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Measurement of Protein Using Bicinchoninic Acid[1]

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Bicinchoninic acid, sodium salt, is a stable, water-soluble compound capable of forming an intense purple complex with cuprous ion (Cu^{1+}) in an alkaline environment. This reagent forms the basis of an analytical method capable of monitoring cuprous ion produced in the reaction of protein with alkaline Cu^{2+} (biuret reaction). The color produced from this reaction is stable and increases in a proportional fashion over a broad range of increasing protein concentrations. When compared to the method of Lowry *et al.*, the results reported here demonstrate a greater tolerance of the bicinchoninate reagent toward such commonly encountered interferences as nonionic detergents and simple buffer salts. The stability of the reagent and resulting chromophore also allows for a simplified, one-step analysis and an enhanced flexibility in protocol selection. This new method maintains the high sensitivity and low protein-to-protein variation associated with the Lowry technique.

KEY WORDS: protein determination; bicinchoninic acid.

The widely used method of Lowry *et al.* (1) for protein determination relies on the Folin–Ciocalteau reagent to enhance the sensitivity of the biuret reaction. The instability of this reagent in an alkaline medium demands that exacting technique be exercised in the timing of both reagent addition and mixing with sample in order to obtain accurate results. The two-step nature of the Lowry method is mechanically cumbersome and tedious, and adds considerable complexity to efforts to attempt to utilize it in an automated or post-column-detection format (2). Additionally, nonionic detergents as well as some buffer salts used at levels useful for protein solubilization can interfere by forming insoluble precipitates with the Folin–Ciocalteau reagent. It seems apparent that most of the difficulties associated with the Lowry method are due to the peculiarities of the detection reagent used.

In this paper we describe a new protein measurement method based upon an alternative detection reagent, namely bicinchoninic acid (BCA).[3] In the form of its water-soluble sodium salt, BCA is a sensitive, stable, and highly specific reagent for Cu^{1+} (3). Previously, this attribute has been utilized to monitor the levels of other substances capable of reducing Cu^{2+} to Cu^{1+} such as uric acid (4) and glucose (5). To our knowledge, no one has exploited a BCA indicator system to monitor Cu^{1+} produced during the biuret reaction.

MATERIALS AND METHODS

Chemicals. Bicinchoninic acid, sodium salt, was synthesized by the Pfitzinger reaction of isatin and acetoin (Aldrich), substituting sodium hydroxide for potassium hydroxide in the method of Lesesne and Henze (6). The

[1] This paper is dedicated to the memory of Dr. E. Melvin Gindler, a long-time friend and colleague. Without Mel's knowledge and love of chemistry which he so generously shared, this work would not have been possible.

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[3] Abbreviations used: BCA, bicinchoninic acid; BCA-Na_2, bicinchoninic acid, disodium salt; RIBO, ribonuclease A; CHYMO, chymotrypsinogen; IgG, human immunoglobulin G; BSA, bovine serum albumin; S-WR, Standard Working Reagent.

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crude product obtained was subjected to recrystallization from a minimum amount of 75°C water until the absorbance blank of the freshly prepared working reagent (described below) was less than 0.065 at 562 nm. Three recrystallizations were usually required. The hydrated, nearly colorless needles isolated revert to amorphous, cream-colored anhydrous powder upon drying at 60°C. Technical-grade BCA currently available from various sources (Sigma, Hach, Pierce) gives a somewhat higher blank reading and has not been further evaluated. Inorganic salts used in the reagent formulations were of reagent-grade quality (J. T. Baker or Fisher). All prepared solutions were passed through 1-μm filters to remove insoluble debris associated with the salts. Folin–Ciocalteau reagent (2 N) was purchased from Fisher and diluted to 1 N prior to use. Compounds used in the interfering substances studies were of the highest quality commercially available, while nonionic detergents were further purified by described methods (7,8). All water used was deionized, but care must be taken to avoid obtaining water from systems containing copper lines and fittings, because enough dissolved copper as Cu^{1+} may be present to contribute appreciably to blank readings. We found that deionized water (18 Mohm-cm) delivered from all plastic cartridged units (Millipore or Barnsted) was satisfactory.

Proteins. BSA (crystallized) and IgG (human) were obtained from Miles. Ribonuclease, chymotrypsinogen, and human insulin were from Boehringer–Mannheim, and avidin (egg white) was of affinity-purified grade obtained from Pierce. Standard protein solutions were prepared in either isotonic saline or, in the case of the interfering substances studies, in a solution containing the particular compound under study.

Lowry reagent and protocol. The Lowry reagent formulations and protocol used in this study were as described (1).

Standard BCA reagent and protocol. Reagent A consists of an aqueous solution of 1% BCA-Na_2, 2% $Na_2CO_3 \cdot H_2O$, 0.16% Na_2 tartrate, 0.4% NaOH, and 0.95% $NaHCO_3$. If needed, appropriate addition of NaOH (50%) or solid $NaHCO_3$ is made to reagent A to adjust the pH to 11.25. Reagent B consists of 4% $CuSO_4 \cdot 5H_2O$ in deionized water. Reagents A and B are stable indefinitely at room temperature and are commercially available (Pierce). Standard Working Reagent (S-WR) is prepared weekly or as needed by mixing 100 vol of Reagent A with 2 vol of Reagent B. S-WR is apple green in color.

The standard assay procedure consists of mixing 1 vol of sample (standard or unknown) with 20 vol of S-WR in a test tube. For convenience we routinely use a 100-μl sample and 2 ml S-WR; however, any multiple of these volumes may be used depending upon the total volume needs of a particular spectrophotometer or considerations based on the availability of the protein to be assayed. Color development proceeds immediately, even at room temperature, but it can be greatly accelerated by incubating the tubes in a constant-temperature water bath. In this respect, the temperature chosen for the color development is directly related to the desired sensitivity. The incubation protocols used to generate the bulk of the data in this report were (i) room temperature for 2 h, (ii) 37°C for 30 min, and (iii) 60°C for 30 min. After the chosen incubation step, the samples are cooled to room temperature and their absorbances measured at 562 nm versus a reagent blank. The concentration of unknowns can be then determined from a plot of concentration vs absorbances obtained for the standard protein solutions.

pH optimum. The pH optimum of the assay was determined by adjusting the pH of Reagent A with either NaOH (50%) or solid $NaHCO_3$, preparing the corresponding working reagents, and assaying a set of BSA standards (100–1200 μg/ml) using an incubation protocol of 30 min at 37°C.

Color stability. Final color stability was determined for all three selected incubation protocols. After the incubation period, the assay tubes were cooled to room temperature as needed and the absorbances recorded imme-

diately. Additional readings were made over a 1-h period in order to monitor any subsequent changes in absorbance values.

Interfering substances. A stock solution of BSA at a concentration of 1000 μg/ml was prepared in deionized water. Stock solutions of each compound listed in Table 1 were also prepared in deionized water, but at twice the concentration shown. An automated pipetting device (Micro Lab P from Hamilton) was used to dispense 0.05 ml of the BSA stock solution (or deionized water) and 0.05 ml of the appropriate interfering compound stock solution (or deionized water) into each of the tubes for the assays. The tubes marked "water blank" contained no BSA or potentially interfering compound, while those marked "interference blank" contained no BSA but did contain the potentially interfering compound. The tubes marked "reference" contained the BSA in water plus an additional aliquot of water so that the sample volumes in all tubes were identical (0.1 ml). All the tubes were then carried though the BCA method using the 37°C/30 min protocol or the Lowry method as single runs. The amount of BSA found was then calculated from the net absorbance at the appropriate wavelength after subtracting the "water blank" or "interference blank."

Protein-to-protein variability. The standard BCA 37°C/30 min protocol was used to conduct the protein-to-protein variation study. All of the proteins assayed were prepared in sets of standard concentrations in the range 100–1200 μg/ml. The same sets of protein standards were also assayed using the Lowry method.

Working reagent stability. A standard curve plotting absorbance vs concentration of BSA was made using freshly prepared working reagent for both the standard BCA and Lowry formulations. The analysis was then repeated after 7 days of storage at room temperature using the same BSA standards.

Micro BCA Reagent and protocol. Extremely dilute protein solutions (0.5–10 μg/ml) can be efficiently assayed by a 60°C/60 min protocol which employs a more concentrated reagent formulation. Micro-Reagent A (MA) consists of an aqueous solution of 8% $Na_2CO_3 \cdot H_2O$, 1.6% NaOH, 1.6% Na_2 tartrate and sufficient $NaHCO_3$ to adjust the pH to 11.25. Micro-Reagent B (MB) consists of 4% BCA-Na_2 in deionized water. Micro-Reagent C (MC) consists of 4 vol of 4% (aq) $CuSO_4 \cdot 5H_2O$ plus 100 vol of Micro-Reagent B. Micro-Working Reagent (M-WR) consists of 1 vol of MC plus 1 vol of MA. MA and MB are stable indefinitely at room temperature, but MC and M-WR should be prepared as needed.

To run the assay, mix 1 volume of sample or standard with 1 volume of M-WR in a test tube and incubate the tubes for 60 min at 60°C, cool to room temperature, and measure the absorbance vs the blank at 562 nm. Determine the concentration of unknowns from a standard curve.

RESULTS AND DISCUSSION

When protein is placed in an alkaline system containing Cu^{2+}, a colored complex can form between the peptide bonds of the protein and the copper atoms. The properties of this "biuret" reaction have been used for quite some time to measure the quantity of protein present within a solution (9–10). Unfortunately, the low sensitivity of this technique severely limits its usefulness for measuring the concentration of dilute protein solutions. With the development of the Lowry assay which successfully applied the Folin–Ciocalteau reagent to enhance the color response of the biuret reaction, a sensitive protein detection system resulted. While the mechanism of the Lowry reaction is not well understood, we have theorized that it involves a reduction of the Cu^{2+} to Cu^{1+} at the complexation sites within the protein molecule. This may then be followed by reaction of the generated Cu^{+1} with Folin–Ciocalteau reagent to form the final intense color. Using this reasoning, we decided to replace the Folin–Ciocalteau reagent with a compound known for its specificity in complexing Cu^{1+} in hopes of overcoming some of the lim-

TABLE 1

EFFECT OF SELECTED POTENTIAL INTERFERING COMPOUNDS

Sample (50 μg BSA) in the following:	BCA assay (μg BSA found)		Lowry assay (μg BSA found)	
	Water blank corrected	Interference blank corrected	Water blank corrected	Interference blank corrected
50 μg BSA in water (reference)	50.00	—	50.00	—
0.1 N HCl	50.70	50.80	44.20	43.80
0.1 N NaOH	49.00	49.40	50.60	50.60
0.2% sodium azide	51.10	50.90	49.20	49.00
0.02% sodium azide	51.10	51.00	49.50	49.60
1.0 M sodium chloride	51.30	51.10	50.20	50.10
100 mM EDTA (4 Na)		No color	138.50	5.10
50 mM EDTA (4 Na)	28.00	29.40	96.70	6.80
10 mM EDTA (4 Na)	48.80	49.10	33.60	12.70
50 mM EDTA (4 Na), pH 11.25	31.50	32.80	72.30	5.00
4.0 M guanidine HCl	48.30	46.90		Precipitated
3.0 M urea	51.30	50.10	53.20	45.00
1.0% Triton X-100	50.20	49.80		Precipitated
1.0% SDS (lauryl)	49.20	48.90		Precipitated
1.0% Brij 35	51.00	50.90		Precipitated
1.0% Lubrol	50.70	50.70		Precipitated
1.0% Chaps	49.90	49.50		Precipitated
1.0% Chapso	51.80	51.00		Precipitated
1.0% octyl glucoside	50.90	50.80		Precipitated
40.0% sucrose	55.40	48.70	4.90	28.90
10.0% sucrose	52.50	50.50	42.90	41.10
1.0% sucrose	51.30	51.20	48.40	48.10
100 mM glucose	245.00	57.10	68.10	61.70
50 mM glucose	144.00	47.70	62.70	58.40
10 mM glucose	70.00	49.10	52.60	51.20
0.2 M sorbitol	42.90	37.80	63.70	31.00
0.2 M sorbitol, pH 11.25	40.70	36.20	68.60	26.60
1.0 M glycine		No color	7.30	7.70
1.0 M glycine, pH 11.	50.70	48.90	32.50	27.90
0.5 M Tris	36.20	32.90	10.20	8.80
0.25 M Tris	46.60	44.00	27.90	28.10
0.1 M Tris	50.80	49.60	38.90	38.90
0.25 M Tris, pH 11.25	52.00	50.30	40.80	40.80
20.0% ammonium sulfate	5.60	1.20		Precipitated
10.0% ammonium sulfate	16.00	12.00		Precipitated
3.0% ammonium sulfate	44.90	42.00	21.20	21.40
10.0% ammonium sulfate, pH 11.	48.10	45.20	32.60	32.80
2.0 M sodium acetate, pH 5.5	35.50	34.50	5.40	3.30
0.2 M sodium acetate, pH 5.5	50.80	50.40	47.50	47.60
1.0 M sodium phosphate	37.10	36.20	7.30	5.30
0.1 M sodium phosphate	50.80	50.40	46.60	46.60
0.1 M cesium bicarbonate	49.50	49.70		Precipitated

itations of the Lowry procedure. As shown in Fig. 1, bicinchoninic acid can form a 2:1 complex with Cu^{1+}, resulting in a stable, highly colored chromophore with an absorbance maximum at 562 nm (3). We have found that it is possible to make use of this reaction to

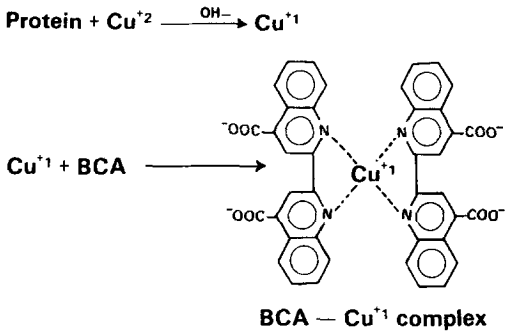

FIG. 1. Formation of purple complex with BCA and cuprous ion generated from the biuret reaction.

formulate a new reagent system for the quantitation of protein in solution which has significantly improved properties over that of the Lowry method.

Protocol optimization. Studies were conducted at various incubation times and temperatures to ascertain suitable conditions for assaying the broadest possible range of protein concentrations (100–1200 μg/ml), while at the same time remaining within the acceptable absorbance range of most common spectrophotometers (0–2 AU). Care was also given to determining those specific conditions which would result in stable color formation. The protocols ultimately chosen allow for multiple assays to be run at one time without the need to maintain precisely timed reagent additions.

The excellent stability of the BCA reagent to elevated temperatures has opened up the possibility of working out several different incubation protocols. We chose room temperature, 37°C, and 60°C as these should be applicable to most laboratory situations. This type of protocol flexibility allows the investigator to "fine tune" the sensitivity needed for a particular assay. Figure 2 shows the results of assaying a standard set of protein concentrations after incubating the tubes for several time periods at each selected temperature. The incubation protocols give essentially equiva-

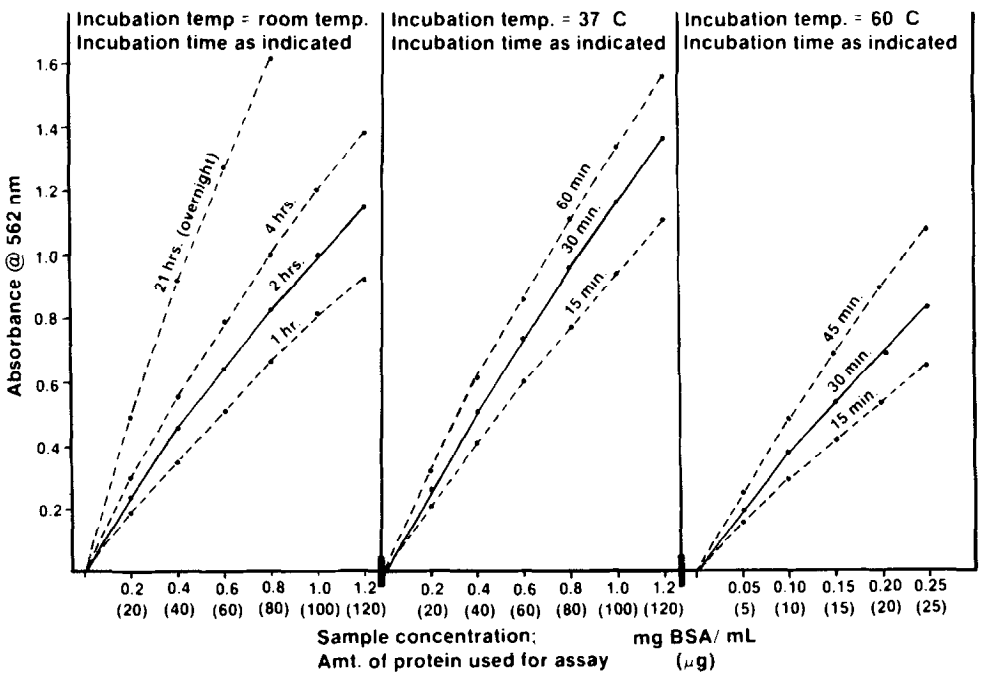

FIG. 2. Color response curves generated at indicated incubation temperatures for various times using standard BCA formulation. The top numbers along the horizontal axis refer to protein concentration in mg BSA/ml in sample before dilution 20:1 with reagent, while the numbers in parentheses refer to amount (μg) of BSA used in the assay.

lent standard curves, but with variable slopes. The relative linearities resulting from these assay protocols are about the same as that seen in the Lowry technique. Although not following Beer's Law exactly, it is nonetheless adequate for measuring the protein concentration in a wide range of potential samples. It is noteworthy to point out that the room temperature incubations can be extended overnight, allowing the preparation of samples in the late afternoon and measuring of absorbances the following morning. This makes running the assay very relaxed, especially since the incubation timings are not critical.

An unexpected characteristic of the BCA system is the effect of pH on the rate of color formation (see Fig. 3). The fundamental reaction being monitored, the biuret reaction, shows a decrease in color development as the pH is lowered from 12 to 11 (9), but the BCA system shows greatly accelerated rates of color generation with a maximum appearing at pH 11.25. When adjusting the pH to 11.25, the color of the BCA working reagent turns from blue (tartrate complex) to an apple green which we assume is a complex of BCA and Cu^{2+}. We speculate that Cu^{2+} in this form is more readily available to react with protein and may account for the observation of an increased reaction rate at pH 11.25. The working reagent has sufficient buffering capacity such that 1 vol of sample containing 0.1 N HCl or 0.1 N NaOH mixed with 20 vol of working reagent will not alter the pH enough to significantly change the assay result (see Table 1). The combination of buffer salts and NaOH in Reagent A was arrived at empirically with the goal of optimizing color formation. Other combinations are possible and perhaps more desirable and logical so long as the resulting pH is approximately 11.25. One of the reviewers has suggested a more expedient formulation consisting of 0.25 M sodium carbonate and 0.01 M sodium bicarbonate and no NaOH.

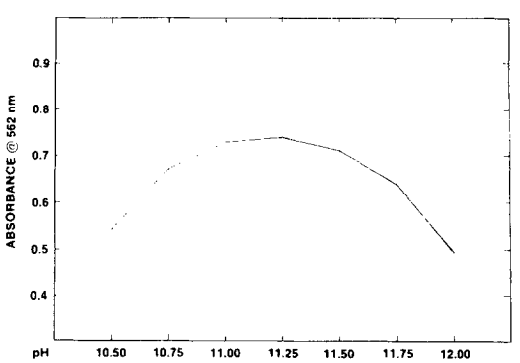

FIG. 3. pH optimum profile for the BCA working reagent. The chosen formulation has sufficient buffering capacity such that the sample may be in 0.1 N HCl or 0.1 N NaOH without significantly altering the rate of color formation from optimum.

Protein-to-protein variability. Since the mechanism of the Lowry assay seems to be closely related to that of the BCA technique, one might expect them to show similar response characteristics. Figure 4 shows a comparison of the Lowry and BCA assays with respect to the color response from seven proteins. As can be seen, the BCA assay retains almost exactly the same response characteristics as the Lowry technique with the curious exception of avidin. The two also show very similar appearances in their relative linearities and sensitivities. Another aspect of this study supporting the mechanistic similarities of the BCA and Lowry methods is the response of the two toward the protein gelatin. It is known (1) that the Lowry assay typically gives a depressed response for this protein, and BCA mimics this finding.

Limited studies conducted to date comparing BSA and gelatin using the BCA method show that as the temperature of the assay is increased to 60°C, the difference in response for the two proteins lessens and, additionally, the standard curves become more linear. For example, at room temperature for 4 h (or 37°C for 30 min) gelatin yields approximately 50% of the color yield as an equivalent amount of BSA. However, the assay conducted at 60°C for 30 min produces a response for gelatin that is nearly 70% of the response for BSA. These data suggest that total color yield is a sum of contributions from at least two sources. We speculate that one source consists of contri-

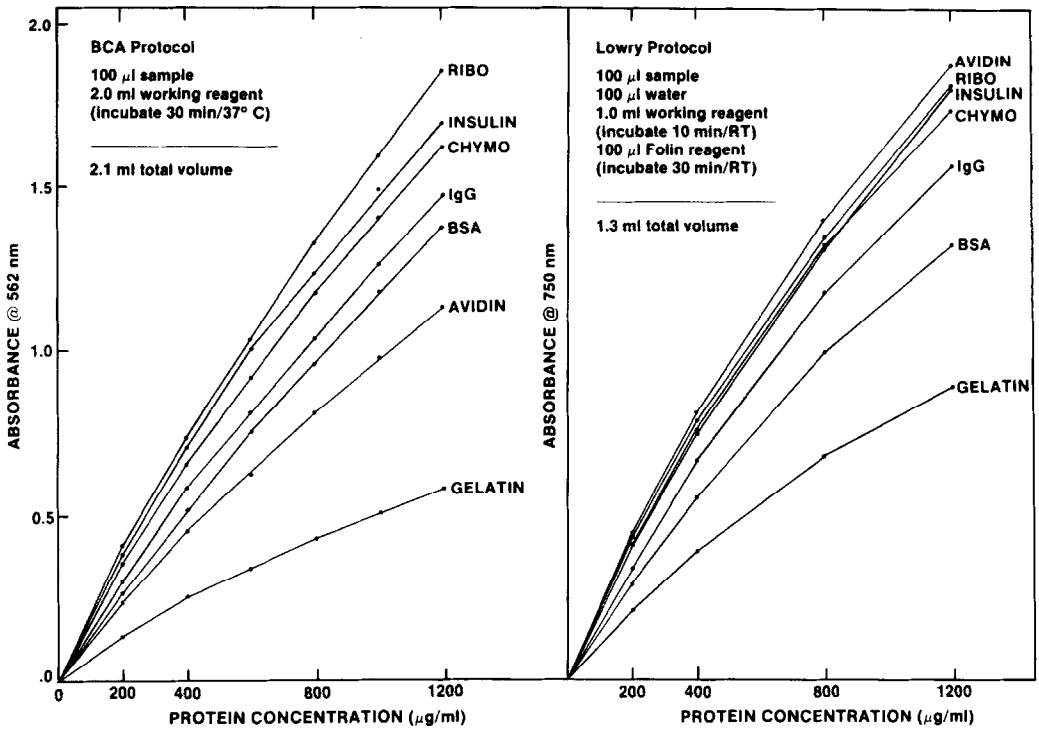

FIG. 4. Protein-to-protein variation for the BCA method compared to the Lowry method for seven proteins. The results shown for the BCA method were obtained using the 37°C/30 min protocol.

butions from readily oxidizable protein components such as tyrosine, cysteine, and tryptophan and that this color contribution is relatively temperature independent. A second, more temperature-dependent color contribution probably arises from peptide bonds reacting with divalent copper. The net contribution to total color due to peptide bonds appears to be more significant at 60°C than at room temperature, thus explaining the reduction in protein-to-protein variation between BSA and gelatin observed for the 60°C protocol. In terms of practical considerations, we feel that BSA behaves in a sufficiently nominal manner in the BCA method such that it can be employed as a reference standard in any of the described protocols without fear of grossly over- or underestimating the concentration of an unknown protein sample.

Interfering substances. The potentially interfering compounds selected for inclusion in this study were chosen on the basis of being frequently encountered during the purification and isolation of proteins or because they were already known to interfere in the Lowry method (11). The data presented in Table 1 demonstrate that most of the compounds studied interfere to a significant extent in the Lowry method, while the BCA method is more tolerant of these compounds. Of particular importance is the tolerance of the BCA method to detergents. Since detergents are frequently used for solubilizing proteins and their complete removal prior to assaying with the Lowry method is often difficult (11), this becomes a significant advantage of the BCA technique. Also noteworthy is the fact that denaturing reagents (4.0 M guanidine–HCl or 3.0 M urea) are fairly well tolerated by the BCA method, while they interfere with the Lowry. The BCA assay is, however, more sensitive to interference from reducing sugars, possibly

because the protocol allows the sugars more time to reduce Cu^{2+} to Cu^{1+}. As expected, copper-chelating reagents such as EDTA cause problems for both methods, as do buffer solutions which change the pH of the BCA or Lowry working reagents. These results indicate that the BCA reagent can be used in many applications where the Lowry method fails. Caution should be taken, however, to prepare the standard and sample protein solutions in the same matrix in order to minimize the affect of potential interfering compounds. Alternatively, the use of trichloroacetic acid to precipitate protein and thereby avoid the effect of certain interfering substances is a technique which works equally well for either the Lowry or the BCA method (data not shown).

Reagent and color stability. One of the most severe limitations of the Lowry formulation is its lack of reagent stability over time. Since the assay response falls off dramatically after only a few days of storage, in order to ensure optimal results fresh reagent must be prepared daily. Figure 5 shows a comparison of the Lowry vs BCA stability by assaying a set of standard protein solutions with freshly prepared working reagents and then subsequently running the assay again after storing the working reagents for 1 week at room temperature. As can be seen, the Lowry reagent shows approximately a 40% decrease in assay response after a week of storage, while the BCA formulation shows no significant change. This attribute of the BCA method allows for working reagent to be prepared in quantities sufficient to run at least 1 week of protein assays without fear of losing sensitivity or precision.

The end-point color stability of the BCA assay was assessed by performing the BCA method at the indicated temperatures, cooling to room temperature as needed, and observing any further absorbance changes at 562 nm. Results are shown in Fig. 6. Using the 60°C/30 min assay, the sample color which developed remained stable throughout the range of observation time, showing no appreciable up-

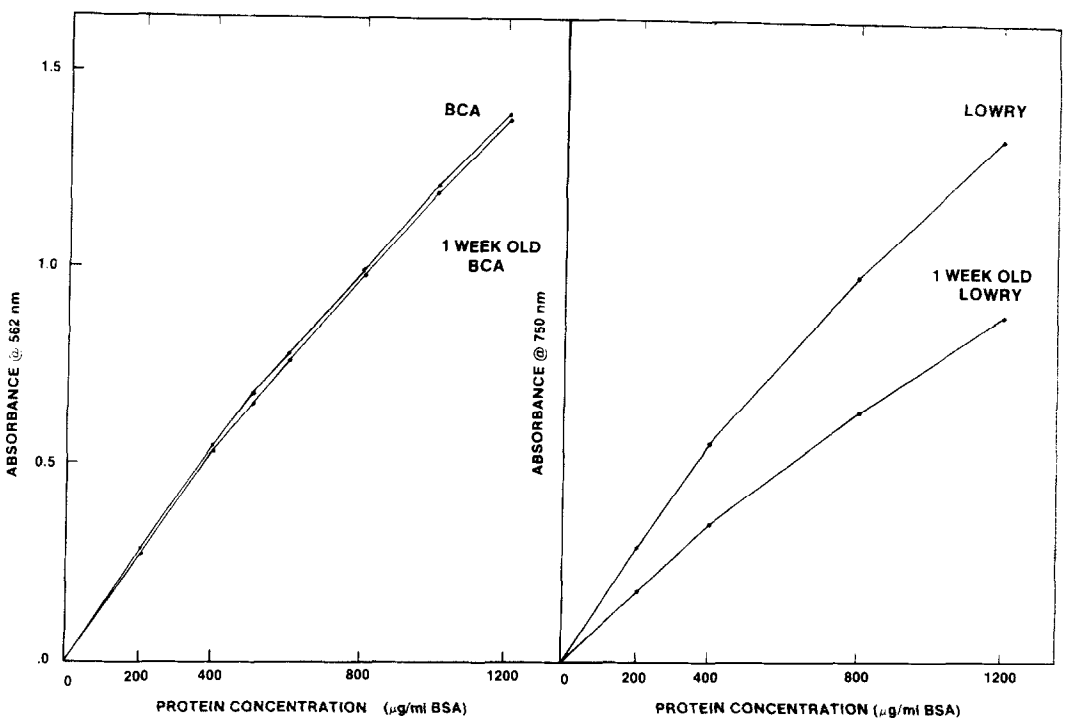

FIG. 5. Performance of fresh and 1-week-old BCA and Lowry working reagents.

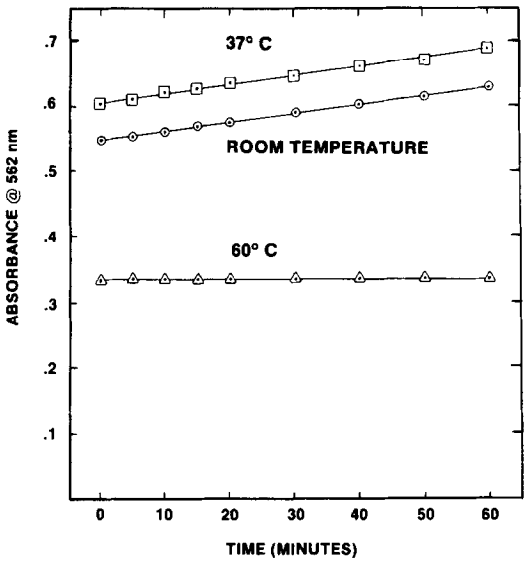

FIG. 6. Endpoint color stability of the BCA method using three different temp/time protocols. The room temperature (⊙, 50 μg BSA) and 37°C (□, 50 μg BSA) protocols show a 2.5% drift per 10 min while the 60°C protocol (△, 10 μg BSA) shows no drift in absorbance after 1 h.

ward drift. This indicates that the color reaction has essentially gone to completion using this incubation timing. However, with the 37°C/30 min protocol, as well as that for room temperature/2 h, a true endpoint is not completely reached in the allotted incubation period, and the absorbance continues to increase at a rate of about 0.25% per minute. Since color development has not reached a maximum in these cases, it is important to read all absorbances in the shortest possible time interval. In actual practice, we found that 30 tubes can be easily read within 10 min without significant loss of accuracy or precision.

Micro method. We also found that a slightly more concentrated reagent formulation would result in the ability to assay extremely dilute protein solutions. Figure 7 illustrates the "micro" method option. Using this reagent, protein solutions containing about 0.5 to 10 μg/ml can be accurately measured. The linearity resulting from the micro protocol is even better than for the standard reagent formulation. However, we have not examined the effect of interfering substances with this protocol.

CONCLUSIONS

The data presented here show the BCA method to be a superior alternative to the Folin–Ciocalteau reagent for monitoring and greatly enhancing the response of the biuret reaction with protein. The BCA technique offers manipulative simplifications, more tolerance toward interfering substances, greater working reagent stability, increased sensitivity, and greater protocol flexibility when compared to the standard Lowry assay. The protein-to-protein variability of the two methods appears to be very similar and quite acceptable.

The flexibility of the proposed method in terms of formulation and protocol options is a direct result of the stability of BCA reagent and its copper I chromophore. This allows the investigator the freedom to optimize conditions for a wide range of protein measurement problems. For instance, preliminary work in our laboratory suggests that simple modifications of the BCA formulations given here can successfully overcome the limitations of either

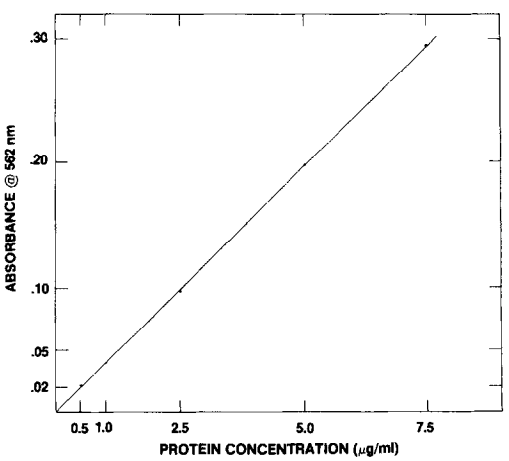

FIG. 7. Calibration curve of BSA using the micro protocol and formulation. In this example 1 ml of BSA standard at the indicated concentration was mixed with 1 ml of Micro-Working Reagent and incubated at 60°C for 60 min.

the Lowry or biuret reagents when used in the post-column HPLC detection of proteins (2). Further studies indicate that BCA can be also utilized to measure the amount of protein covalently immobilized on beaded agarose or passively adsorbed on plastics. These investigations will be the basis of future reports.

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REFERENCES

1. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
2. Schlabach, T. D. (1984) *Anal. Biochem.* **139,** 309–315.
3. Tikhonov, V. N., and Mustafin, I. S. (1965) *Zh. Anal. Khim.* **20,** 390–392.
4. Gindler, E. M. (1970) *Clin. Chem.* **16,** 536.
5. Gindler, E. M. (1970) *Clin. Chem.* **16,** 519.
6. Lesesne, S. D., and Henze, H. R. (1942) *J. Amer. Chem. Soc.* **64,** 1897–1900.
7. Chang, H. W., and Bock, E. (1980) *Anal. Biochem.* **104,** 112–117.
8. Ashani, Y., and Catrvas, G. N. (1980) *Anal. Biochem.* **109,** 55–62.
9. Weichselbaum, T. E. (1946) *Amer. J. Clin. Pathol.* **7,** 40.
10. Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) *J. Biol. Chem.* **177,** 751–766.
11. Peterson, G. L. (1979) *Anal. Biochem.* **100,** 201–220.