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Determination of Protein Concentration Using the Varian DMS 90 Spectrophotometer

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Introduction

Determination of the concentration of protein in a solution is fundamental to virtually all biochemical investigations. Many methods have been devised which vary in the sensitivity of the procedure and the convenience with which they may be performed. In addition to sensitivity and convenience, interference by other substances present in the solution also becomes an important consideration for accurate determination of protein concentration. Of the methods that are generally used, measurement of the absorbance of the solution at 280 nm is the simplest and most convenient; however, contamination of solutions by nucleotides or materials that scatter light can render this measurement inaccurate. Correction for the contribution of nucleotides to the absorbance at 280 nm can be made by making a second absorbance measurement at 260 nm and then correcting the 280 nm measurement for the contribution of the nucleotide (1,2). This procedure may give very inaccurate results because of the variability in extinction coefficients for proteins and poly, oligo and mononucleotides and thus cannot be recommended for general use.

Correction for light scattering is much more tedious and requires measurements of the apparent absorbance at several wavelengths at which the protein does not absorb light, and then calculating a correction factor for the absorbance at 280 nm based on either a plot of the apparent absorbance versus the reciprocal of the wavelength to the 4th power or a plot of the logarithm of the apparent absorbance versus the logarithm of the wavelength. The correction factor is determined by extrapolation of this line to 280 nm and the subtraction of the absorbance due to scattering from the raw absorbance value (3). Even in the absence of nucleotide contamination and turbidity, measurements of protein concentration at 280 nm cannot be expected to be accurate unless the extinction coefficient for the particular protein is known. This is due to the fact that the absorbance at this wavelength reflects principally the tryptophan content of the protein with smaller contributions from tyrosine and phenylalanine and not total protein. A simple proportionality between one absorbance unit and a protein concentration of 1 mg/mL of protein is frequently assumed for rough initial estimations of protein concentration. If the protein of interest is one of a member of a family of proteins that have well characterized extinction coefficients, then mean values for proteins in that family may be used to improve the estimation of protein concentration from the simple absorbance measurement at 280 nm. Tables of extinction coefficients for a large number of proteins have been provided by Kirschenbaum (4). Second derivative spectrophotometry is a method which greatly aids in measurements in mixtures and in turbid solutions. Use of the second derivative of the absorption spectrum for such measurements is discussed in references 10 and 11

A method which will estimate protein concentration without the biases introduced as a result of variable content of aromatic amino acids is the biuret method (5). This method, which takes advantage of the formation of the chromophoric copper complex with peptide bonds in proteins is, however, subject to interference by several commonly used buffers, particularly those containing primary amines. The biuret method lacks the sensitivity of many of the other methods and thus is limited in its usefulness.

A popular method for protein concentration determination is an extension of the biuret procedure, the "Lowry" protein determination method (6). Although more tedious than either measurement at 280 nm or the biuret reaction, it is more sensitive than the biuret reaction alone. The Lowry method, however, like the biuret method, is subject to interference by other components which may be present in the protein solution. The "Lowry" procedure is biased in that the colour yield and total protein concentration is not the same for all proteins.

A method with sensitivity comparable to the "Lowry" method uses the binding of the Coomassie brilliant blue G-250 by proteins, which is accompanied by a shift in the maximum wavelength at which the bound dye absorbs light (7). This method is less susceptible to interference by other compounds present in the protein solution than the "Lowry" procedure and is as easy to perform as the biuret reaction. But, like simple absorbance at 280 nm and the "Lowry" procedure, it does not generate a chromophore with the same extinction coefficient for all proteins. Based on a comparison of the response of various proteins in the Coomassie blue dye-binding assay, this procedure appears to be more variable than the "Lowry" procedure (8). However, it must be noted that if proteins such as cytochromes, myoglobin, or haemoglobin are eliminated from the list of proteins that have been compared, the variability in the dye-binding assay is very dramatically reduced.

Experimental

Reagents and Materials

Reagents for the determination of total protein using the biuret reaction were purchased from Sigma Chemical Company, St. Louis, Missouri. Biuret reagent stock No. 540-2, protein blank reagent stock No. 540-3, and protein standard solution stock No. 540-10 were employed. This reference protein solution contains approximately 68% albumin and 37% plasma globulins.

The phenol reagent for use in the "Lowry" protein assay was from Fisher Chemical Company, Phenol reagent solution, 2N stock No. SO-P-25. Reagent A for the "Lowry" protein determination was prepared by dissolving 100 g of sodium carbonate in 1 litre of 0.5 N sodium hydroxide. Reagent B was prepared by dissolving 1 g of cupric sulphate pentahydrate in 100 mL of deionized water. Reagent C was prepared by dissolving 2 g of sodium potassium tartrate in 100 mL of deionized water. These three reagents may be stored almost indefinitely.

The reagents for the dye-binding assay of total protein using Coomassie Brilliant Blue G-250 employed the Bio-Rad Protein Assay Kit, Bio-Rad catalogue No. 500-0001.

Equipment

Varian Model DMS 90 UV-Vis Spectrophotometer (Part No. 00-100222-01)
Varian Model 325 Recorder (Part No. 00-100123-01)
Semi Micro Cuvette, 4 mm width, quartz (Part No. 00-998798-54).

Procedure

Protein concentration at 280 nm was determined using the DMS 90 spectrophotometer with a spectral bandwidth of 1.0 nm.

Total protein using the biuret method was performed as described in Sigma Technical Bulletin No. 540. 0.1 mL of the sample to be assayed was placed in a 15 mm x 150 mm test tube, 5.0 mL of biuret reagent was added and the solution mixed on a Vortex mixer. 0.1 mL of Tris buffered saline (0.05 M Tris, 0.15 M NaCl, pH 7.5) was used as a reagent blank. After 15 minutes, absorbances were read at 540 nm (with a spectral band width of 1 nm) using the reagent blank to zero the spectrophotometer. The protein solution used was the Sigma protein standard.

The "Lowry" protein assay was performed as described in reference (9). Into thirty 16 mm x 150 mm test tubes were placed aliquots of a 0.3 mg/mL solution of bovine serum albumin. All samples were done in triplicate. Aliquot volumes varied from 0 — 1.0 mL. The volume in each of the individual sample tubes is then brought to 1.0 mL by adding the appropriate volume of Tris-buffered saline. A working reagent which is prepared from 30 mL of Reagent A, 1.5 mL of Reagent B, and 1.5 mL of Reagent C is prepared in an Erlenmeyer flask. After thoroughly mixing the working reagent, 1.0 mL of this reagent is added to each of the samples described above. All tubes are mixed thoroughly on a Vortex mixer and then permitted to stand for 15 minutes at room temperature. The Folin reagent working solution is then prepared by mixing 15 mL of the 2 N Folin reagent with 150 mL of deionized water. To each of the protein samples described above is added 3.0 mL of the Folin working solution.

This sample is Vortex-mixed immediately upon addition of the Folin reagent. After 45 minutes at room temperature, the absorbance of each sample is determined at a wavelength of 540 nm. If greater sensitivity is required, absorbances may be determined at 660 or 750 nm.

Protein concentration determination by the Coomassie Brilliant Blue G-250 dye-binding procedure was performed as described in the instructions to the Bio-Rad Protein Assay Kit. 0.1 mL samples of bovine plasma albumin varying from 0.2 to 1.4 mg/mL were placed in 16 mm x 150 mm test tubes, 0.1 mL of Tris buffered saline was used as the

reagent blank. 5.0 mL of the diluted dye reagent (stock reagent diluted 1:5 and then filtered) were added to each of the tubes and the tubes mixed on a Vortex mixer. Care was taken to avoid excess foaming. After approximately 15 minutes, the absorbance at 595 nm was measured.

Results and Discussion

The 280 nm absorbance versus protein concentration curve for bovine plasma albumin is shown in Figure 1. Sensitivity of the assay can be described in terms of absorbance per mg/mL protein in the sample from which the protein aliquot has been taken. The sensitivity at 280 nm for this albumin preparation is 0.64 absorbance units per mg/mL. Other proteins which contain more tryptophan may have sensitivity factors at 280 nm in the range of 2.0 or more absorbance units per mg/mL.

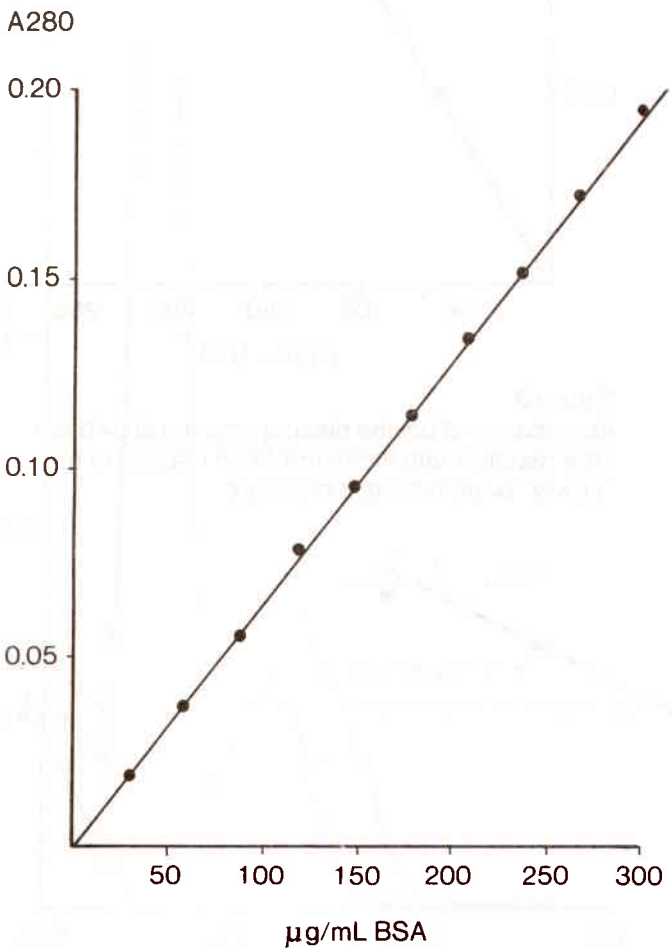

Figure 1
Absorbance of bovine plasma albumin as a function of concentration.
Absorbance was measured at 280 nm with a spectral bandwidth of 1.0 nm.

The standard curve obtained from the biuret procedure is shown in Figure 2. A sensitivity of 0.93 absorbance units at 280 nm was observed for this mixture of plasma albumin and globulins. For the biuret assay, the sensitivity at 540 nm is 0.56 absorbance units per mg/mL protein calculated for the protein sample from which the aliquot was removed for biuret assay.

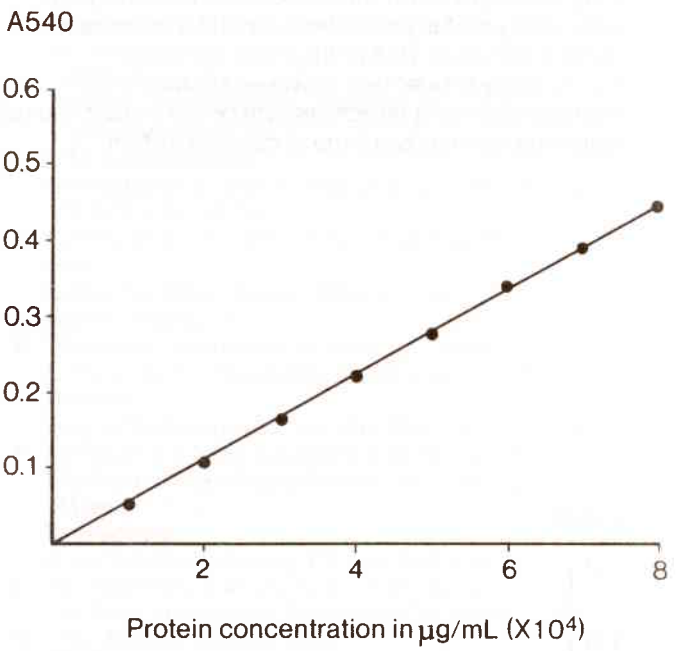

Figure 2
Absorbance of the Sigma protein standard at 540 nm after reaction with the biuret reagent.

The standard curve obtained, using the "Lowry" assay with bovine plasma albumin as described above, is shown in Figure 3. Sensitivity of the assay when measurements are made at 540 nm can be described as 1.67 absorbance units per mg/mL. If the absorbance at 280 nm of the same sample is measured, a sensitivity of 0.64 absorbance units per mg/mL is observed, making the "Lowry" protein assay approximately three times more sensitive than the absorbance at 280 nm. If the absorbance measurements are made at 750 nm, the "Lowry" procedure is approximately two times more sensitive than when the chromophore is measured at 540 nm.

Figure 4 shows the data obtained using the Coomassie Brilliant Blue G-250 dye-binding protein assay. A sensitivity at 595 nm of 0.98 absorbance units per mg/mL is observed with the "Bio-Rad Standard Assay Procedure". If the Bio-Rad microassay procedure were employed in the Coomassie Brilliant Blue G-250 method, then the sensitivity of this procedure would be increased almost 20 times. Use of the more sensitive microassay procedure, however, results in the consumption of a larger amount of the protein sample and thus its disadvantage is clearly evident.

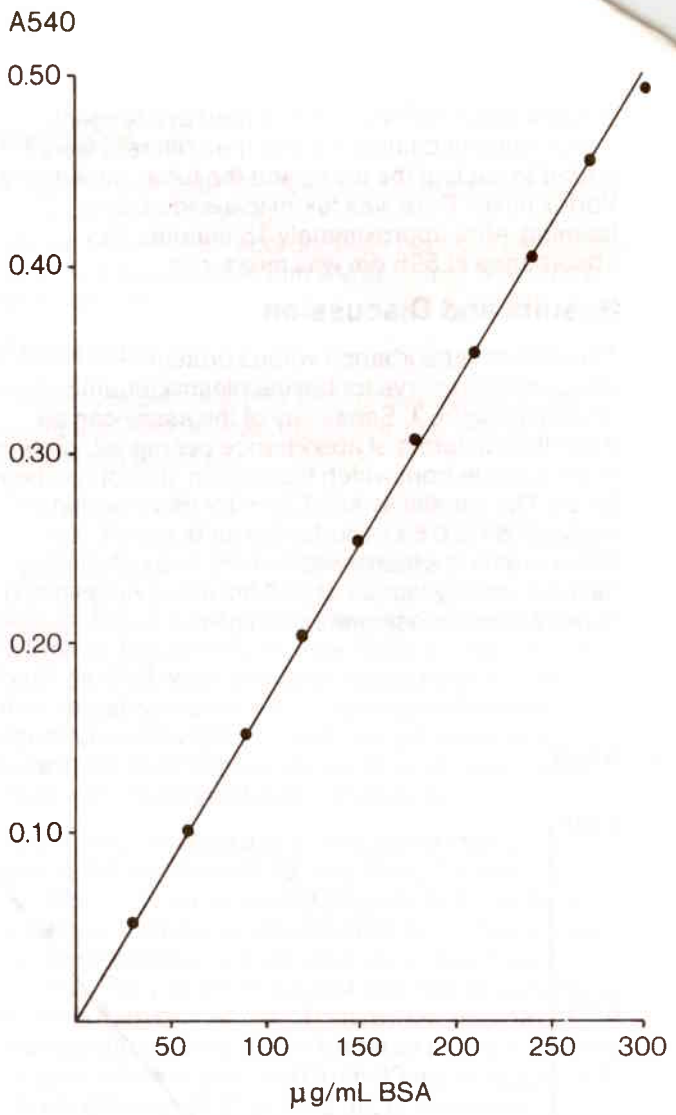

Figure 3
Absorbance of bovine plasma albumin at 540 nm after reaction with the biuret-Folin reagent in the "Lowry" protein assay procedure.

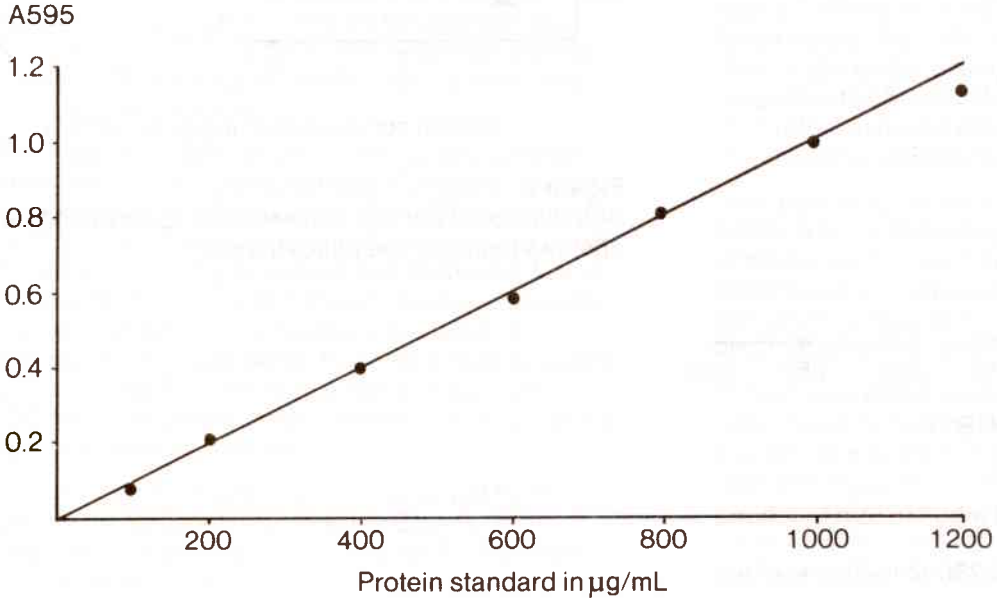

Figure 4
Absorbance at 595 nm as a function of protein concentration after reaction with Coomassie Brilliant Blue G-250 in the Bio-Rad dye-binding protein assay.

"Naive" use of measurements at 280 and 260 nm for protein determination in the present of a nucleotide, adenosine monophosphate is illustrated in Figure 5. If the equation derived by Kalckar (2) is used to correct for the nucleotide contribution to the absorbance at 280 nm, a protein concentration of 0.09 mg/mL is calculated. The actual protein concentration was 0.5 mg/mL. The shortcomings of this dual wavelength method can be seen from the disparity in the calculated protein concentration and the actual protein concentration in this sample. The extent of the disparity varies from protein to protein because of the difference in the extinction coefficients for individual proteins at 280 nm and the differences in extinction coefficients for nucleotides of different structure and degrees of polymerization. The superiority of the other colorimetric procedures in which nucleotides or other absorbing compounds are present in the protein solution is thus very evident. However, the fact that no protein is consumed when making measurements at 280 nm, whereas all of the other procedures do consume the protein, make it possible to do so with the required accuracy. The use of the second derivative of the absorption spectrum for measurements in mixtures is described in references 10 and 11.

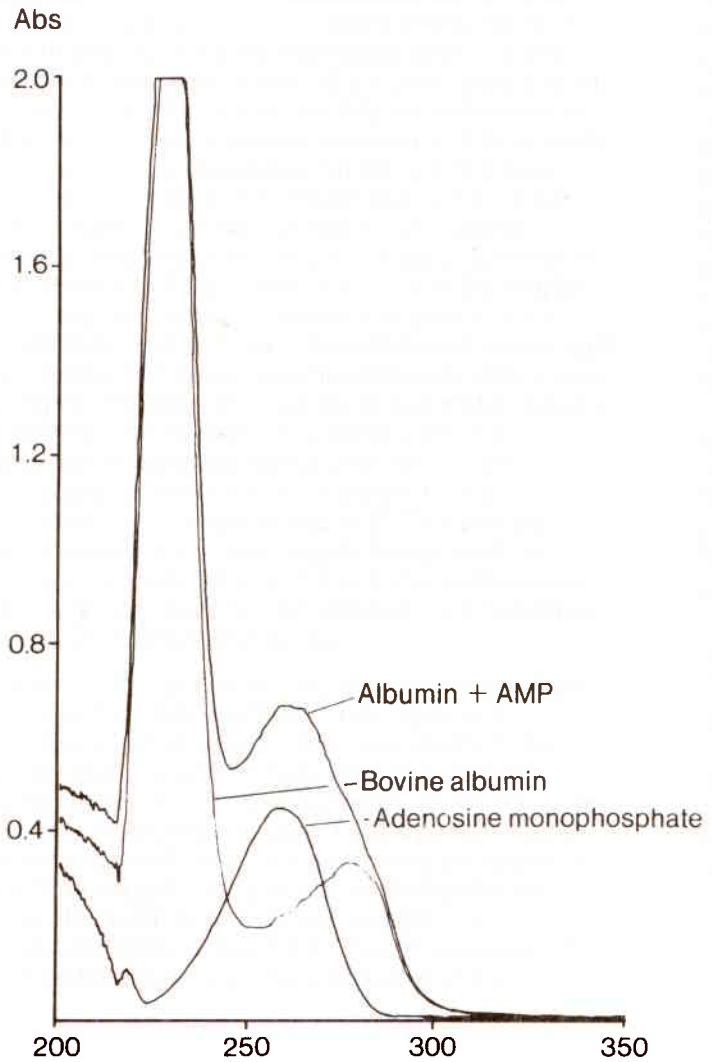

Figure 5
Absorption spectra for bovine plasma albumin (absorption maximum at 280 nm), adenosine monophosphate (absorption maximum at 260 nm) and a mixture of both albumin and adenosine monophosphate with the same final concentrations as in the two samples for which individual spectra are shown.

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