

A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding

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A protein determination method which involves the binding of Coomassie Brilliant Blue G-250 to protein is described. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 365 to 595 nm. and it is the increase in absorption at 595 nm which is monitored. This assay is very reproducible and rapid with the dye binding process virtually complete in approximately 2 min with good color stability for 1 hr. There is little or no interference from cations such as sodium or potassium nor from carbohydrates such as sucrose. A small amount of color is developed in the presence of strongly alkaline buffering agents, but the assay may be run accurately by the use of proper buffer controls. The only components found to give excessive interfering color in the assay are relatively large amounts of detergents such as sodium dodecyl sulfate, Triton X-100, and commercial glassware detergents. Interference by small amounts of detergent may be eliminated by the use of proper controls.

Laboratory practice in protein purification often requires a rapid and sensitive method for the quantitation of protein. Methods presently available partially fulfill the requirement for this type of quantitation. The standard Lowry procedure (1) is subject to interference by compounds such as potassium ion (2), magnesium ion (3), EDTA (4), Tris (3) thiol reagents (2), and carbohydrates (5). The relatively insensitive biuret reaction (6) is subject to interference by Tris (7), ammonia (8), and glycerol (9). Even the modified procedure for eliminating problems with the Lowry and biuret assays (10,11) present problems since more complications and time are involved in the modified procedures. The dye binding techniques in the literature are for the most part insensitive assays involving the binding of Orange **G** to protein (12-16). The exception to this rule is the Amidoschwarz 10-B binding assay (17). This procedure, too, has its drawbacks since the precipitation of the protein by trichloroacetic acid followed by filtration on Millipore membranes is required.

The protein assay herein described eliminates most of the problems involved in the procedures described above, and is easily utilized for

processing large numbers of samples, as well as adaptable to automation. It is based on the observation that Coomassie Brilliant Blue G-250 exists in two different color forms, red and blue (18). The red form is converted to the blue form upon binding of the dye to protein (18). The protein-dye complex has a high extinction coefficient thus leading to great sensitivity in measurement of the protein. The binding of the dye to protein is a very rapid process (approximately 2 min), and the protein-dye complex remains dispersed in solution for a relatively long time (approximately 1 hr), thus making the procedure very rapid and yet not requiring critical timing for the assay.

MATERIALS AND METHODS

Reagents. Coomassie Brilliant Blue G-250 was obtained from Sigma, and used as supplied. 2-Mercaptoethanol was obtained from Sigma. Triton X-100 was obtained from Schwartz/Mann. Sodium dodecyl sulfate was obtained from BDH Chemicals Ltd., Poole, England. Hemosol was obtained from Scientific Products. All other reagents were of analytical grade or the best grade available.

Protein preparation. Bovine serum albumin (2x crystallized), chymotrypsinogen A, and cytochrome *c* (horse heart) were obtained from Schwartz/Mann. Hemoglobin and human serum albumin were obtained from Nutritional Biochemicals Corporation. Protein solutions were prepared in 0.15 M NaCl. Concentrations were determined for bovine serum albumin, human serum albumin, chymotrypsinogen A, and cytochrome *c* spectrophotometrically in a Bausch and Lomb Spectronic 200 uv spectrophotometer based on $\epsilon_{280}^{1\%} = 6.6$ (19,20), 5.3 (19,21), 20 (19,22) and 17.1 (23,24) respectively. Hemoglobin solutions were prepared gravimetrically.

Preparation of protein reagent. Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml 95% ethanol. To this solution 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter. Final concentrations in the reagent were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid.

Protein assay (standard method). Protein solution containing 10 to 100 μg protein in a volume up to 0.1 ml was pipetted into 12 x 100 mm test tubes. The volume in the test tube was adjusted to 0.1 ml with appropriate buffer. Five milliliters of protein reagent was added to the test tube and the contents mixed either by inversion or vortexing. The absorbance at 595 nm was measured after 2 min and before 1 hr in 3 ml cuvettes against a reagent blank prepared from 0.1 ml of the appropriate buffer and 5 ml of protein reagent. The weight of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in unknown samples.

Microprotein assay. Protein solution containing 1 to 10 μg protein in a volume up to 0.1 ml was pipetted into 12 x 100 mm test tubes. The volume of the test tubes was adjusted to 0.1 ml with the appropriate buffer. One milliliter of protein reagent was added to the test tube and the contents mixed as in the standard method. Absorbance at 595 nm was measured as in the standard method except in 1 ml cuvettes against a reagent blank prepared from 0.1 ml of the appropriate buffer and 1 ml of protein reagent. Standard curves were prepared and used as in the standard method.

RESULTS

Reproducibility, sensitivity, and linearity of the assay. Triplicate standard assays of bovine serum albumin as a standard result in a highly reproducible response pattern. Statistical analysis gives a standard deviation of 1.2% of mean value for the assay. There is extreme sensitivity in the assay with 25 μg sample giving an absorbance change of 0.275 OD units. This corresponds to 5 μg protein/ml in the final assay volume. 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. This presents no real problem since the degree of curvature is only slight. If the assay is run with a set of standards and unknowns measured against the response curve of the standards instead of calculated by Beer's Law, there is no difficulty in obtaining satisfactory results.

Accuracy of the assay. Figure 1 shows the results of various proteins assayed in the system as to individual responses. There is a scattering of points around the line drawn in the graph. The scattering is believed to be a multifaceted function composed of difficulties in determining the exact amount of protein present in a given sample due to variation of extinction coefficients in the literature, the methods used to determine the exact amount of protein used in measuring extinction coefficients, and some degree of variation in the efficiency of dye binding to various proteins. Figure 2 shows the response pattern obtained from Lowry (1) assays of the same proteins. The degree of scatter in protein response to Lowry (1) assay is similar to that shown for the dye-binding assay presented here. The sensitivity of the Lowry (1) method is an absorbance of 0.110 OD units for the 25 μg standard corresponding to 8 μg protein/ml of final assay volume. By calculation, then, the dye binding assay is approximately four times more sensitive than the Lowry (1) assay. The degree of scatter around the Lowry (1) assay plot also points to the difficulty in establishing a quantitative value for a protein in standard solutions.

Stability of the protein-dye complex color. Figure 3 shows the rate of

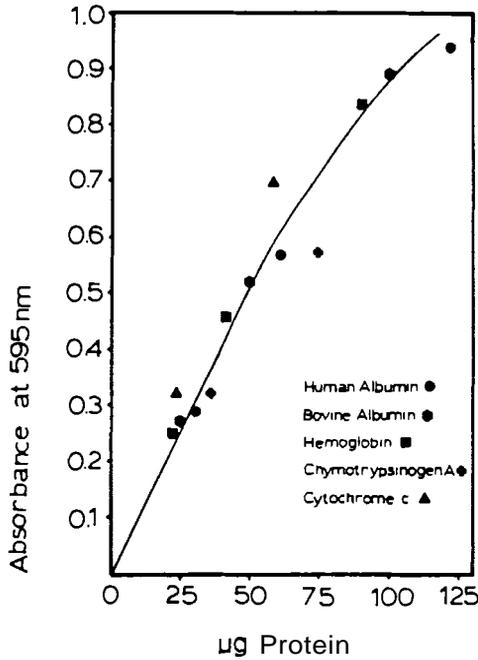


FIG. 1. Protein dye binding response pattern for various proteins.

formation of protein-dye complex in the assay system and the stability of the color complex. The absorbance was monitored at 7.5 sec intervals for 2 min and then at 1 min intervals for a period of 1 hr. As seen from the graph, the color development is essentially complete at 2 min, and remains stable plus or minus 4% for a period of 1 hr. Since the protein-dye complex has a tendency to aggregate with time, there is a decrease in color after this period of time simply by the physical removal of the pretein-dye complex from solution. If very precise determinations are required, investigators should take precaution to read the absorbance of

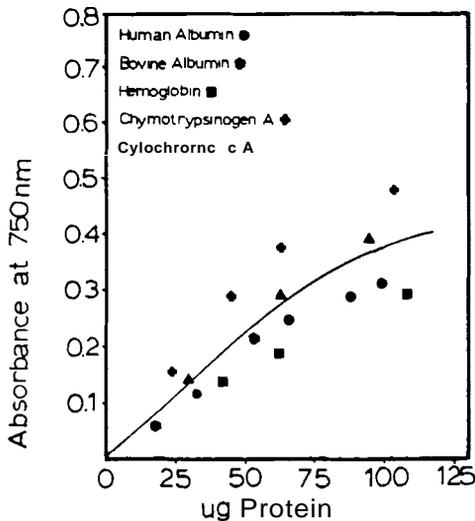


FIG. 2. Lowry (1) response pattern for various proteins.

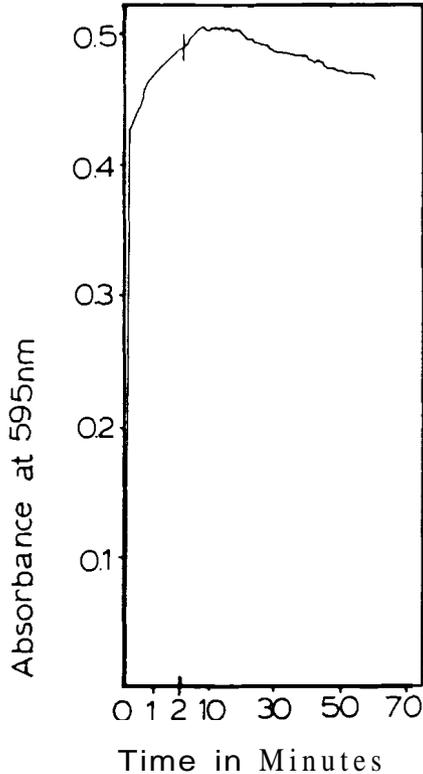


FIG. 3. Protein-dye complex formation rate and color stability.

samples during one of the flatter portions of the color stability curve between 5 and 20 min after reagent addition. This still gives ample time to read a relatively large number of samples.

Microassay system sensitivity. When bovine serum albumin is used as the standard in the micro assay system the degree of nonlinearity is similar to that found in the standard assay. There is a loss in protein-dye complex response as compared with the standard assay, i.e., 5 μg protein/ml gives an absorbance change of 0.1 vs 0.27 in the standard assay. Perhaps this results from increased dilution of the protein reagent.

Interference by nonprotein components. As indicated earlier, there is some interference in the assay system by strongly alkaline buffering agents. This may be overcome by running the appropriate buffer controls and subtracting the value for the control either mathematically or spectrophotometrically. A wide spectrum of components was tested for effects on the protein dye binding assay (Table 1). A lack of effect on the assay by magnesium chloride, potassium chloride, sodium chloride, ethanol, and ammonium sulfate was observed. The small effects due to Tris, acetic acid, 2-mercaptoethanol, sucrose, glycerol, EDTA, and trace quantities of the detergents, Triton X-100, sodium dodecyl sulfate, and Hemosol, can be easily eliminated by running the proper buffer control with the assay. However, the presence of large quantities of the detergents present abnormalities too great to overcome.

TABLE I
EFFECT OF VARIOUS LABORATORY REAGENTS ON COOMASSIE BRILLIANT
BLUE-G-250-PROTEIN COMPLEX ASSAY^a

Substance	Change in OD 595	(μ g) Equivalent BSA
1 M KCl	0.000	0.00
5 M NaCl	0.000	0.00
1 M $MgCl_2$	0.000	0.00
2 M Tris	0.026	2.34
0.1 M EDTA	0.004	0.36
1 M $(NH_4)_2SO_4$	0.000	0.00
99% Glycerol	0.012	1.08
1 M 2-Mercaptoethanol	0.004	0.36
1 M Sucrose	0.013	1.17
95% Ethanol	0.000	0.00
Acetone	0.069	6.21
5% Phenol	0.046	4.14
0.1% Triton X-100	0.013	1.17
1% Triton X-100	0.590	53.10
0.1% Sodium dodecyl sulfate	0.011	0.99
1% Sodium dodecyl sulfate	0.495	44.55
0.1% Hemosol	0.004	0.36
1% Hemoml	0.108	9.72

^a The above values were obtained when 0.1 ml of each substance was assayed in the standard assay.

A difficulty observed in performing the assay is the tendency of the protein-dye complex in solution to bind to cuvettes. This results in a blue colored cuvette. The amount of binding is negligible as far as assay readings are concerned, i.e., less than 1% error, as indicated by the standard deviation of triplicate assays in the reproducibility section. The blueness of the cuvettes after assay does present problems in other uses of the cuvettes so the following directions for cleaning the blue complex from cuvettes is included:

Method 1: Rinse cuvettes with concentrated glassware detergent, followed by water and acetone. (Gives immediate removal.)

Method 2: Soak cuvettes in 0.1 M HCL. (Removes complex in a few hours.)

The binding of the protein-dye complex has been observed only with quartz cuvettes and may be eliminated by using either glass or plastic cuvettes.

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