ANALYTICAL BIOCHEMISTRY 86, 193–200 (1978)

A Simplified Method of Quantitating Protein Using the Biuret and Phenol Reagents[1]

S. TSUYOSHI OHNISHI AND JAMES K. BARR[2]

Biophysics Laboratory, Department of Anesthesiology, Department of Biological Chemistry, Hahnemann Medical College, Philadelphia, Pennsylvania 19102

Received September 28, 1976; accepted November 5, 1977

A new modification of the Lowry method of quantitating protein is introduced, whereby the protein sample is mixed first with a diluted biuret reagent and later with 2 N phenol reagent (undiluted) for color development. The method is superior to the original in (i) extremely stable color development (0.3% change from 20 min to 2 hr), (ii) good reproducibility (±2% for 50–600 μg/ml of protein), (iii) elimination of the need to mix reagents for each assay, (iv) good storability (the diluted biuret reagent is storable for months), (v) simplicity (both reagents are available commercially), and (vi) the biuret method can be immediately converted to the Lowry method if the former does not yield a sufficient absorbance. It was found that the relationship between absorbance and protein concentration is expressed by a straight line with a slope of 1 in the Hill plot.

For the quantitation of protein, the biuret method (1–4) and the Lowry method[3] (5), which incorporates the use of phenol reagent (6–8), have been widely used as standard procedures. The biuret method has a more linear correlation between protein concentration and absorbance but lacks sensitivity. Therefore, the sensitive Lowry method is more commonly used. However, the following problems are present in the latter method: (a) the necessity of preparing chemicals for each protein measurement due to poor stability of the combined reagents, (b) instability of color development, (c) the standard curve is not reproducible, especially at low concentrations (below 15 μg), and (d) nonlinearity between absorbance and protein concentration.

Ever since the development of the Lowry method, several studies on interfering substances (9–14) and modification of the method (15) have

[1] Preliminary results were presented at the 67th Annual Meeting of the American Society of Biological Chemists (June 1976, San Francisco, California).

[2] Present address: Box 79, Hampshire College, Amherst, Massachusetts 01002.

[3] Abbreviations used: BS_7, mixture of one part biuret reagent and seven parts 2.3% Na_2CO_3; BSA, bovine serum albumin; Lowry method, the method developed by Lowry *et al.* (5); phenol reagent, Folin–Ciocalteu reagent (phospho-18-molybdic-tungstic acid) developed by Folin *et al.* (6–8).

0003-2697/78/0861-0193$02.00/0

been reported, but little has been done in terms of simplification of the procedure and improvement of reagent storability. Noting that similar chemicals are being used in the biuret and Lowry methods, we have tested the possibility of using a common reagent for each method. We have found that a biuret reagent properly diluted with Na_2CO_3 can be employed in the Lowry method. It was found that this new method (entitled the "dilution method" in this paper) can solve most of the problems existing in the Lowry method mentioned above, except for the nonlinearity which is inherent in the reaction mechanism.

The use of the biuret reagent in the Lowry method also permits an interesting application of converting the biuret method into the Lowry method when the sample does not provide sufficient absorbance in the former (entitled the "conversion method").

In this communication, various aspects of the new methods are studied, such as the dilution factor, reaction time, quantity of phenol reagent, effect of temperature, and stability of color development. A comparison with the classic Lowry method is included.

MATERIALS AND METHODS

I. Chemicals

Commercially prepared biuret and phenol reagents, both manufactured by Sigma Chemical Co. (St. Louis, Missouri), were used. The biuret reagent was also prepared in our laboratory for comparison with the commercial reagent. We have used BSA as a protein to test the method. The solution of BSA was also purchased from Sigma Chemical Co.

II. Spectrophotometric Measurements

Absorbances were measured by an Aminco DW-2 spectrophotometer. Temperature of the cuvette holder of the spectrophotometer was kept constant (25 ± 0.1°C) by circulating water from a thermostatic bath (Lauda K-2/R).

III. Assay Procedures

1. Biuret method. To a 1-ml protein sample, 4 ml of biuret reagent (4) is added. After a waiting time of 30 min, the absorbance is read at 550 nm against a blank.

2. Classical Lowry method (5). To 0.8 ml of the sample, 4 ml of Lowry reagent C (a mixture of two stock solutions: cupric sulfate in sodium potassium tartrate and Na_2CO_3 in NaOH) is added, and the solution is allowed to stand for 10 min at room temperature. Then, 0.4 ml of phenol reagent (diluted to 1 N) is pipetted rapidly into the mixture with thorough mixing by a vortex mixer (total volume is 5.2 ml). After 30 min, the

absorbance is read at any wavelength between 550 and 750 nm against a blank.

3. Dilution method. A standard biuret solution is diluted eight times with 2.3% Na_2CO_3 (i.e., 1 volume of biuret reagent plus 7 volumes of Na_2CO_3; abbreviated as BS_7). To a 1-ml protein sample (protein concentration range is 5 to 600 μg/ml in this study), 4 ml of BS_7 is added. The mixture is allowed to stand for 10 min at room temperature, and 0.125 ml of 2 N phenol reagent (undiluted) is added while the solution is mixed on a vortex mixer (total volume is 5.125 ml). The absorbance is read as in the Lowry method. Alternate volumes of 0.8 ml of the sample, 3.2 ml of BS_7, and 0.1 ml of phenol reagent can also be used to ease pipetting of the phenol reagent.

4. Conversion method. When working with the biuret method, the protein concentration at times is too low to yield sufficient absorbance for an accurate reading. In such a case, both the color-developed sample and a blank are diluted eight times with 2.3% Na_2CO_3, and, to 4 ml of these mixtures, 0.1 ml of 2 N phenol reagent is added, with the absorbance read as in the Lowry method. The following can be used as an alternative method: to a 0.1-ml sample add 0.4 ml of biuret reagent (undiluted), wait 10 min, then add 3.5 ml of 2.3% Na_2CO_3 and 0.1 ml of 2 N phenol reagent.

RESULTS AND DISCUSSION

I. Effect of Concentration of the Biuret Reagent on the Stability of Color Development

As shown in Table 1, a biuret reagent diluted eight times with 2.3% Na_2CO_3 contains the same chemicals with similar concentrations as in Lowry reagent C (not including KI which acts as a preservative in the biuret reagent). For the sake of simplicity, an attempt was made to use

TABLE 1

CONCENTRATIONS (PERCENTAGE) OF CHEMICALS IN BIURET REAGENTS AND LOWRY REAGENT C

Chemicals	Biuret		Lowry reagent C
	Original	×8 Dilution with 2.3% Na_2CO_3	
$CuSO_4 \cdot 5H_2O$	0.15	0.018	0.01
Tartrate	0.6	0.075	0.02
NaOH	3.0	0.375	0.4
Na_2CO_3	—	2.01	2.0
KI	0.1	0.0125	—

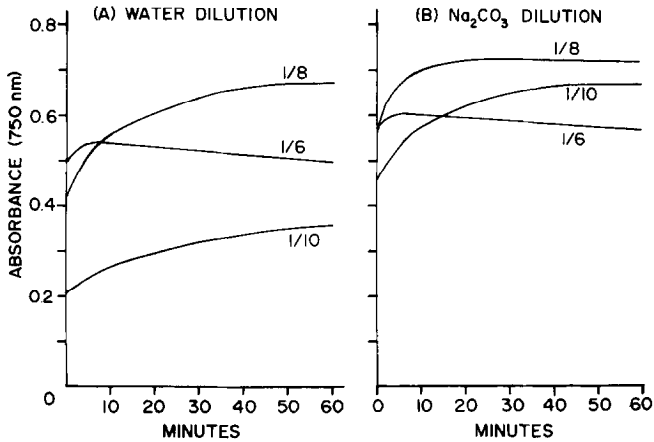

FIG. 1. Effect of the dilution ratio of the biuret reagent on the stability of color development. Conditions for (A): using the dilution method a 200-μg/ml protein sample was treated with various water dilutions of the biuret reagent. Conditions for (B): same as in (A), except the sample was treated with biuret reagents diluted with 2.3% Na_2CO_3. Dilution ratios are indicated in the figure.

water instead of Na_2CO_3 in diluting the biuret reagent, but, as shown in Fig. 1A, the color development is relatively slow with all dilutions. As shown in Fig. 1B, the Na_2CO_3-diluted reagents produce more color development than the corresponding water-diluted reagents in Fig. 1A, with the 1/8 dilution providing maximum stability and color development. Therefore, we have used this reagent (BS_7) for the following tests.

II. Effect of the Quantity of Phenol Reagent on Color Development and pH

The amount of phenol reagent employed greatly affected the linearity, stability, and rapidity of color development. As shown in Fig. 2A, the absorbance increases with the increase of phenol reagent. However, 0.125 ml of phenol reagent provided the most stable color development.

Using two diluted biuret solutions (one with Na_2CO_3 and another with water), the effect of the amount of phenol reagent employed in the method was examined (Fig. 2B). As seen in Fig. 2, the reagent diluted with Na_2CO_3 is much less sensitive to small changes in acidity.

III. Comparison of the Lowry Method and the Dilution Method

The dilution method provides a more stable color development than the Lowry method. At 25°C, the absorbance of the dilution method reaches a plateau 20 min after the addition of phenol reagent and maintains this value for 2 hr (change is less than 0.3%), then slowly decreases. The decrease at 6 hr deviates 1.7% from the plateau value. The reproduci-

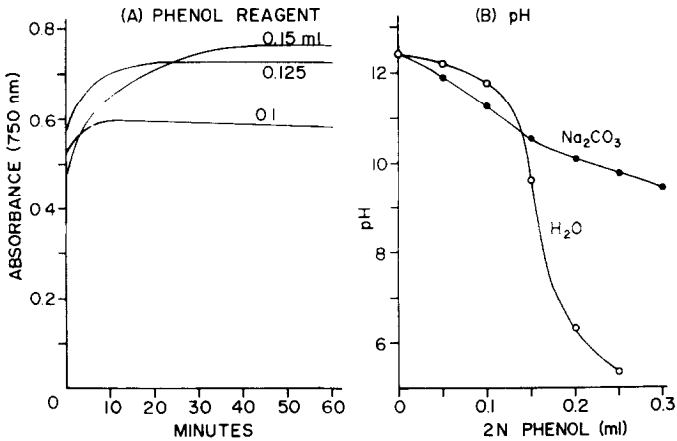

FIG. 2. Effect of the quantity of phenol reagent on (A) stability of color development and (B) pH. Conditions for (A): a 400-μg/ml protein sample was treated with the dilution method, using various amounts of phenol reagent (volumes are indicated in the figure). Conditions for (B): a 1-ml protein sample (200 μg/ml) was mixed with 4 ml of diluted biuret reagent (solid circles, BS_7; open circles, biuret reagent diluted eight times with water), after which the mixture was titrated with 2 N phenol reagent.

bility of the dilution method is $\pm 2.0\%$ at protein concentrations between 50 and 600 μg/ml.

The absorbance of the Lowry method constantly increased for a period of 1 hr, followed by a faster decrease than that observed in the dilution method. The absorbance change between 20 min and 2 hr was 9.2%, and the decrease at 6 hr was 3.0% from the peak value.

IV. Effect of Reaction Time on Color Development

The dashed line in Fig. 3 shows the color development of the biuret reaction. As seen in Fig. 3, the color development measured at 550 nm was a slow process. It took 30 min to reach the point of saturation.

However, the reaction between protein and copper itself was fast. Filled circles in Fig. 3 are the results of a series of experiments where 1 volume of protein and 4 volumes of BS_7 were mixed at time 0, after which 4 ml of the mixture were withdrawn at certain time intervals (as shown on the abscissa); 0.1 ml of phenol reagent was then added to each sample immediately, with the absorbance measured 30 min later. As seen from the data, 98% of the reaction was completed 10 sec after the protein was mixed with BS_7, and the reaction seems to be fully complete in 10 min.

V. Stability and Storability

According to the original paper (5), the Lowry method was most stable at pH 10. Therefore, they used Na_2CO_3 as a buffer to stabilize the pH.

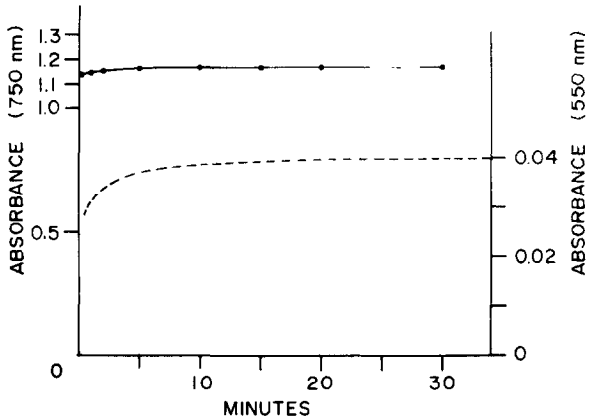

FIG. 3. Stability of the color development of the biuret method (dashed line, absorbance at 550 nm shown on the right scale), and the relation between the protein–copper reaction time and the absorbance of the dilution method (filled circles, absorbance at 750 nm shown on the left scale). A 600-μg/ml protein sample was used. See section IV under Results and Discussion for further details.

However, it was found that the dilution method gave the most stable color development at a pH of about 10.9. Na_2CO_3 also serves to stabilize the pH in our case.

A chemical which is not involved in the original method is KI, used as a preservative in the biuret reagent. In order to test the effect of KI, we have prepared biuret solutions with different concentrations of this compound and found that it has little effect on color development after the addition of phenol reagent, as long as the concentration is less than 0.1%. The amount of KI present as a preservative in the commercial reagent is sufficient to make the diluted biuret reagent storable for a long period of time (6 months at least).

VI. Effect of Temperature in the Dilution Method

At temperatures between 20 and 25°C, the maximum absorbance and stability of color development are the same. However, at 30°C, the maximum absorbance was reached faster (within 10 min), with a decrease in absorbance 40 min after the addition of phenol reagent. The maximum absorbance was approximately 13% less than that at 20–25°C. Therefore, running the dilution method above 25°C is not recommended. Also, when working with a spectrophotometer without a temperature control system, it is not advisable to leave the mixture in the spectrophotometer since the temperature in the cuvette housing is normally higher than room temperature.

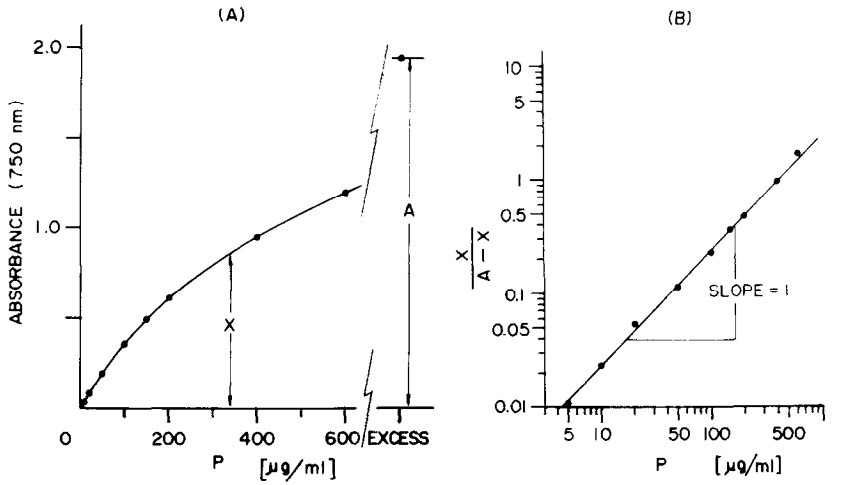

FIG. 4. (A) Relation between absorbance and the concentration of protein as measured by the dilution method (concentration range 5–600 μg); (B) Hill plot [data taken from (A)]. See section VII under Results and Discussion for further details.

VII. Nonlinearity of the Method

Figure 4A shows the relation between absorbance (X) and the concentration of protein (P). As pointed out previously (15,16) the relation is not linear. Since the curve shows a tendency to saturate, the saturation value A was measured using an excess amount of protein (100 mg/ml). It was found that the relationship between log $(X/(A - X))$ and log P [Hill plot (17)] is expressed by a straight line with a slope of 1 (Fig. 4B). This suggests that the basic mechanism of color development is expressed as

$$R + F \rightleftharpoons RF$$

where R stands for chromogenic protein-bound coppers and tyrosine and tryptophan residues, all of which are able to reduce phenol reagent F to yield the complex RF (molybdenum blue). From this, it is clear that the nonlinearity of the Lowry method is inherent to the reaction mechanism.

ACKNOWLEDGMENTS

The authors thank Dr. H. L. Price of the Department of Anesthesiology, Hahnemann Medical College, for his encouragement, and Dr. M. Sono of the Childrens Hospital of Philadelphia for his discussions. This work was supported in part by NIH Grant No. HL 15799-02 (PHRA).

REFERENCES

1. Kingsley, G. R. (1942) *J. Lab. Clin. Med.* **27,** 840.
2. Mehl, J. (1945) *J. Biol. Chem.* **157,** 173.
3. Weichselbaum, T. E. (1946) *J. Clin. Pathol. Tech. Suppl.* **10,** 40.
4. Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) *J. Biol. Chem.* **177,** 751.
5. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol Chem.* **193,** 265.
6. Folin, O., and Ciocalteu, V. (1927) *J. Biol. Chem.* **73,** 627.
7. Wu, H. (1920) *J. Biol. Chem.* **43,** 189.
8. Wu, H. (1922) *J. Biol. Chem.* **51,** 33.
9. Gerhardt, G., and Bievers, H. (1968) *Anal. Biochem.* **24,** 337.
10. Eichberg, J., and Makrasch, L. C. (1969) *Anal. Biochem.* **30,** 386.
11. Peters, M. A., and Fouts, J. R. (1969) *Anal. Biochem.* **30,** 299.
12. Gregory, J. D., and Sajdera, S. W. (1970) *Science* **169,** 97.
13. Turner, L. V., and Manchester, K. L. (1970) *Science* **170,** 649.
14. Herd, J. K. (1971) *Anal. Biochem.* **44,** 404.
15. Hartree, E. F. (1972) *Anal. Biochem.* **48,** 422.
16. Bates, W. K., and McAllister, D. F. (1974) *Anal. Biochem.* **59,** 190.
17. Hill, A. J. (1913) *Biochem. J.* **7,** 471.