the appropriate volume of 6% NaOH, as determined in step 1, to neutralize the H_2SO_4 used in hydrolysis.
4. Add 2 ml ninhydrin reagent to the neutralized sample, and to equal volumes of diluted calibration standards, and of the buffer (reference standard) in separate test tubes. Incubate the mixture 20 min in 100°C (boiling) water bath. Cool to room temperature.

Calibration and reference standards should be light blue, samples a darker purplish blue. Very dark samples should be diluted by a factor of 2 to 5 with isopropanol diluent

(dilute all standards to the same extent). Dilution may be **performed** after incubation with the reagent, and it is not necessary to repeat the **assay** in such cases.

6. Measure the net **absorbance** of sample and calibration and reference standards at 570 nm. **If** the spectrophotometer does not automatically give net absorbance readings, subtract the value for the reference standard from those obtained for sample and calibration standards.

The absorbance of a sample dominated by hydroxyproline and proline may be read at 440 nm; a separate set of calibration standards should be prepared with these amino acids.

7. Prepare a **calibration** plot by plotting the adjusted values versus protein concentration ($\mu\text{g protein/ml f.a.v.}$). Determine the protein concentration of the sample by interpolation from the curve.

The slope of ninhydrin color yield (A_{570}) with most amino acids is 20 to 25 $A_{570} \mu\text{mol amino acid/ml f.a.v.}^{-1} \text{cm}^{-1}$. As a general guide, use 0.02 to 0.05 mg protein per assay.

SUPPORT PROTOCOL 1

HEAT SEALING GLASS TUBES

For acid hydrolysis at 100° to 104°C, air (oxygen) should be excluded and evaporation should be prevented by carrying out the hydrolysis in sealed tubes. Ordinary glass test tubes, –10 to 12 mm diameter and 10 cm long, can be used for this purpose. Figure 3.4.4 summarizes the procedure.

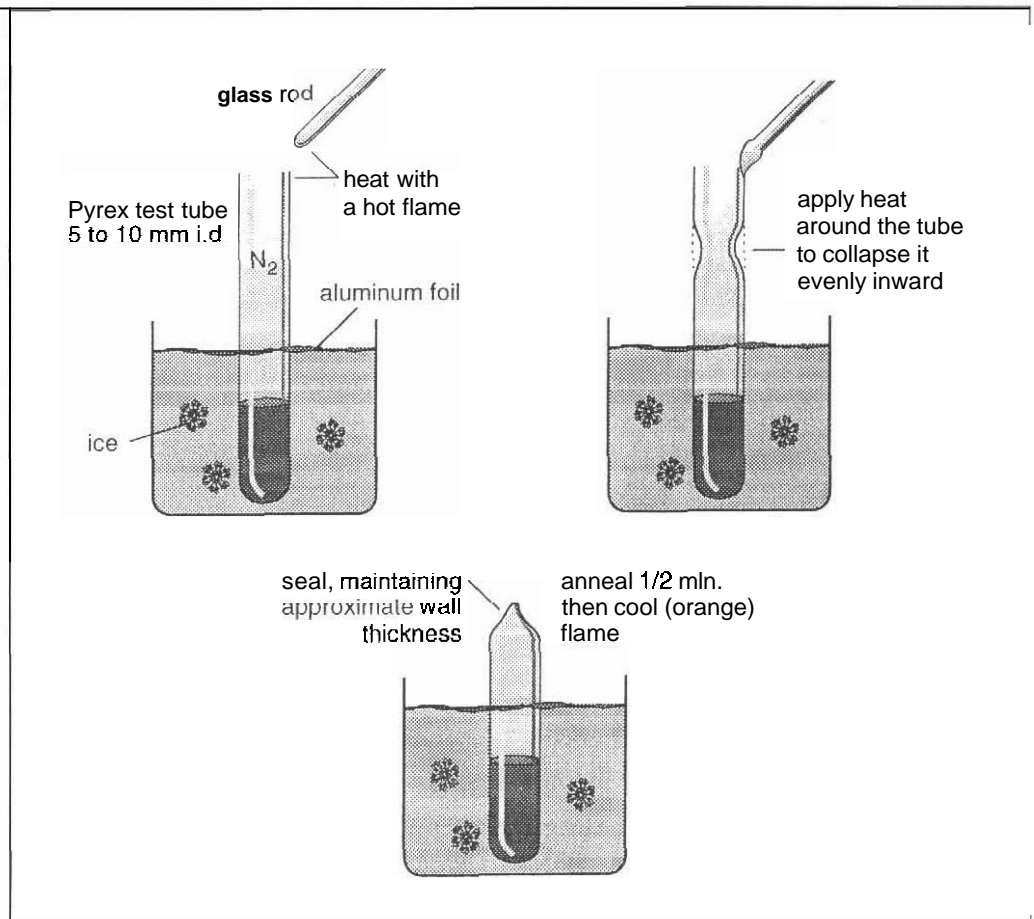


Figure 3.4.4 Heat sealing test tubes for hydrolysis.

Materials

Pyrex glass test tube, 10 to 12 mm diameter x 10 cm long
Nitrogen gas
Glass rod
Gas-oxygen flame source
Triangular file

1. Set a Pyrex test tube containing sample to be hydrolyzed in ice and briefly blanket the tube with nitrogen gas.

CALJTION: Adjust the force of the nitrogen stream carefully to avoid splashing the acid solution. out of the tube or up the sides of the tube.

2. Using a gas-oxygen flame, fuse a small glass rod to the lip of the test tube to act as a handle.
3. Use the flame to apply heat around the body of the tube, ~2 cm below the lip until the tube softens and collapses.
4. Slowly withdraw the "handle" and the scrap upper end, maintaining test tube wall thickness around the seal.
5. When the seal is fused, reduce the flame temperature down to an orange, then a red flame and continue to flame the seal for ~1/2 min.

This should anneal the glass. Do not build a thick seal, which is likely to crack, or draw out the seal so that it becomes thin and likely to blow out.

6. After acid hydrolysis, break the tubes open as follows. First make a deep scratch with a sharp triangular file, about one-third of the way around the tube. Then heat a glass rod until it is white hot, wet the scratch, and apply the white-hot glass rod to the scratch.

This quire reliably cracks the tube all round and the top easily lifts off.

ULTRAVIOLET ABSORPTION TO MEASURE TOTAL PROTEIN

Proteins contain tyrosine and tryptophan side chains that are fairly strong absorbers of light in the 275- to 280-nm (ultraviolet) region. Consequently, after suitable dilution to produce on-scale absorbance readings, total protein can be estimated from UV absorbance spectra using quartz or fused silica cuvettes. Phenylalanine, which has an aromatic side chain, is only weakly absorbing and is usually neglected for most purposes. Weight absorption coefficients, $E^{1\%}$ values (concentration of protein in dry grams per 100 ml volume or weight/volume percent) range between ~3 and 30 A_{280} units $(\text{gm}/100 \text{ ml})^{-1} \text{ cm}^{-1}$ for most proteins. A useful survey of $E^{1\%}$ values for individual proteins at 275 to 280 nm is available in Sober (1970). If the molecular weight of proteins is known, the molar absorption coefficient, ϵ_{λ} in moles/liter is related to the $E^{1\%}$ values at the same wavelength, by Equation 3.4.2.

$$10 \epsilon_{\lambda} = E_{\lambda}^{1\%} \times \text{molecular weight}$$

Equation 3.4.2

Molar absorption coefficients can be calculated from amino acid composition based on tyrosine and tryptophan contributions to the total molecule. The $\epsilon_{275-280}$ values for tyrosine (in neutral or acidic solution) and for tryptophan are close to 1470 and 5700 absorbance

BASIC
PROTOCOL 5

Detection and
Assay Methods

3.4.9

units $M^{-1} \text{ cm}^{-1}$, respectively. There are minor variations in these values depending on the peptide and the solvent (Bencze and Schmid, 1957).

For example, an 0.04% to 0.12% solution of protein having 4 to 12 tyrosine groups, 2 to 6 tryptophans, and an ϵ_{280} value of -8 , should have an A_{280} of -0.2 to 1 with a 1-cm path length cuvette. Hence UV absorption around the 280-nm band provides a fairly sensitive, convenient means for detecting and quantitating pure proteins or mixtures of pure proteins having concentrations in the range of 0.1 to 1 mg/ml.

Materials

Protein sample, pH <8
Fused-silica or quartz cuvettes

1. Turn on the spectrophotometer, set the wavelength to 280 nm, and allow it to warm up 30 min.
2. Dilute the protein sample, if necessary, to give an A_{280} between 0.2 and 1.

Some spectrophotometers may give precise measurements of A_{280} up to 2 absorption units but in general, conditions and concentrations should be adjusted so the instrument is not forced to read absorbances >0.9 to 1.0.

3. Read the absorbance of sample and reference standard (buffer or solvent) at 280 nm in fused-silica or quartz cuvettes.

Glass and plastic cuvettes are opaque below the near UV range, $\sim 350 \text{ nm}$.

A complete UV absorption spectrum, ~ 230 or 240 nm to $>310 \text{ nm}$, may be very helpful for detecting interfering compounds. For a solution of one or more pure proteins at acid pH, there should be a deep trough at 250 nm, a peak around 275 to 280 nm, and very little absorption above 310 nm, unless the proteins contain cofactors or prosthetic groups.

4. If necessary, dialyze or precipitate the sample to remove contaminants and interfering compounds and reread the A_{280} .

BASIC PROTOCOL 6

COOMASSIE DYE-BINDING ASSAY (BRADFORD ASSAY) TO MEASURE TOTAL PROTEIN

Coomassie dye (Brilliant blue G250) binds to protein molecules in acid pH by two means. The triphenylmethane group binds to nonpolar structures in proteins, and the anion sulfonate groups interact with protein cationic side chains (e.g., arginine and lysine side chains) in acid pH (Lovrien et al., 1995). The color change produced when the dye binds to proteins provides a measure of total protein, which is quite sensitive in the case of albumin and certain globular proteins (Bradford, 1976; Sedmak and Grossberg, 1977). The Coomassie dye-binding assay, or Bradford assay, also responds to some interfering substances which are generally unknown unless specifically tested for (Van Kley and Hale, 1977; Kirazov et al., 1993). Nevertheless, because of its apparent simplicity and sensitivity towards many proteins, the Bradford assay is popular and widely used. Using bovine serum albumin (ideally well behaved) to calibrate the Bradford assay produces a calibration plot with a slope of 4.5 to $5.5 \times 10^{-2} (\mu\text{g protein/ml f.a.v.})^{-1} \text{ cm}^{-1}$. Hence, it is somewhat more sensitive, by a factor of roughly two or three, than the values generally quoted for Lowry, Hartree-Lowry, or BCA assays (see Fig. 3.4.2 and see Table 3.4.2).

Materials

Calibration standards: 1.5 mg/ml BSA and 1.5 mg/ml lysozyme
Buffer or solvent used to prepare the protein-containing sample
Sample containing protein
Coomassie dye reagent (see recipe) or commercial Coomassie reagent (Bio-Rad, Pierce)

1. Prepare a **dilution** series of calibration standards in the buffer or solvent used to prepare the sample to cover the range 150 to 750 $\mu\text{g protein/ml}$.

Depending on the kind of protein being measured, it may be useful to calibrate with various proteins including proteins related to or even a purified preparation of the protein being analyzed. For example, if collagen is the analyte, various collagens, high-glycine-hydroxyproline polymers, should be used as calibrating standards. Bovine serum albumin (BSA) is often used as a calibration standard, but it has greater general dye-binding capacity than most proteins.

2. Add 100 μl of protein-containing sample, calibration standard, or of buffer used to prepare the sample (reference standard) to 5 ml Coomassie dye reagent. Mix. Incubate 10 min at room temperature.

The ratio of sample to reagent may range from 1:20 to 1:50 (v/v). A micro assay can be performed using 5 to 10 μl sample in 250 to 500 μl Coomassie dye reagent.

If a commercially prepared Coomassie reagent is used, follow the manufacturer's instructions.

3. Measure the absorbance of the sample, calibration standards, and reference standard at 595 nm (A_{595}). If the spectrophotometer does not automatically give net absorbance readings, subtract the values for the reference standard from those obtained for the sample and calibration standards.
4. Prepare a calibration plot by graphing the net A_{595} values for the standards versus protein concentration ($\mu\text{g protein/ml f.a.v.}$). Determine the protein concentration of the sample by interpolation from the plot.

At maximal sensitivity (with BSA as a calibration standard) the Coomassie dye-binding assay produces a calibration plot with a slope (sensitivity) of $4 \times 10^{-2} A_{595} (\mu\text{g/ml f.a.v.})^{-1} \text{cm}^{-1}$ and falls to a third or half of that value with less responsive proteins.

DRY WEIGHT DETERMINATION TO MEASURE TOTAL PROTEIN

Drying a protein-containing sample in a 104° to 106°C oven for a few hours removes water and volatile materials. The sample is then weighed on a balance to measure the aggregate weight of protein plus whatever nonvolatile material remains, such as salts and many buffers.

Materials

Protein sample
Small weighing bottle or beaker
104° to 106°C oven

1. Dry a small weighing bottle or small beaker by heating it 10 min in a 104° to 106°C oven.
2. Cool the weighing container in a desiccator 10 min.
3. Weigh the container on a balance to the nearest 0.1 mg (tare weight).

BASIC PROTOCOL 7

Detection and
Assay Methods

3.4.11

4. Add a **0.5-** to **3-ml** sample of protein solution to the container.

*Buffersalts, polysaccharides, the **salt forms** of aminoacids, and **high** molecular weight pigments and sugars may not be driven off in a 4- to 6-hr drying cycle (see step 5). They should be **removed from the sample** by dialysis, ion-exchange chromatography, or precipitation before it is dried (see Support Protocols 2 and 3).*

5. **Dry** the sample and container 4 to **6** hr (or overnight) at 104° to 106°C.

*Usually it is not **necessary** to carry out more **than** one cycle of drying, cooling, and weighing to approach a constant weight.*

Overnight drying is required for samples to 5 to 10 ml.

6. Cool the container and reweigh it (dried weight).

7. Calculate the dry weight of the sample as: dry sample weight = dried weight of container and protein – tare weight.

*The **first** dry weights **measured after 6 hr** usually remain constant, but if volatile salts such as ammonium chloride and **ammonium formate** are present, the sample may have to be dried longer **or for more cycles** to completely drive them off*

*Dry weight is reliable to within **-3%** for net weights of 2 to 4 mg protein and to within **1% to 2%** for **≥5 mg protein**.*

SUPPORT
PROTOCOL 2

GEL DIALYSIS OF PROTEIN SAMPLES

This protocol describes a simple method for dialyzing small interfering molecules and salts away from protein samples using an agarose or polyacrylamide gel (see Fig. 3.4.5; Freifelder and Better, 1982). Somewhat dense, well cross-linked gels are used to keep proteins from penetrating the gel while unwanted salts and low-molecular-weight compounds diffuse (am "dialyzed away") into the gel. This is a micro to semimicro method, an alternative to conventional dialysis (Craig, 1967), and to the use of small pressure-membrane disposable kits or microcentrifuge filtration units with MWCO values of 5,000 to 10,000.

Materials

Polyacrylamide gel (UNIT 10.1)

Sample containing protein

Small glass rods or test tubes

Beaker

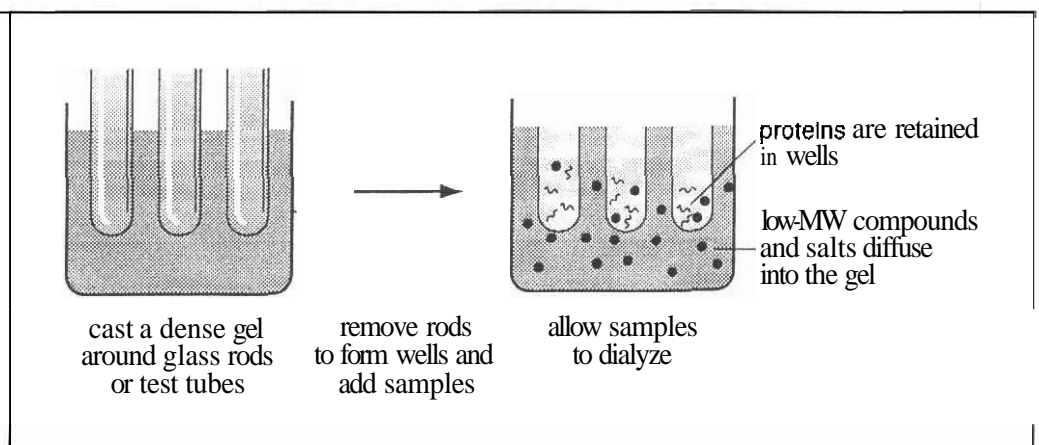


Figure 3.4.5 Dialysis by out-diffusion of low-molecular-weight compounds into gels cast in small cavities (Beyer, 1983).

1. Suspend small glass rods or test tubes inside a beaker.

The glass rods or test tubes are used to form wells that will hold the protein sample. Use a size that will create a well of sufficient volume to hold the sample.

2. Prepare a 12% to 15% polyacrylamide gel cross-linked with 0.8% to 1.5% bisacrylamide and pour it into the beaker to a depth that will provide wells of sufficient volume to hold the protein sample. Allow the gel to polymerize. Remove the glass rods or test tubes.

In this procedure, the intent is to prevent the protein from entering the gel. Alternative & 2% to 5% agarose gels may be used.

If the gels are to be stored, fill them with water to prevent drying. Remove the water and blot out any excess before adding a protein sample.

3. Add the protein sample to the well and let stand 30 min to 1 hr at room temperature.

If the wells have a diameter >5 mm, dialyze for 1 to 2 hr or longer. The rate of dialysis is temperature dependent, so dialysis should be carried out at room temperature or higher if it does not adversely affect the protein.

4. Use a pipet to transfer the dialyzed protein sample from the well to a clean test tube suitable for the selected total protein assay.

TRICHLOROACETIC ACID PRECIPITATION OF PROTEIN SAMPLES

Trichloroacetic acid (TCA) precipitation can be used to precipitate proteins away from TCA-soluble, low-molecular-weight compounds that may interfere with assays for total protein. The procedure may also be used to concentrate protein from a dilute aqueous solution.

Add 10% (w/v) TCA to a protein sample to give a final concentration of 3% to 4% (v/v). Let stand 2 to 5 min at room temperature. Remove the supernatant and resuspend the precipitate in neutral buffer or alkali, depending on the method for further analysis.

If the sample is to be acid hydrolyzed (see Basic Protocol 4), carry out TCA precipitation in the heat-sealable tube, remove the supernatant, and dissolve the precipitate in 3% H₂SO₄.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

BCA reagent A

- 1 g 4,4'-dicarboxy-2,2'-biquinoline, disodium salt (Na₂BCA; Pierce or Sigma; 26 mM final)
- 2 g Na₂CO₃·H₂O (0.16 M final)
- 160 mg disodium sodium tartrate (7 mM final)
- 0.4 g NaOH (0.1 M final)
- 0.95 g NaHCO₃ (0.11 M final)
- 100 ml H₂O

After mixing and dissolving the components, adjust the pH to 11.3 ± 0.2, using solid NaOH to increase pH or NaHCO₃ to decrease pH. Store this alkaline reagent in a plastic container 1 to 3 weeks at room temperature, longer at 4°C.

Only the disodium salt of Na₂BCA is soluble at neutral pH: the free acid is not readily soluble, even in alkali.

SUPPORT PROTOCOL 3

Detection and Assay Methods

3.4.13

BCA reagent B

4 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (16 mM final)

100 ml H_2O

Store up to a few months at room temperature

BCA reagent A/reagent B mix

Mix 50 vol BCA reagent A with 1 vol BCA reagent B (see recipes). Prepare only sufficient mix for a few days' work because the mixture is not-stable.

BCA reagent A/reagent B mix is light apple-green in color.

Biuret reagent

1.5 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (6 mM final)

6 g sodium potassium tartrate tetrahydrate ($\text{C}_4\text{H}_4\text{KNaO}_6 \cdot 4 \text{H}_2\text{O}$, Rochelle salt; 21 mM final)

300 ml 10% (w/v) NaOH (0.75 M final)

Reboiled, cooled H_2O to 1 liter

Store at room temperature in plastic bottles

The water should be preboiled and cooled to remove dissolved CO_2 .

Biuret reagent ordinarily is stable for several months. If a black color (due to cupric oxide) develops, discard the reagent.

CAUTION: Discarded reagent and assay samples may be stored in open plastic buckets in a chemical fume hood to reduce volume (evaporate off water). The salts, especially copper; should be disposed of as hazardous waste.

Coomassie dye reagent

100 mg Coomassie brilliant blue G250 [0.01% (w/v)]

50 ml 95% ethanol (5% final)

100 ml 85% phosphoric acid (8.5% final)

H_2O to 1 liter

Filter through Whatman no. 2 filter paper

Store up to 1 month at room temperature in a glass container

Coomassie brilliant blue G250 is available from Sigma (as brilliant blue G1), Bio-Rad, Pierce, and others.

Hartree-Lowry reagent A

2 g sodium potassium tartrate-4 H_2O (Rochelle salt; 7 mM final)

100 g Na_2CO_3 (0.81 M final)

500 ml 1 N NaOH (0.5 N final)

H_2O to 1 liter

Store 2 to 3 months at room temperature in a plastic container

Hartree-Lowry reagent B

2 g sodium potassium tartrate-4 H_2O (0.07 M final)

1 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.04 M final)

90 ml H_2O

10 ml 1 N NaOH

Store 2 to 3 months at room temperature in a plastic container.

Hartree-Lowry reagent C

Dilute 1 vol Folin-Ciocalteu reagent (Sigma) with 15 vol water,

Prepare this solution daily in 16-ml quantities or multiples thereof: Do not adjust the pH.

Ninhydrin reagent

Solution 1:

0.2 M citrate buffer, pH 5.0
21 g citric acid monohydrate (reagent grade; 0.22 M final)
200 ml 1 N NaOH (0.4 N final)
H₂O to 500 ml
Store at 4°C

Solution 2

0.8 g SnCl₂·2H₂O (7 mM final)
500 ml 0.2 M citrate buffer, pH 5.0 (Solution 1; 0.1 M final)

Solution 3

20 g ninhydrin, crystalline [Sigma; 4% (w/v) final]
500 ml 2-methoxyethanol (methyl cellosolve)

Working solution: Mix 500 ml Solution 2 and 500 ml Solution 3. Purge with nitrogen and store in dark bottles at 4°C.

The quantities of reagents can be scaled down.

Methyl cellosolve (2-methoxyethanol) often forms peroxides that need to be removed e.g., by refluxing over tin metal and distilling. Alternatively, it can be purchased protected under nitrogen (e.g., from Aldrich). In any case, ninhydrin solutions in methyl cellosolve should be purged briefly with nitrogen after each use to exclude air:

CAUTION: *Methyl cellosolve has a boiling point of 124°C and is rather toxic, though not very volatile. Large quantities should be handled in a chemical fume hood.*

CAUTION: *Ninhydrin solution stains everything with an intense purple color. Always wear gloves and a lab coat when mixing and transferring ninhydrin solutions. Avoid spills. If skin becomes stained wash with soap and water several times per day; color will persist for several days.*

COMMENTARY

Background Information

Many assays for quantitating total protein exist. Several are reliable and straightforward. How to choose the most suitable or optimal method is a recurring problem; the solution frequently requires the use of more than one method or protocol. A good strategy is to compare the results of two methods, such as A₂₈₀ measurements and one of the copper-based chromogenic methods — assays that rely on different chemical properties. Very large differences in total protein estimates from two or more methods occur with crude preparations from bacterial cells, cell cultures, tissues, and food extracts, all of which may be laden with interfering substances. The principle question is often not how sensitive a particular protein assay happens to be, but rather how the assay is affected by interfering substances. The primary concern is how to maneuver around the interfering compounds or how to eliminate them altogether, using analytical protocols to follow the progress. Methods for dealing with some of the more common interfering substances are discussed below.

Individual total protein analytical methods commonly disagree with one another by as much as 5% to 20% even in the case of a well-behaved protein not laden with interfering compounds. Disagreement in the case of crude samples may be much greater. There are two general ways to deal with such discrepancies. The first is to remove interfering compounds that are likely to upset one, or perhaps both, analyses, or to resort to a third method, such as biuret analysis, if there is enough sample to resolve the differences. The second and ultimate is to conduct total nitrogen analysis, e.g., by the Kjeldahl method or modern versions of it. It is a generally reliable practice to assume that all proteins and polypeptides contain very close to 16.5% nitrogen by weight, so multiplication of the weight of nitrogen obtained from Kjeldahl analysis by a factor of 6.0 should provide a valid benchmark measure of the weight of protein in an ammonium salt-free sample.

The protocols and related commentaries in this unit describe specific, frequently used methods for quantitating total protein. How-

ever, this field is still evolving. *Analytical Biochemistry* frequently contains reports of new analytical methods, variants of existing methods, and methods for removing interfering compounds.

Recording and reporting data for total protein analysis

Calibration curves for spectrophotometric (colorimetric) assays for total protein are almost always plotted with the vertical axis in units of absorbance at the optimum wavelength (A_h), which usually ranges between 0 and 1.00 or 0 and 2.0, depending on the spectrophotometer. The horizontal axis is usually labeled in units of protein such as μg or mg , often without specifying the related volume—the volume of sample introduced into the assay or the concentration per milliliter of sample or per milliliter of total assay volume. If a 0.50-ml sample is analyzed in a colorimetric assay that adds 3 to 6 ml of reagents, there is a >10-fold concentration difference involved. For this reason, it is very important to be clear and specific in defining the appropriate volume units for the values on the horizontal axis. Clear specification of units for calibration curves is also important because the numerical value of the slope is a direct measure of the sensitivity of the method and a reflection of the molecular composition of the calibrating protein(s). Molar absorption coefficients at specified wavelengths (ϵ_λ) are defined in Equation 3.4.3, where l is cuvette pathlength in cm, c is the molar concentration of the chromogen (moles liter⁻¹), and A_λ is the absorbance as read from the spectrophotometer. In most cases, path length $l = 1$ cm.

$$\epsilon_\lambda = \frac{A_\lambda}{c \times l}$$

Equation 3.4.3

Because spectrophotometry-based total protein analyses produce calibration plots of A_λ versus concentration (c), the slopes of the plots are either directly equal to molar absorption coefficients (if c is in units of molarity) or directly proportional to molar absorption coefficients (if other units are used for the horizontal axis). A well-characterized pure protein (e.g., bovine serum albumin, BSA) used for calibration should produce a calibration curve with a slope (absorption coefficient) that is reasonable based on the composition of the protein for the chromogen or prochromogen.

BSA has close to 18 phenolic (tyrosine) groups per protein molecule. Calibration of a procedure such as the Folin assay that depends on phenolic side chains should respond at least semiquantitatively to the protein's composition as well as its concentration variation. Folin assays for phenolic (and cresol) compounds produce ϵ_{700} values in the range 7500 to 8500 $\text{M}^{-1} \text{cm}^{-1}$; the Folin "color yield" for BSA should be within $\pm 5\%$ of the value for an equivalent number of cresol side chains.

Careful performance of total protein assays and recording of the results are helpful for two reasons. First of all, information from the calibration curve can be compared to known absorption coefficients for purified or partially purified proteins to evaluate the accuracy of the assay. Second, the results can be used as a guide in planning additional experiments to minimize the amount of sample protein required for analysis. Even approximate calculations of the amount of protein, numbers of tyrosine side chains in individual proteins, useful dilution ranges, or amounts of lyophilized protein to use can be helpful in the planning stages and initial benchwork.

It is not necessary for the horizontal axis to be plotted in units of molarity. However, it is desirable both for planning and for cross-checking results that units include a clear statement of volume so they can be readily converted to other units as needed for reporting and planning. One appropriate unit is μg or mg/ml of final assay volume (per ml f.a.v.), which specifies the concentration of the analyzed protein in the operational volume of a solution for spectrophotometric reading after the sample, reagents, and diluents have all been combined.

Table 3.4.2 lists the slopes of calibration curves, equivalent to absorption coefficients and assay sensitivities, for proteins and sugars in some commonly used assays. For some total protein assays, it is a good strategy to monitor sugars in parallel to evaluate removal of interfering compounds. Details for total sugar and reducing sugar assays are outlined in Lovrien et al. (1987) and references therein.

Proteins are frequently investigated and put into large-scale production and use before their molecular weights are known. However, their dry weights may be available. Hence the relationship between molar absorption coefficients (ϵ_λ) and weight absorption coefficients ($E^{1\%}$) at a stated wavelength is useful for converting data using Equation 3.4.2. The molar absorption coefficient and the weight absorption coefficient are related to absorbance readings at

Table 3.4.2 Slopes of Calibration Plots for Spectrophotometric Assays¹

Assay	Calibrating compound	Measured slope
Dinitrosalicylate (DNS)	Glucose	$5.50 A_{575} (\text{mg sugar/ml f.a.v.})^{-1} \text{ cm}^{-1}$
Nelson-Somogyi reducing sugar	Glucose	$6.3 \times 10^{-3} A_{520} (\text{nmol glucose/ml f.a.v.})^{-1} \text{ cm}^{-1}$
Phenol-sulfuric acid neutral sugar	Mannose	$8.6 \times 10^{-2} A_{485} (\text{pg sugar/ml f.a.v.})^{-1} \text{ cm}^{-1}$
Biuret protein	BSA	$2.3 \times 10^{-4} A_{550} (\mu\text{g protein/ml f.a.v.})^{-1} \text{ cm}^{-1}$
Hartree-Lowry protein	BSA	$1.7 \times 10^{-2} A_{650} (\text{pg protein/ml f.a.v.})^{-1} \text{ cm}^{-1}$
Bicinchinonic acid protein	BSA	$1.5 \times 10^{-2} A_{562} (\text{pg protein/ml f.a.v.})^{-1} \text{ cm}^{-1}$
Colorimetric microkjeldahl nitrogen	Ammonium sulfate	$1.3 A_{660} (\mu\text{g nitrogen/ml f.a.v.})^{-1} \text{ cm}^{-1}$

¹Abbreviations: BSA, bovine serum albumin; f.a.v., final assay volume.

the stated wavelength λ (often, the wavelength of an absorbance maximum or peak), to concentrations, and to cuvette pathlength (l , in cm) as stated in Equations 3.4.3 and 3.4.4, where c

$$E^{1\%} = \frac{A_{\lambda}}{c \times l}$$

Equation 3.4.4

is concentration in dry g/100 ml

Copper ion-dependent assays for total protein

The biuret, Lowry, and bicinchinonic acid (BCA) assays are all dependent on copper ions—cupric and cuprous ions. Prochromogenic reagents used in each copper-based assay—Folin reagent, Folin-Ciocalteu, and BCA reagent—depend on the extent of reduction of cupric ion (Cu^{2+}) to cuprous ion (Cu^+) to develop their color (Smith et al., 1985). The brilliant color that BCA reagent develops is the result of formation of a complex with Cu^+ but not Cu^{2+} . In turn, the amount of Cu^+ produced depends on the amount of protein present, so

color development is a measure of the amount of protein (see Fig. 3.4.6).

Compounds that affect either cupric or cuprous ion chemistry will interfere with the assays. In the BCA assay, the reagent chelates the cuprous ion. In the biuret assay, alkaline cupric ion interacts with polypeptide chains. The biuret reaction which is based on the interaction of the cupric ion with the imide form of the polypeptide in strong base to ionize the proton off the polypeptide amide group, may be a precursor to more strongly chromogenic, later-developed color-producing reactions. Aliphatic amines and ammonia or ammonium ion are strong ligands for copper, at least for Cu^{2+} . Reducing agents such as glucose can certainly be expected to interfere with these assays. Proteins rich in disulfide and sulfhydryl groups such as keratin interfere because reduced sulfur compounds powerfully bind to and reduce Cu^{2+} . Cupric ions in general are avid coordinating metals for foreign compounds, precipitating with them or becoming reduced by them. Large concentrations of ammonium sulfate and some of the phosphates used in purification schemes interfere with cupric ion-based chemistries. A list of interfering compounds, many

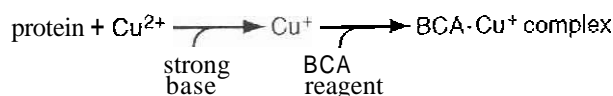


Figure 3.4.6 Reactions in the BCA assay.

Table 3.4.3 Interfering Compounds for Assays to Quantitate Total Protein"

Assay	Interfering compounds
Biuret	Ammonium sulfate Glucose Sulfhydryl compounds Sodium phosphate
Hartree-Lowry	EDTA Guanidine-HCl Triton X-100 SDS Brij 35 >0.1 M Tris Ammonium sulfate 1 M sodium acetate 1 M sodium phosphate
Bicinchinonic acid	EDTA >10 mM sucrose or glucose 1.0 M glycine >5% ammonium sulfate 2 M sodium acetate 1 M sodium phosphate
Acid digestion-ninhydrin	Ammonium sulfate Amino sugars
W adsorption	Pigments Phenolic compounds Organic cofactors
Bradford	>0.5% Triton X-100 >0.1% SDS Sodium deoxycholate
Dry weigh	All buffer salts

*See Smith et al. (1985) for a more complete discussion.

of them used as buffers or found as metabolites in protein technology, is presented in Smith et al. (1985) and briefly summarized in Table 3.4.3.

Biuret assay

The biuret assay (see **Basic Protocol 1**) is periodically reinvestigated, using different formulations for the reagents and different proteins as calibrating standards. There are a number of modifications for the method scattered through the literature (Goshev and Nedkov, 1979). Some of these recommend the addition of KI, -5 g per liter of reagent (Layne, 1957). Differences in apparent slopes from laboratory to laboratory may occur because of varying amounts of inert materials such as NaCl or water from incompletely dry proteins used for calibration. The average slope for the biuret assay, obtained using several globular proteins

as calibration standards, is $-2.5 \pm 0.3 \times 10^{-4} A_{550} (\mu\text{g protein/ml of f.a.v.})^{-1} \text{ cm}^{-1}$, about 100 times smaller than the Hartree-Lowry and BCA assays.

A perennial question in total protein analysis is the choice of calibration standard: should the protein be a purified sample of the protein under study or will BSA suffice? Although it is true that some total protein assays, notably UV-based A_{280} assays, are dependent on the amino acid composition of the protein, biuret chemistry is based on polypeptide structure, not the composition of side chain residues. Accordingly biuret assays are rather independent of the protein used as the calibration standard.

The Hartree-Lowry assay

The original Lowry method for total protein analysis (Lowry et al., 1951) has been reinves-

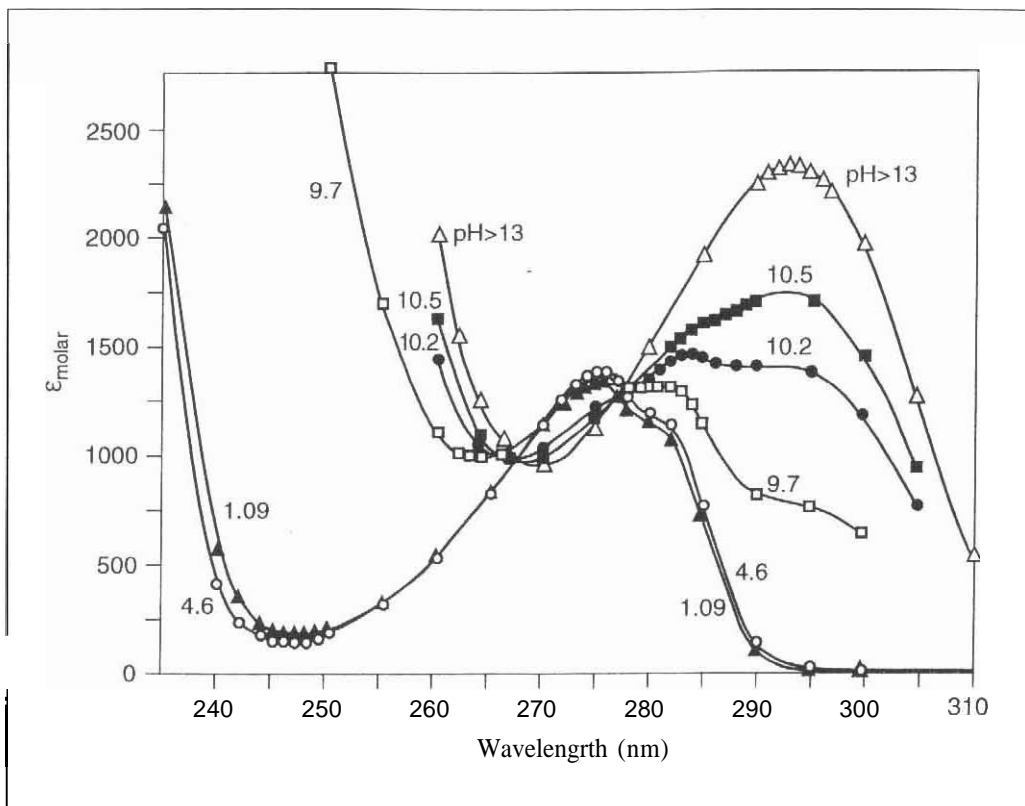


Figure 3.4.7 Absorption spectra of glycyl-L-tyrosine as a function of pH (Craig, 1967).

tigated many times to evaluate the effects of interfering compounds and the ability of detergents to solubilize otherwise insoluble proteins (reviewed by Peterson, 1983). The Hartree-Lowry assay (see Basic Protocol 2) yields linear results over a wider range of protein concentration, maintains the sensitivity, and is superior in the formulation of reagents (Hartree, 1972).

The bicinchoninic acid assay

The bicinchoninic acid (BCA) assay (see Basic Protocol 3 and Fig. 3.4.6) developed by P.K. Smith et al. (1985) uses cupric ion in a biuret reaction with proteins in a strong base. The biuret reaction produces cuprous ion from cupric ion, and the cuprous ion is chelated with the BCA reagent. The Cu^+ -BCA chelate is brilliantly colored with an absorption peak, λ_{max} , at 562 nm. Therefore, A_{562} is directly dependent on protein concentration, when the reaction conditions outlined by Smith et al. (1985; see Basic Protocol 3) are maintained.

The BCA method compares favorably with the older Lowry or Hartree-Lowry methods in sensitivity and convenience. The sensitivity of the BCA assay can be increased somewhat by using a higher concentration of the BCA reagent (i.e., of bicinchoninic acid disodium salt).

However, the BCA reagent is rather expensive, and the fractional increase in sensitivity may not be worth the increase in cost.

The BCA assay is less susceptible than other assays to interference by a number of detergents. For this reason the BCA assay is sometimes favored with detergent-loaded samples, e.g., membrane and cellular proteins extracted by detergent solubilization. On the other hand, the BCA assay is susceptible to interference by reducing sugars, and even by ostensibly non-reducing sugars such as sucrose (because sucrose releases reducing sugars on partial hydrolysis; Spies, 1957). Some experimental systems use 10^{-2} to 10^{-4} M sulfhydryl reagents to protect proteins. These reagents can contain significant quantities of reduced sulfhydryl reagents such as mercaptoethanol that react with cupric ion. Dialysis or other methods for removing organic -SH compounds from the sample may be required.

In order to avoid interfering substances in general, and also to increase protein concentrations, powerfully precipitating agents such as TCA (trichloroacetic acid) may be useful (Anand and Romeo, 1976; Beyer, 1983; also see Support Protocol 3). Most proteins in even dilute solution are quantitatively precipitated by TCA (at a concentration of a few percent),

which also concentrates them. The supernatant is discarded, which rids the sample of most low-molecular-weight impurities. The TCA precipitate should be redissolved in base for subsequent BCA assay.

Acid digestion–ninhydrin assay

The acid digestion/ninhydrin assay (see Basic Protocol 4; Rosen, 1957) takes considerably more time than other methods that depend on starting with protein samples in solution. This assay is useful for dried solid samples such as plant material because the whole sample is hydrolyzed under conditions that convert peptides and proteins to amino acids suitable for conventional chromatographic analysis (Marks et al., 1985). Nucleic acid bases such as adenine apparently do not interfere with the ninhydrin reaction, which can also be advantageous with complex samples.

UV spectrum analysis

The ultraviolet (UV) absorption spectrum for a sample protein can be used to quantitate the protein and to evaluate the purity of a sample. Figure 3.4.7 shows the UV absorption spectra for glycyl-L-tyrosine. The molar absorption coefficient E as a function of wavelength is dependent on pH. The glycyl moiety has negligible effect on the spectra, so they are close in general shape to those of tyrosine side chains in proteins. Tryptophan side chains (indole groups) have similar spectra at acid pH. However, tryptophan absorption is considerably more intense at 275 to 280 nm, by a factor of nearly 4. Thus, although different proteins have very variable tyrosine–tryptophan compositions, the general shape of their UV absorption spectra in acid are fairly close to those of tyrosine at neutral and acid pH.

Proteins in neutral and acid solution exhibit a deep trough in absorption at 245 to 250 nm. Most proteins, except for heme proteins and proteins with cofactors such as NADH, do not absorb from either tyrosine or tryptophan above 300 to 310 nm. Increased absorption at ≤ 250 nm and/or 2300 nm is a strong indication of the presence of compounds that interfere with A_{280} measurements and their interpretation. The effectiveness of techniques such as dialysis and size-exclusion chromatography for removing interfering UV-absorbing compounds can be monitored by analyzing the sample for absorption at 250 nm (trough), 280 nm (peak), and 300 to 310 nm (residual). Ratios of these absorbances are often used to determine whether the UV spectrum is that of pure

or contaminated protein. For example, the peak/trough ratio (A_{280}/A_{250}) for proteins in neutral and acid solutions is almost always ≥ 2 .

When a protein solution is shifted from acidic to alkaline pH, the absorption spectrum for tyrosine changes radically due to alkaline ionization of the tyrosine hydroxyl group. The maximum change in absorption occurs at 295 nm, with a $\Delta\epsilon_{295}$ of $2470 \text{ M}^{-1} \text{ cm}^{-1}$. Proteins absorb rather little at 295 nm, except for some contribution by tryptophan groups. However, titration of tryptophan side chains does not produce a shift in A_{295} , so observation of changes in A_{295} with changing pH can be ascribed to tyrosine in the absence of interfering compounds that behave similarly to tyrosine. Analysis of UV absorption spectra, especially at 280 and 295 nm, for a purified protein under acid and alkaline conditions can be used to characterize the tyrosine and tryptophan content of the protein (Bencze and Schmid, 1957). With care, both tyrosine and tryptophan can be quantitated by the Bencze–Schmid method to within 3% to 4% of values obtained from complete analysis of amino acid content by hydrolysis and chromatography (Stein and Moore, 1954; Chang, 1992).

A number of sophisticated UV spectrum–based methods for protein quantitation and characterization use absorption at 205 nm and 224 to 236 nm (Bencze and Schmid, 1957), and second-derivative spectra of enzymatic digests of proteins (Bewley, 1982). See UNIT 3.1 for additional information and discussion. It is necessary to remove oxygen from the spectrophotometer by nitrogen purging for measurements at very low wavelengths ("hard" UV, < 220 nm).

All proteins absorb strongly at 205 nm, so at first glance this seems an attractive means for quantitating protein. However, most published methods describing hard UV absorbance are based on the behavior of pure, mostly single-subunit proteins. Buffers with carbonyl groups and many other impurities also become strongly absorbing in the hard UV range, and the problems of conventional UV absorbance are magnified in the hard UV range.

Coomassie dye binding assay (Bradford assay)

Coomassie blue dye (see Basic Protocol 6) produces a rather large absorption spectrum shift in the visible-light range when it binds to many globular proteins. The strong binding forces are partly, but not entirely, due to electrostatic attraction between dye molecule sulfonate groups (two on the G250 dye molecule)

and **cationic** groups of proteins when they are made quite acidic. The pH of the reaction mixture is low enough to thoroughly convert proteins to cations, but not so low that any **sulfonate** groups can become neutralized. The low pH of -0.8 M phosphoric acid also causes most proteins to expand so **that they** become exposed to the **triphenylmethane**, organic, and nonpolar sectors of the dye to enhance binding (Craig, 1967). Proteins that do not bind anionic dye, even in very low pH, may not bind Coomassie dyes. Early research **on Coomassie dye**-based protein **assays** used globular proteins, which are known to avidly bind dye and other similar anions such as detergent anions (Edsall and Wyman, 1958). Albumin, **hemoglobin**, **chymotrypsinogen**, and **cytochrome c**, which Bradford used to calibrate the **method** (Bradford, 1976), are particularly able proteins in this regard. However, other classes of proteins are quite unlike these globular, organic **anion-binding** proteins and these differences may be at the root of some of the failures reported for the Bradford method (Pierce and Suelter, 1977). At any rate, the Bradford method is especially simple and has good sensitivity when it is calibrated with proteins to which it is sensitive (e.g., albumin).

Jernejc et al. (1986) compared four different methods for determining total protein—Kjeldahl nitrogen, biuret, Lowry, and Coomassie dye binding—for each of eight stages in the isolation of proteins from mycelia. They found that the Coomassie dye binding method often grossly underestimated protein content, by factors >2 -fold, relative to the other methods tested. Although Coomassie dye binding is an easy and convenient assay, its reliability must be verified for each experimental system.

A similar **method** is the Udy dye binding method, which uses acid orange 12 dye, a sulfonated **azobenzene** dye. The technique was originally developed for food proteins, and it apparently compares well with Kjeldahl nitrogen analytic criteria (Udy, 1956).

Critical Parameters

There are two **endemic** problems in total protein assays. First, there is the issue of interfering compounds and how to remove them or minimize their effects. **Second** is the intense barrage of advertisements for reagents for measurement of total protein; these promise simplicity, sensitivity, and by implication suggest there is little need to consider what **interfering** compounds may do, how they operate, or in

what **concentrations** they operate. In **crude** proteins and at many stages in protein purification, interfering compounds often are present in concentrations one, two, or even three orders of magnitude larger than protein concentrations. Some of the well-advertised means for measuring total proteins are based on calibration with pure proteins such as crystalline bovine serum albumin, **which can be expected** to behave well. However, good performance in calibration with single, fairly pure proteins frequently does not translate into reliable **performance** with real samples—tangled mixtures of carbohydrates, lipids, and nucleic acids with proteins and **glycoproteins**. In some cases, when samples are crude, approximately half the effort needs to be devoted to controls and to simple steps that can be taken to either eliminate interference or at least help **understand** it. Support Protocols 2 and 3 **describe** two techniques—dialysis and TCA precipitation—for removing **low-molecular-weight** interfering compounds.

It should be noted that calibrating a total protein assay using a pure protein, usually bovine serum albumin, as a reference standard is **common** practice. However, that does not ensure that data from crude samples, taken through the same procedures, accurately quantitates total protein in **crude** samples.

Variability in BCA analysis

There are a number of factors that contribute to variability in the **BCA assay**: the temperature and duration of the incubation step just before **spectrophotometry**, the use of too much protein, the choice of calibration protein, and the presence of interfering compounds in samples of unknown character. It may be helpful to experiment with the temperature and duration of incubation. Sometimes incubating the sample 30 min at 60°C can accelerate color development without loss of color stability (Smith et al., 1985; Goldschmidt and Kimelberg, 1989). However, incubated samples must be **cooled** adequately before making **spectrophotometric** measurements, because residual temperature gradients in the cuvettes can cause **refractive index gradations** or striations, which distort the optical path producing anomalies and errors. These errors can be neutralized by remixing samples until their temperatures are close to the temperature of the cuvette and they appear **uniform** by simple visual inspection.

RCA calibration plots (see Fig. 3.4.3) show **definite** curvature downward for most proteins in excess of -90 to $100\ \mu\text{g protein/ml}$ final assay volume. Such nonlinearity can occur

when the protein or polypeptide are present in excess with respect to the amount of reagents (Cu^{2+} and BCA) available to develop color.

There is considerable variation in the slope of calibration plots and therefore in apparent sensitivities, depending on the protein used as a calibration standard. For conventional proteins—e.g., BSA, chymotrypsin, and ribonuclease—slopes can vary by as much as a factor of two (Smith et al., 1985). If gelatin is used for calibration with an incubation of 30 min at 37°C, the variation can be up to 3-fold. Some preparations of proteins, even “crystalline” and lyophilized proteins, still contain appreciable amounts of water and/or salts used in isolation or crystallization. Water contents may be measured by the dry weight method (see Basic Protocol 7). Some freeze-dried preparations are actually only 95% protein or even less. Accordingly, water and other contaminants in supposedly dried proteins may contribute to lowered slopes, sensitivities, and apparent absorption coefficients.

UV absorption

UV absorption spectra are useful for quantitating and characterizing pure proteins (also see UNIT 3.1). However, UV absorption is probably the most profoundly and easily distorted analytic method of quantitating proteins because concentrations of UV-absorbing contaminants as low as 10^{-4} to 10^{-5} M can affect the spectra. Thus caution is necessary when analyzing protein samples that are not very pure. Pigments, oxidation products, tannin-like substances, Maillard and other condensation products—all manner of biochemical compounds and natural products—all absorb at ultraviolet wavelengths, sometimes strongly, potentially causing very large errors (at least an order of magnitude). If a protein sample absorbs UV above ~320 nm, it is likely that appreciable and

perhaps unacceptably large amounts of interfering compounds are present. Elaborate means have been proposed by a number of authors for UV analysis of total protein (reviewed by Peterson, 1983); nearly all are built on the supposition that no UV-absorbing interfering compounds are present in the protein sample. In practice, this supposition is very shaky, particularly at early and intermediate stages in protein purification of proteins. It may be valid for rather well-purified proteins. In general, UV absorption should be used in conjunction with other methods for analysis of total protein to correlate results. Additionally, UV absorption spectra can be used in a reverse sense, in conjunction with other analyses, to estimate the amounts of absorbing impurities (Beyer, 1983).

TCA precipitation

Trichloroacetic acid precipitation may also be affected by reagents used to prepare the sample. Chang (1992) describes a procedure for TCA precipitation of a solution containing large amounts of the strong anionic detergent sodium dodecyl sulfate (SDS). Sometimes it is useful to precipitate the interfering compounds leaving the proteins in solution. Polyethyleneimines (PEI; Sigma) can be used to precipitate nucleic acids, which have a UV absorption peak at 260 nm (Jendrisak, 1987). PEI does not absorb in the ultraviolet range. However, neither TCA nor PEI is free of cross-precipitation, so it may be necessary to experiment with the concentration of precipitant, pH, and temperature to optimize a particular precipitation step.

Anticipated Results

Table 3.4.4 presents a summary of the detection ranges to expect for the assays described here.

Table 3.4.4 Summary of Assays for Quantitating Total Protein

Assay	Sample size	Detection range ($\mu\text{g/ml}$)
Biuret	1 ml	1000–10,000
Hartree-Lowry	1 ml	100–600
BCA	100 μl	200–1000
Ninhydrin	1 ml	20–50
UV absorption	1 ml	30–300
Coomassie dye binding	100 to 200 μl	60–300
Dry weight	0.5 to 10 ml	2000–10,000

Time Considerations

Basic Protocols 1, 2, and 3 (biuret, **Hartree-Lowry**, and BCA assays) require one hour for measuring sample, mixing, incubation, and reading the absorbance. Basic Protocol 4 (acid digestion—ninhydrin detection) requires one hour on the first day to **measure** the sample and seal the tubes, followed by an overnight hydrolysis. On the second day, 1 to 2 hr are required for opening the tubes, neutralization, mixing reagents, heating, and reading the resulting color. Basic Protocol 5 (**W** absorption) is the simplest of the methods described in this unit, taking only about 30 min to make dilutions, **warm** up the spectrophotometer, and measure the absorbance curve. Basic Protocol 6 (Coomassie dye binding) is also fast, requiring 20 to 30 min. Basic Protocol 7 (dry weight determination) requires a long drying time (4 hr to overnight) plus 10 to 20 min each time the container is weighed.

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Contributed by **Rex Lovrien** and
Daumantas Matulis
University of Minnesota
St. Paul, Minnesota