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Linearization of the Bradford Protein Assay Increases Its Sensitivity: Theoretical and Experimental Studies

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Determination of microgram quantities of protein in the Bradford Coomassie brilliant blue assay is accomplished by measurement of absorbance at 590 nm. However, an intrinsic nonlinearity compromises the sensitivity and accuracy of this method. It is shown that under standard assay conditions, the ratio of the absorbances, 590 nm over 450 nm, is strictly linear with protein concentration. This simple procedure increases the accuracy and improves the sensitivity of the assay about 10-fold, permitting quantitation down to 50 ng of bovine serum albumin. Furthermore, protein assay in presence of up to 35-fold weight excess of sodium dodecyl sulfate (detergent) over bovine serum albumin (protein) can be performed. A linear equation that perfectly fits the experimental data is provided on the basis of mass action and Beer's law.

The Coomassie brilliant blue protein assay, known as the Bradford assay (1), is widely used because of its ease of performance, rapidity, relative sensitivity, and specificity for proteins. As Bradford herself observed, however, "There is a slight nonlinearity in the response pattern. The source of the nonlinearity is in the reagent itself since there is an overlap in the spectrum of the two different color forms of the dye. The background value for the reagent is continually decreasing as more dye is bound to protein" (1). Over a broad range of protein concentrations the degree of curvature is quite large; therefore, only a narrow range of relatively high protein concentrations, 2–10 μg/ml BSA,[2] is used for assay and construction of the calibration graph. This nonlinearity presents a serious problem outside this narrow range of protein concentrations and when microgram amounts of protein are not available. Over 100 works have attempted to improve the Bradford assay (for review, see (2)). Some have addressed the nonlinearity problem (3–5), but all offered only a partial solution.

Three charge forms of the Coomassie brilliant blue dye are present in equilibrium at the usual acidic pH of the assay. The red, blue, and green forms have absorbance maxima at 470, 590, and 650 nm, respectively (6). The blue is the form that binds the protein, forming a complex that intensely absorbs light at 594 nm (7, 8). Therefore one may monitor the decrease in concentration of the free blue form in the presence of protein as reflected by the decrease in absorbance at 450 nm that is proportional mainly to the concentration of the red dye form. Following this procedure, we have found that in contrast to Bradford's proposal the decreasing background could not fully account for the nonlinearity. The decrease in dye concentration, however, does produce another distortion of the linear response because binding to the protein is in equilibrium (9); thus complex formation is dependent not only on protein concentration but also on dye concentration. This notion had been previously described by Chial and Splittgerber (7).

The present study shows that when these two interferences are taken into account in Beer's law, an equation can be written that describes a linear relationship between protein concentration and the ratio of absorbances, 590 nm over 450 nm. This equation was experimentally tested and found to yield a full-scale linear calibration line over the entire range studied. Furthermore, the sensitivity of the Bradford assay is increased by approximately one order of magnitude, making it possible to determine as little as 50 ng BSA in the 0.25-ml microplate assay. The

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[2] Abbreviations used: BSA, bovine serum albumin; CBBG, Coomassie brilliant blue G-250; DDW, double-distilled water; SDS, sodium dodecyl sulfate.

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improved sensitivity results also in reduced interference by detergents.

MATERIALS AND METHODS

Reagents

Coomassie brilliant blue G-250 (CBBG) was obtained from Merck. Crystallized bovine serum albumin was purchased from Schwarz/Mann (Spring Valley, NY). α-Chymotrypsin (C-3142) was obtained from Sigma. All reagents were of the highest available grade. Deionized, double-distilled water (DDW) was used. Proteins were dissolved in DDW.

Dye Reagent

Dye reagent was prepared according to Bradford (1). Coomassie brilliant blue G-250 (100 mg) was dissolved in 50 ml 95% ethanol. A volume of 100 ml phosphoric acid (85% w/v) was added and the solution was diluted to 1 liter with DDW and immediately filtered twice. The dye reagent was stored at 4°C, protected from light. Only when specifically mentioned in the text or figure legend, dye reagent from Bio-Rad was used.

Protein Determination

1-ml assay. Protein determination was performed with a slight modification to the original assay (1). A volume of 0.8 ml dye reagent was added to duplicate 0.2-ml protein samples in disposable plastic cuvettes, the tube content was thoroughly mixed, and absorbance was measured after 5–60 min against DDW as blank. All protein concentrations shown correspond to the final assay volume.

0.25 ml microplate assay. The procedure was performed as described for the 1-ml assay, reducing all volumes to $\frac{1}{4}$.

Equipment

A Varian Cary 1E UV/VIS spectrophotometer was used for the 1-ml assay. Microplate autoreader EL309 of BIO-TEK Instruments was used for the 0.25-ml assay.

RESULTS AND DISCUSSION

Theoretical Considerations

The spectrum (Fig. 1A) of the dye–protein complex (λ_{max} = 594 nm) was obtained by addition of the dye reagent to a large molar excess of protein. Under this condition, there are practically no free dye molecules, as demonstrated by the complete disappearance of the red dye form that absorbs at λ_{max} = 466 nm (Fig. 1B). The free dye exists as the acid–base equilibrium of the red (λ_{max} = 466 nm) form, the blue form that reacts

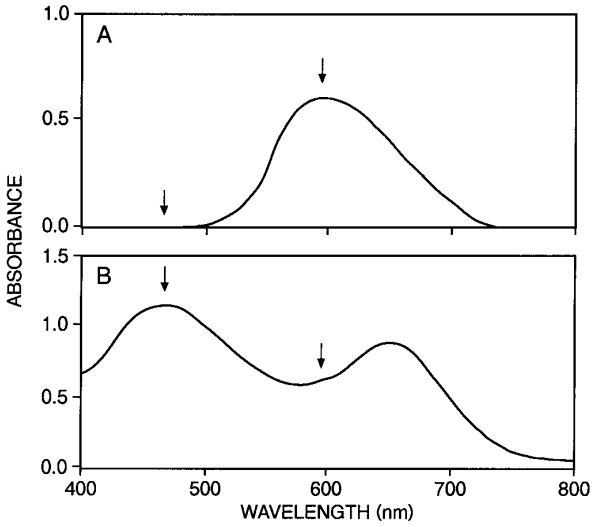

FIG. 1. Spectra of the dye–protein complex (A) and the dye (CBBG) alone (B) were obtained by addition of 0.8 ml dye reagent to 0.2 ml of 5 mg/ml BSA (A) or to 0.2 ml DDW (B). The length of the optical path is 0.1 cm in A and 1 cm in B. The arrows point at the absorbance maxima of 594 and 466 nm.

with the protein, and the green form that peaks at 650 nm but absorbs significantly at 590 nm, the λ_{max} of the dye–protein complex (6). Since the dye–protein complex does not absorb at 466 nm, it was possible to test Bradford's suggestion (1) that the nonlinearity of the protein calibration curve is due to a decrease in the background value of the reagent as more dye binds to the protein when protein concentrations are increased. Beer's law states that $A = \epsilon cl$, where A is the absorbance at any wavelength, ϵ is the molar absorption coefficient at the same wavelength, c is the molar concentration of the chromophore, and l is length of the optical path in centimeters. Using Beer's law, the expression for the concentration-corrected blank is the noncorrected blank (A_{594} without protein) multiplied by the ratio of absorbances at 466 nm with and without protein. We found that subtraction of a corrected blank from A_{594} obtained in the presence of 0–20 μg/ml BSA does not fully linearize the response curve, indicating an additional cause for the nonlinearity (results not shown).

Since dye–protein complex formation is not limited by the amount of protein, yet the dye is not in a large excess (10), we concluded that the formation of the dye–protein complex will not be proportional to protein concentration and will be dependent on dye concentration and on the coefficient of the equilibrium reaction

$$D + P \overset{K}{\leftrightarrow} DP \qquad [1]$$

in which P is the protein and D is the dye. The equilibrium constant is defined as

$$K = \frac{[DP]}{[nP] \times [D] \times f_b} \quad [2]$$

where f_b, the fraction of the reactive blue dye form, equals 0.053 under assay conditions (6) and n is the number of binding sites on a protein molecule, being primarily arginine residues (8). For simplicity the binding sites are assumed to be homogeneous and noninteracting, although binding has some unclear dependence on macromolecular structure (8). Substituting the conservation equation of protein binding sites, $[nP]_t = [nP] + [DP]$, where t indicates total, into Eq. [2] and rearrangement yields

$$[DP] = \frac{K \times f_b \times [D]}{1 + K \times f_b \times [D]} \times [nP]_t. \quad [3]$$

Further rearrangement gives the following expression for the complex concentration:

$$[DP] = \frac{1}{\left(\frac{1}{K \times f_b \times [D]} + 1\right)} \times [nP]_t. \quad [4]$$

The absorbance at 590 nm, composed of the independent absorbances of both the dye–protein complex and the free dye, is given by

$$A_{590} = \epsilon^{DP}_{590} \times l \times [DP] + \epsilon^{D}_{590} \times l \times [D], \quad [5]$$

where the superscript of ϵ defines the compound and the subscript refers to the wavelength.

Substitution of Eq. [4] into Eq. [5] leads to

$$A_{590} = \frac{\epsilon^{DP}_{590} \times l}{\left(\frac{1}{K \times f_b \times [D]} + 1\right)} \times [nP]_t + \epsilon^{D}_{590} \times l \times [D]. \quad [6]$$

According to Eq. [6], both components of A_{590} depend upon free dye concentration. Thus the correction for the absorbance of the free dye alone will not lead to linear dependence between A_{590} and protein concentration.

Because the dye–protein complex has no contribution to the absorbance at 450 nm, $[D]$ can be calculated as follows:

$$A_{450} = \epsilon^{D}_{450} \times l \times [D]. \quad [7]$$

This wavelength, like 590 nm, was chosen rather than the λ_{max} (466 and 594 nm) to make the assay compatible with the microplate autoreader instrument. It should be pointed out that ϵ^D, at both 590 and 450 nm, is a weighted average of the molar absorption coefficients of the three dye forms. The addition of protein does not change the combined ϵ^D since the fraction of each dye form depends only on pH and not on total concentration or consumption of one dye form. The substitution of Eq. [7] into Eq. [6] results in

$$A_{590} = \frac{\epsilon^{DP}_{590} \times l}{\left(\frac{\epsilon^{D}_{450} \times l}{K \times f_b \times A_{450}} + 1\right)} \times [nP]_t + \frac{\epsilon^{D}_{590} \times l}{\epsilon^{D}_{450} \times l} \times A_{450} \quad [8]$$

which is rearranged to become

$$\frac{A_{590}}{A_{450}} = \frac{\epsilon^{DP}_{590} \times l}{\left(\frac{\epsilon^{D}_{450} \times l}{K \times f_b} + A_{450}\right)} \times [nP]_t + \frac{\epsilon^{D}_{590}}{\epsilon^{D}_{450}}. \quad [9]$$

To reach a linear equation, in which protein concentration is the X variable, the following assumption is made

$$\frac{\epsilon^{D}_{450} \times l}{K \times f_b} \gg A_{450}. \quad [10]$$

Substitution of Eqs. [2] and [7] into Expression [10] and rearrangement gives

$$\frac{[nP]}{[DP]} \gg 1. \quad [11]$$

The critical assumption given by Expressions [10] and [11] was verified by the elegant experiments of Splittgerber and colleagues (6, 9). According to these experiments, the ratio $[nP]/[DP]$ equals approximately 4 when considering only high-affinity binding sites. In this case, the value of the expression preceding $[nP]_t$ in Eq. [9] changes by less than 5% over the protein concentrations range assayed. Furthermore, when considering low-affinity binding sites, the ratio $[nP]/[DP]$ is indeed far larger than 1 as assumed in Expression [11] and the value of the expression preceding $[nP]_t$ in Eq. [9] is practically constant. Since under assay conditions a substantial part of the absorbance is contributed by low-affinity binding sites (9), it can be concluded that the assumption given by Expressions [10] and [11] is fully justified.

Therefore, when assuming relationship [10], Expression [9] becomes

$$\frac{A_{590}}{A_{450}} = \frac{\epsilon_{590}^{DP} \times K \times f_b \times n}{\epsilon_{450}^{D}} \times [P]_t + \frac{\epsilon_{590}^{D}}{\epsilon_{450}^{D}} . \qquad [12]$$

Equation [12] predicts a linear relationship between the ratio A_{590}/A_{450} and total protein concentration, $[P]_t$, when the binding is far from saturation (Expression [11]).

As explained above, for simplicity the binding sites were assumed to be homogeneous and noninteracting. Under assay conditions, however, binding to a heterogeneous population of binding sites occurs (9). Yet, we found that the same form of linear equation (Eq. [12]) is obtained. The slope of this equation includes the parameters of all different binding sites: equilibrium constants (K) and number of binding sites on a protein molecule (n).

Experimental Support of the Theory

The following observations are consistent with the linear Eq. [12]:

(a) While the regular Bradford calibration graph (Fig. 2A) markedly deviates from linearity, Fig. 2B shows a straight line obtained upon plotting A_{590}/A_{450} as a function of BSA concentration.

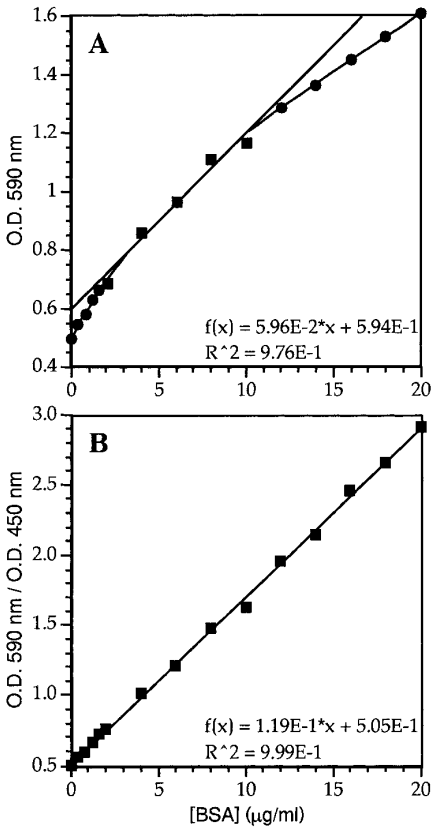

FIG. 2. Calibration graphs of 0–20 μg/ml BSA. (A) The conventional Bradford calibration graph. The linear regression line was calculated for the narrow range of 2–10 μg/ml BSA (■). (B) Linearized Bradford calibration graph. The same set of samples was used for both plots.

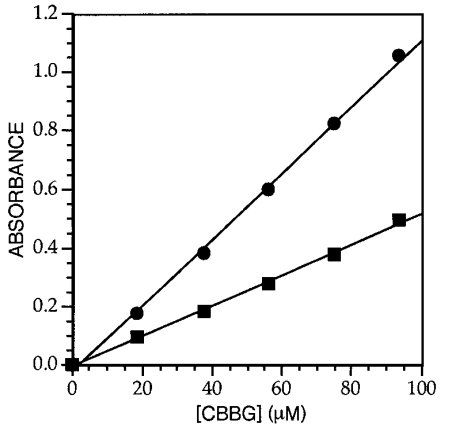

FIG. 3. Plots for the free CBBG dye at 450 nm (●) and at 590 nm (■) under the usual assay conditions.

(b) The theoretical equation predicts that the linear curve will intercept the Y axis at a value that equals the ratio of the dye's molar absorption coefficient at 590 nm over that at 450 nm. These constants were determined and found to be 5200 and 11,300 M^{-1} cm^{-1}, respectively (Fig. 3). The ratio of the molar absorption coefficients is 0.46 ± 0.01. This value is in excellent agreement with the experimental data of the calibration graph which is 0.48 ± 0.02 (average of 15 independent determinations, cf. Figs. 2B, 4, 5, and 6)

Calibration Graph

The regular Bradford calibration graph (Fig. 2A) shows distinct curvature in the range of 0–20 μg/ml

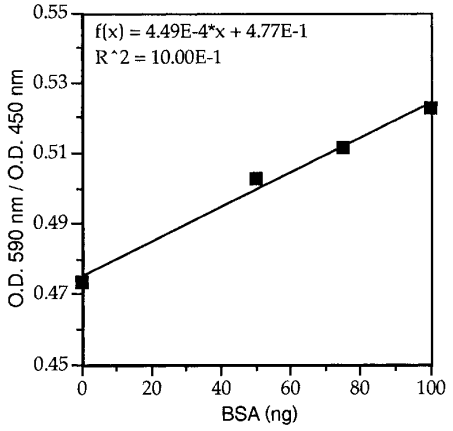

FIG. 4. Linearization of the Bradford calibration graph in the 0.25-ml microplate assay. The straight line was produced by the values derived of 7 points using 0–4 μg BSA. Only the range of 0–100 ng BSA is shown for the demonstration of sensitivity. The slope of the graph is smaller than that of Fig. 2B by approximately 250 due to the 4-fold smaller protein quantities and the 3 orders of magnitude difference in X-axis units.

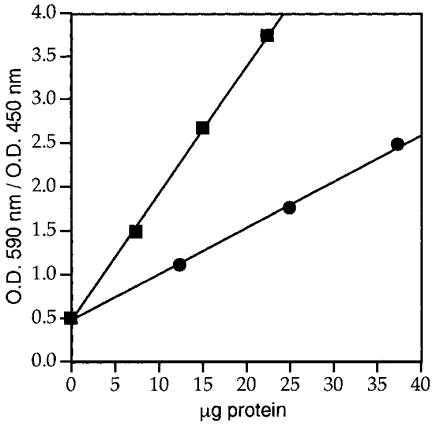

FIG. 5. Linearization of the Bradford calibration graph using α-chymotrypsin (●) and BSA (■). The linear equations are $Y = 0.053X + 0.474$ with $R^2 = 0.998$ for α-chymotrypsin and $Y = 0.146X + 0.457$ with $R^2 = 0.999$ for BSA.

BSA. However, in a curved graph, a semilinear relationship can be seen over a narrow range of points. The "close to linear" range of the Bradford calibration graph is considered to be 2–10 μg/ml BSA since smaller protein concentrations are characterized by a small signal to noise ratio and therefore cannot be accurately determined from a nonlinear graph. It is important to note that the linear regression in Fig. 2A was calculated using only 5 points (shown as squares) within the "linear range." Figure 2B shows that a linear correlation exists between A_{590}/A_{450} and BSA concentration in the range of 0–20 μg/ml, for the same set of samples used in plotting the nonlinear Fig. 2A. Statistical analysis gives standard deviations of 1.2 and 0.9% of error in the narrow range of 2–10 μg/ml BSA, for the regular and corrected calibration curves, respectively. The correlation parameter, R^2, is increased from 0.976 in Fig. 2A to 0.999 in Fig. 2B. The difference is even more obvious in the full-scale graphs, where the standard deviation is decreased from 3.9% error for the regular calibration curve to 1.2% error for the corrected calibration curve. The statistical analysis proves the improvement in linearity over the standard concentrations range and demonstrates the linear relationship between A_{590}/A_{450} ratio and protein concentration over the entire range, 0–20 μg/ml.

In order to determine the limits of sensitivity, an extended range of protein concentrations was tested. A quantity of 0.2 μg BSA can be accurately determined in the 1-ml assay while Fig. 4 shows that an amount as low as 50 ng BSA is accurately determined in the 0.25-ml microplate assay. The linear relationship exists up to 20 μg BSA in the 1-ml assay and up to 4 μg BSA in the microplate assay. It can be concluded that the procedure presented here produces a full-scale linear graph.

The procedure described can be applied to any standard protein, as shown in Fig. 5. Linear curves were obtained for both α-chymotrypsin and BSA, while the response of BSA is 2.8-fold higher than the response of α-chymotrypsin, a value that is in very good agreement with previous reports (11, 12). For both proteins the regular calibration graph was nonlinear (results not shown). It should be kept in mind that protein determination is always relative to the standard and not absolute. BSA is a commonly used standard in the Bradford assay because of its high color yield and availability while other standards may more closely resemble the proteins under determination (Fig. 5 and Ref. (13)).

Determination of Unknown Protein Samples

Although Fig. 4 shows that 0.2 μg/ml BSA can be accurately determined, the limits of detection of an unknown protein sample must also be established. To this end, rabbit serum was diluted to give 3–110 nl per

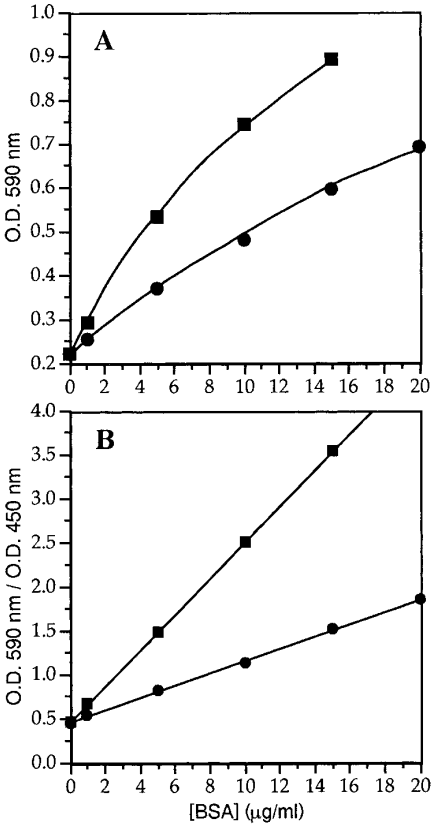

FIG. 6. Calibration graphs in the absence (■) or presence (●) of 0.002% SDS. A volume of 0.8 ml dye reagent from Bio-Rad was added to 0.2 ml protein sample without (■) or with (●) SDS. (A) Conventional Bradford calibration graph. (B) Linearized Bradford calibration graph. The same set of samples was used for both plots. The linear equations are $Y = 0.205X + 0.457$ with $R^2 = 1.000$ (■) and $Y = 0.070X + 0.452$ with $R^2 = 0.999$ (●).

TABLE 1

Determination of Serum Protein[a]

No.	Serum (μl)	A_{590}/A_{450}[b]	Serum protein[c]			SD percentage[f]	
			μg in assay[d]	mg/ml in the serum[e]			
				By one point	By slope[e]	By one point	By slope[e]
1	0	0.462					
2	0.003	0.480	0.17	51.0	53.0	7.3	4.8
3	0.010	0.530	0.57	61.5	62.8	5.7	7.3
4	0.014	0.547	0.80	54.9	57.3	2.4	0.6
5	0.070	0.898	3.98	57.0	57.1	0.1	0.2
6	0.110	1.144	6.26	56.8	56.9	0.1	0

[a] 1-ml assay.
[b] Average of duplicate samples.
[c] Equation of the BSA standard curve: $Y = 0.109X + 0.463$.
[d] The concentration used for the calculation is 56.9 mg/ml, determined by the ratio of the slopes.
[e] The slope was determined by the curve of samples $1-N$ ($N = 2-6$).
[f] Standard deviation of the concentration from 56.9 mg/ml.

assay and these were determined in parallel to BSA in a standard calibration curve. The linear equations of BSA and serum are $Y = 0.109X + 0.463$ and $Y = 6.20X + 0.463$, respectively, with correlation parameters R^2 of 0.996 and 0.999, respectively. The Y values are A_{590}/A_{450} and the X values are micrograms of BSA or microliters of serum. In general, the most accurate method to determine protein concentration in an unknown sample is to divide the slope of several sample dilutions by the slope of the calibration curve. The product representing concentration units is the protein concentration of the unknown sample before dilution. Table 1 shows the results of these calculations. The error is increasing gradually from 0.1% for 6.26 μg up to 7.3% for the sample containing only 0.17 μg in the 1-ml assay. The error is markedly reduced when the concentration is calculated by the slope of the unknown sample curve made of 2–6 points, including the blank. Table 1 shows that it suffices to determine the slope between one point of protein concentration and the blank value at zero protein concentration, to significantly improve the accuracy (Table 1, line 2). Usually, above 1 μg protein, calculation by one point is accurate enough and a slope is not needed. Similar results were obtained in the microplate assay, testing 42–1560 ng protein of rabbit serum, although the errors were somewhat higher, due to instrumental optical limitations.

Interference by Detergents

Detergents are known to interfere with the Bradford assay (1). Protein determination of solubilized proteins by Bradford assay can be carried out when glucopyranoside detergents are used (14) or following exclusion of detergents from the sample (15). The interference becomes evident when final SDS concentration exceeds 0.002% (15). Diluting the solubilized protein sample in order to reach noninterfering detergent concentration frequently results in protein content that is below the threshold of detection. Figures 6A and 6B show the regular and corrected calibration graphs, respectively, in presence of 0.002% SDS. A linear relationship exists between the ratio A_{590}/A_{450} and protein quantity in both the absence and presence of 0.002% SDS. It is therefore possible to determine as little as 0.3 mg/ml of protein solubilized by 1% SDS. The response to protein is reduced by the detergent, while the background is constant. However, the slope of the A_{590}/A_{450} curve in presence of 0.002% SDS is about equal to the slope of the A_{590} curve in the absence of detergents (compare Figs. 2A, 2B, and 6B). Higher SDS concentrations considerably reduce the response to protein and increase the background. Therefore, final SDS concentration that exceeds 0.002% does not allow protein determination. These findings and Eq. [12] are consistent with previous suggestion (16) that a high SDS concentration interferes with the Bradford assay by stabilizing the green dye form while a low SDS concentration interferes by competing with the dye on binding to the protein. As shown in Fig. 6B, the latter interference can be eliminated by measurement of A_{590}/A_{450} in the presence of the detergent for both the standard and the unknown sample.

CONCLUSIONS

The purpose of the present study was to evaluate the reasons for the nonlinearity of the Bradford assay in

order to develop a procedure that would yield a linear relationship between absorbance and protein concentration. We have found that the nonlinearity is due to two factors; both of them originate from the fact that the free dye concentration is decreased by protein addition. The outcome is a continuous decrease in the absorbance contributed by the free reagent on the one hand and a reduced dye–protein complex formation on the other hand. An equation describing absorbance at 590 nm, taking into account these two factors, demonstrates a linear relationship between the ratio of absorbances at 590 nm over 450 nm and total protein concentration. The applicability of this equation for the Bradford assay was established by demonstration of an extended linear range between 0.2 and 20 μg/ml BSA, indicating an improvement of sensitivity of the assay by one order of magnitude. The modified calibration graph makes it possible to accurately determine 50 ng BSA in the 0.25-ml microplate assay or 0.2 μg BSA in the 1-ml assay.

Protein determination by measuring A_{590}/A_{450} ratio is more accurate than a single measurement of A_{590} not only due to the transformation from nonlinearity to linearity, but also due to the higher slope of the resulting calibration curve obtained. In the conventional curve the slope within the concentration range assayed (2–10 μg/ml) is lower than the actual slope which should be measured close to the Y axis since the slope is continuously decreasing with increasing protein concentrations. Moreover, as A_{590} is increasing and A_{450} is decreasing with higher protein quantities, the ratio A_{590}/A_{450} is more sensitive to changes in protein concentration than either of the absorbances alone. This is expressed in the slope of the calibration curve.

In conclusion, the experimental demonstration of a linearized Bradford calibration graph that intercepts the Y axis at $\epsilon_{590}/\epsilon_{450}$ strongly supports our theoretical considerations. We hope that the improved accuracy and sensitivity and the partial elimination of interference by detergents will promote a more widespread use of the assay.

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