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A Candidate Reference Method for Determination of Total Protein in Serum I. Development and Validation

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We developed a candidate Reference Method for measuring total serum protein by use of the biuret reaction. The method involves a previously described biuret reagent (*Clin. Chem.* **21**: 1159, 1975) and Standard Reference Material (SRM) 927 bovine albumin (National Bureau of Standards) as the standard. At 25 °C, color development for 30 or 60 min provides identical serum protein values. Glucose (up to 10 g/L) and bilirubin (up to 300 mg/L) do not interfere. Hemoglobin, at 3 g/L, increases apparent serum protein by 0.4 g/L. The presence of dextran in serum causes easily detected turbidity, but this interference can be eliminated by centrifuging the reaction mixture. Therapeutic concentrations of ampicillin, carbenicillin, penicillin, oxacillin, nafcillin, chloramphenicol, cephalothin, and methicillin in blood do not interfere, nor do triglycerides up to 10 g/L. Within-run and day-to-day standard deviations of the method are 0.1 and 0.4 g/L, respectively.

Additional Keyphrases: *analytical error · reference materials · proteins · reference interval · variation, source of · quality control · spectrophotometry · standards · peptides in serum*

Introduction

In 1974, the Study Group on Total Protein of the American Association for Clinical Chemistry (AACC) Committee on Standards conducted an interlaboratory study on the determination of total serum protein by the biuret reaction (*1*). The success of that cooperative effort provided the impetus for the development of a candidate Reference Method for total serum protein. This was made possible by the re-definition of a Reference Method (*2*) and the release by the National Bureau of Standards (NBS) of a primary protein standard (*3*), prepared according to the recommendations issued in 1968 by the AACC Committee on Standards (*4*).

Definition of a Reference Method

The following definition has been accepted by the participants in the Atlanta conference (*2*): "A reference method is an analytical method with thoroughly documented accuracy, precision, and low susceptibility to interferences. The accuracy and precision shall be demonstrated by direct comparison with the definitive method and SRM or, where not available, with other well-characterized and documented analytical approach(es)." [6]

The variety of proteins present in serum does not allow an exact definition of the analyte. Accuracy can only be documented by demonstrating low susceptibility to interfering tions exhibit similar absorptivities in the biuret reaction (*4*). Absorptivity ($a^{1\,g/L}_{540\,nm,\,10\,mm}$) is defined as the corrected absorbance (corrected for sample and reagent blanks) of a solution containing 1.00 g of the analyte per liter.

The biuret reaction was chosen as the basis of the method because of the relative specificity of the biuret reagent for proteins, the reproducibility of absorbance values when the reaction (color development) is allowed to go to completion, the close similarity in the absorptivity values (color yield) of the main serum protein fractions (*4*), and the relatively few substances that interfere (*5*).

History of the Biuret Reagents

The biuret reaction has been used to quantitate total serum protein for more than 60 years (*6*). Because of the instability of the early reagents, the NaOH and $CuSO_4$ solutions had to be kept separate (*7, 8*). With these reagents, the turbidity that usually developed in the reaction mixture had to be clarified by extraction with ethyl ether (*8*). The first stable biuret reagent, with $CuSO_4$ and NaOH present in a single solution, was introduced by Kingsley (*9*). Precipitation of $Cu(OH)_2$ was prevented by use of a high NaOH concentration (2.9 mol/L) and a low $CuSO_4$ concentration (7 mmol/L). A serious disadvantage of Kingsley's reagent, most likely ascribable to the high content of alkali, is that turbidity develops in the reaction mixture, which must be removed by extracting with ethyl ether before absorbance is measured. The same disadvantage applies to reagents containing ethylene glycol (*10*) or citrate (*8*). To circumvent the turbidity, Keyser and Vaughn (*11*) measured the background absorbance after destroying the biuret color by adding KCN, but this approach results in underestimates of serum protein because the KCN causes a yellow discoloration of the reaction mixture that increases the background absorbance (*8*).

The incorporation of potassium sodium tartrate into the biuret reagent by Weichselbaum (*12*) produced a stable reagent and decreased the incidence of turbidity. KI was added to prevent the autoreduction of Cu(II).

Reagents containing potassium sodium tartrate can be divided into two groups. Those in the first group have a low alkali concentration (0.1–0.2 mol/L) and a $CuSO_4$ concentration of 10–30 mmol/L (*12–14*). Those in the second group have a higher alkali concentration (0.5–0.8 mol/L) and a $CuSO_4$ concentration of 4–6 mmol/L (*15–17*).

The low copper concentration in these reagents provides reagent blanks with low absorbance values—a rather insignificant advantage, because modern spectrophotometers with digital readouts permit accurate measurements of absorbance

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Developed for the Committee on Standards of the American Association for Clinical Chemistry by the Study Group on Proteins, with the cooperation of the Centers for Disease Control and the National Bureau of Standards.

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Ed. note: This paper and the one that follows will be part of the next volume of *Selected Methods of Clinical Chemistry*. Thus criticisms of them are invited and should be addressed to B.T.D.

[6] Nonstandard abbreviations: SRM, Standard Reference Material (from NBS, the U.S. National Bureau of Standards); HSA, human serum albumin; BSA, bovine serum albumin.

up to 1.5 or more. The main disadvantage of the reagents with low $CuSO_4$ concentration is the deviation from Beer's law (nonlinearity) when the protein concentration in the reaction mixture exceeds 1.4 g/L (*18*). Reagents containing EDTA as the copper-complexing agent have also been proposed (*19–20*), but we doubt that they offer any advantage over those containing tartrate.

Principle

Treatment of a protein solution with tartrate-complexed copper and alkali produces a violet color, characteristic of proteins and peptides, with an absorption maximum at 540 nm. The stoichiometry of the reaction was studied by Strickland et al. (*21*), who postulated a structure for the protein–copper complex.

Materials and Methods

Equipment

A Cary 16 spectrophotometer (Varian, Inc., Palo Alto, CA 94303) and "Suprasil (QS)" cuvets (specified lightpath, 10 ± 0.01 mm; Hellma Cells, Inc., Jamaica, NY 11424) were used for absorbance measurements. The photometric accuracy of the instrument was established and regularly checked by the use of standard glass filters (SRM 930) available from the NBS.

Any spectrophotometer may be used if it has a bandpass of less than 8 nm, an accurate wavelength scale, and acceptable photometric accuracy and linearity. An instrument will be considered to meet the requirements for accuracy and linearity if absorbance measurements made on NBS SRM 930 or SRM 931 lie within the NBS tolerances specified for these materials.

A mechanical pipettor (Micromedic Systems, Inc., Philadelphia, PA 19106) was used to pipet protein solutions and reagents. The accuracy and precision of the pipet was checked as described elsewhere (*22*).

Materials

Bovine serum albumin (BSA, Cohn Fraction V, lot nos. M45001, M45703, and N50404) was obtained from Metrix Clinical Diagnostics, Div. of Armour Pharmaceutical Co., Chicago, IL 60616.

Human serum albumin (HSA), L-cystine-treated and deionized (*1*), was donated by the Hyland Division, Travenol Laboratories, Inc., Costa Mesa, CA 92626. HSA solutions (approximately 100 g/L) were purchased from Miles Laboratories, Inc., Elkhart, IN 46515 (code no. 81-017-1), and from Kabi Diagnostica, Stockholm, Sweden.

Protein solutions. Solutions of BSA and HSA were prepared as described elsewhere (*1*).

Serum samples. These were obtained from healthy subjects and from the Clinical Laboratories of the Milwaukee County Medical Complex, Milwaukee, WI.

Bilirubin. Reference-grade bilirubin was obtained from Pfanstiehl Laboratories, Inc., Waukegan, IL 60085.

Dextran. Dextran with an average relative molecular mass of 40 000 was purchased from Sigma Chemical Co., St. Louis, MO 63178.

Antibiotics. Ampicillin, oxacillin, and methicillin (from Bristol Laboratories, Division of Bristol-Myers Co., Syracuse, NY 13201); cephalothin, penicillin-G (from Eli Lilly and Co., Indianapolis, IN 46206); carbenicillin (from Roerig, Co., Division of Pfizer, Inc., New York, NY 10017); chloramphenicol (from Parke, Davis and Co., Detroit, MI 48232); and nafcillin (from Wyeth Laboratories, Inc., Philadelphia, PA 19101) were obtained in the dry form and reconstituted with water.

Reagents

Reagent-grade chemicals were used throughout this study.

Water. Use demineralized or distilled water with a specific resistance of at least 10^6 MΩ cm at 25 °C.

NaOH, 6.0 mol/L. Dissolve 240 ± 2 g of NaOH in freshly distilled water and dilute to 1 L. Store in a tightly closed polyethylene bottle. Use an unopened bottle of NaOH, to assure that it is dry and free of Na_2CO_3.

Biuret reagent. Dissolve 3.00 ± 0.01 g of $CuSO_4 \cdot 5H_2O$ in 500 mL of freshly distilled water. Add 9.00 ± 0.01 g of K Na tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) and 5.0 ± 0.1 g of KI. After solution is complete, add 100 mL of 6 mol/L NaOH and dilute to 1 L with water. Store in a tightly closed polyethylene bottle at room temperature. Although this reagent is stable for at least one year, we recommend that it not be used after six months.

Note: The absorbance (*A*) of the reagent blank (5.0 mL of biuret reagent + 100 μL of H_2O) at 540 nm should be between 0.095 and 0.105; if it is not, prepare new biuret reagent.

Alkaline tartrate: Dissolve 9.0 g of K Na tartrate and 5.0 g of KI in 0.8 L of water. Add 100 mL of 6 mol/L NaOH and dilute to 1 L with water. Store in a polyethylene bottle at room temperature. This solution is stable for approximately six months. Discard the reagent when there is evidence of biological growth.

Protein standard solution: Use the standardized bovine albumin solution, SRM 927, available from the NBS. The protein concentration in the SRM is 70.45 ± 0.2 g/L. A BSA solution from another source may be used, if it is demonstrated by direct comparison to yield a biuret absorbance value and linearity identical with those of the SRM. Directions for preparing such solutions (secondary standards) have been published (*23*).

Procedure

Use any spectrophotometer that meets the specifications described in the Transferability of the Method (*24*). The photometric drift before each run should not exceed 0.002 *A* per hour. Flow-through cuvets, instead of regular cuvets, may be used if carryover is eliminated by thorough rinsing between samples. Use volumetric pipets with a specified tolerance of ±0.3% or less. Mechanical pipettors may be used if the achievable precision of repetitive delivery approaches a relative SD of 0.3%. A procedure for checking such devices has been described elsewhere (*22*).

Glossary

Test: The sample to be analyzed plus biuret reagent; its uncorrected absorbance (*A*) is A_4.

Reagent blank: Biuret reagent plus a volume of water equal to that of the sample; its uncorrected absorbance is A_3.

Sample blank: Alkaline tartrate plus a sample containing protein; its uncorrected absorbance is A_2.

Alkaline tartrate: The biuret reagent without copper; its absorbance is A_1.

Corrected absorbance: The corrected absorbances of the Test, sample blank, and reagent blank are $A_4 - A_3 - (A_2 - A_1)$, $A_2 - A_1$, and $A_3 - A_1$, respectively.

Analyze samples, controls, sample blanks, and reagent blanks in duplicate. Analyze duplicate standards twice (including sample blanks), one pair at the beginning and one pair at the end of each run. To avoid problems of carryover, the sample blanks should be run first as a group, followed by the "Tests."

Sample Blanks

1. Pipet 5.0 mL of alkaline tartrate into a series of 16 × 100 mm tubes.

2. Pipet 100 μL of sample (serum, standard, etc.) into each tube. If a manual pipet is used, make sure all sample is transferred by rinsing the pipet at least three times with the alkaline tartrate. Add the samples to the reagent at timed intervals (e.g., 0.5 or 1 min) and observe the same intervals for the absorbance measurements.

3. Cover the tubes with "Parafilm" and mix the contents gently and thoroughly by inversion immediately after a sample is added to the reagent. Let the tubes stand at 25 ± 1 °C in a water bath for 60 min.

Tests

1. Follow the same procedure and timing schedule as for the sample blanks (Steps 1–3 above) but substitute biuret reagent for alkaline tartrate in Step 1.

2. Prepare a large volume of reagent blank by pipetting 0.5 mL of water into 25 mL of biuret reagent; this will be needed to check for photometric drift.

Absorbance Measurements

Use the same cuvet for all measurements or, preferably, a 10-mm flow-through cuvet. For a regular cuvet, it is recommended that the cuvet be filled, emptied, and rinsed in place; solutions may be added with Pasteur pipets, and removed with a plastic-tipped tube attached to a water aspirator. If conventional "pour and fill" handling must be used, the cuvet must be carefully wiped and placed in the same orientation with respect to the light beam throughout the run.

1. Set the spectrophotometer at zero absorbance (0.000 *A*) at 540 nm with *air* in both the sample and reference beams (i.e., without the cuvet in the cuvet holder).

2. Fill the cuvet with alkaline tartrate solution, wipe off fingerprints with lint-free tissue and then measure the absorbance (A_1) against air in the reference beam. If a flow-through cuvet is used, fill the cuvet with alkaline tartrate and set the absorbance at zero; A_1 in this case will be 0.000 *A*.

3. Carry out the remaining absorbance measurements without moving the cuvet from its holder. Aspirate the solution completely through a plastic-tipped tube. Rinse the cuvet twice by adding, with a clean Pasteur pipet, 0.5- to 1.0-mL aliquots of each solution to be read, and aspirating the wash aliquots each time. Fill the cuvet with the solution and measure its absorbance.

4. In the properly timed sequence, read all sample blanks (A_2), then the reagent blanks (A_3), and the Tests (A_4).

5. Check the photometric drift of the spectrophotometer after every 10 samples, as follows: Rinse the cuvet five times with 1-mL portions of alkaline tartrate (when reading sample blanks) or reagent blank (when reading Tests) and aspirate each time. Then fill the cuvet the sixth time and measure the absorbance (A_1 or A_3). In each case, the reading should lie within ±0.002 *A* of the first A_1 or A_3 value. If the difference is greater, repeat the rinsing and reading. If the drift is greater than ±0.002 *A*, record the new A_1 or A_3; subtract these new values from succeeding samples.

Note: If drift presents an irremediable problem, do not perform the analysis until the instrument is repaired.

Acceptability of Data and Calculations

First condition: The difference between readings of duplicate serum specimens, controls, sample blanks, and reagent blanks should not exceed 0.005 *A*. If differences are greater, re-analyze those specimens.

Second condition: All four readings of the standard should lie within 0.007 *A*, and at least three should lie within 0.005 *A*. If not, repeat the analysis of the standards in quadruplicate but not the entire run. If the two mean absorbance values of the standard (from the initial and repeated runs) differ by more than 0.005 *A*, the drift is unacceptable and its cause must be eliminated.

If the above two conditions are met the run is acceptable.

As already mentioned, the corrected absorbance of the Test is the absorbance of the Test minus the absorbance values of the reagent blank and sample blank:

$$\text{Corrected } A \text{ Test} = A_4 - A_3 - (A_2 - A_1)$$

Note: From each A_4, subtract the mean of the two A_3 values and the corresponding sample blank ($A_2 - A_1$); if there has been a photometric drift, subtract the new A_1 and A_3 values.

The concentration of total protein in the sample is given by the equation:

$$\text{Protein concn. in the sample (g/L)} = \frac{\text{corrected } A \text{ Test}}{\text{corrected } A \text{ standard}} \times \text{protein concn. of the standard* (g/L)}$$

*Calculations should be based on the average of the four corrected absorbance values and the protein concentration of the standard.

Validation of the Method

Time required for completion of the reaction. The reaction time (30 min) for color development at 25 °C, used in the previous study (*1*), was re-examined because complete color yield was one of the requirements for high precision. Solutions of BSA and HSA (100 g/L) and pooled sera were added to biuret reagent, and their absorbances were followed for 70 min. The reaction was complete in 60 min with all protein solutions (Figure 1); we therefore elected to extend the incubation from 30 to 60 min.

Effect of the composition of the biuret reagent on serum protein values. We evaluated the effect of composition of the biuret reagent on serum protein values by analyzing serum samples with five reagents with the following composition:

	$CuSO_4 \cdot 5H_2O$, g/L	NaOH, mol/L	K Na tartrate, g/L	KI, g/L
Reagent 1	2.0	0.12	9.0	5.0
Reagent 2	3.0	0.6	9.0	5.0
Reagent 3	3.0	1.0	9.0	5.0
Reagent 4	5.0	0.6	9.0	5.0
Reagent 5	5.0	1.0	9.0	5.0

Reagent 1 had been used for several years at the Centers for Disease Control for assigning protein values on serum pools used in the Proficiency Testing Program. To determine if reagents 1 and 2 (reference reagent) would provide different protein values, we analyzed 20 serum samples with normal and abnormal concentrations of protein fractions (albumin, 24 to 46 g/L, total globulins, 21 to 43 g/L), using the two reagents and with SRM 927 as the standard. Mean values were 67.70 and 67.67 g/L with reagents 1 and 2, respectively; the maximum difference observed was 1.5 g/L. The difference between means was not significant by the paired *t*-test.

Solutions of HSA from different sources and a BSA solution were analyzed with the five reagents, and several human sera were analyzed with reagents 2–5. Results from this experiment

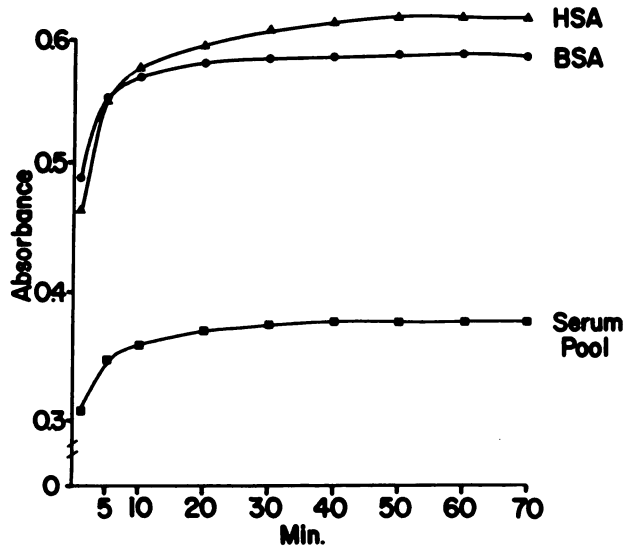

Fig. 1. Rate of color development for human serum albumin, bovine serum albumin, and serum in the biuret reaction

are shown in Table 1. Values for BSA are not affected by the composition of the reagent; this was verified with several BSA preparations from the same manufacturer (Metrix). In contrast, protein values for HSA solutions did vary with reagent composition. Because the most common methods for measuring serum albumin are based on the principle of dye-binding and need to be calibrated with human albumin, the assignment of accurate protein values to HSA solutions used for this purpose is of paramount importance. The question as to which reagent should be used to assign protein values on HSA solutions cannot now be answered with certainty because HSA reference materials are not available. However, based on the experience of two of the authors, reagent 1 (used at CDC) is the reagent of choice; with this reagent, solutions of BSA and HSA, prepared from lyophilized powders after making corrections for moisture and ash contents, provided identical absorptivities (color yield).

Protein values in sera depend less on the reagent composition if the SRM is used as the standard. The large discrepancies in the observed protein values of samples 7 and 8, with reagents 3 and 5, were investigated. These samples were re-analyzed with reagents 3 and 5 and the absorbances were measured at 20, 40, and 60 min. The reaction mixtures remained clear for 40 min, but precipitates formed in the Tests during the next 20 min, while the absorbances of the sample blanks remained unchanged. The higher concentration of NaOH in these reagents is apparently responsible for the turbidity.

Precision. A frozen serum control with a protein concentration of 67 g/L was analyzed 20 times during six months. The within-run and day-to-day standard deviations were 0.1 and 0.4 g/L, respectively. Two lyophilized commercial controls were also used to assess the precision of the method. Each control was analyzed 13 times during two years. Mean protein values were 43.2 g/L for Control A and 60.6 g/L for Control B. The within-run SDs for Controls A and B were 0.07 and 0.2 g/L, respectively; the corresponding day-to-day SDs were 0.6 and 0.4 g/L.

Linearity. We evaluated the relationship between absorbance and protein concentration in the SRM, BSA, and serum. Aliquots of 25 to 200 μL of SRM and of a 70 g/L BSA solution were analyzed by the proposed method. Reagent blanks and sample blanks were prepared in the same manner. Because the volume of the final solution varied from 5.025 to 5.20 mL, corrections to the standard volume (5.10 mL) were made by multiplying the net absorbance of the Test times the volume of the final solution and dividing by 5.1; for example, if the sample aliquot was 200 μL, the absorbance was multiplied by 5.2/5.1. A serum pool was concentrated by repeated freezing and thawing until the protein content was about 150 g/L. Seven dilutions of the pool were made with 9 g/L NaCl solution, and 100 μL of each dilution was analyzed by the proposed method. Figures 2–4 show typical results for absorbance–concentration relationships.

Small peptides in serum. Although it has already been reported that the concentration of non-protein, biuret-re-

Table 1. Effect of Biuret Reagent Composition on Protein Values of HSA, BSA, and Human Sera (Standard: SRM 927)

Reagent no.	Protein concn, g/L 1	2	3	4	5
HSA					
Miles Lot 25	96.8	99.5	100.4	97.2	100.5
Miles Lot 24	98.2	100.8	102.5	100.0	102.2
Kabi Lot 56366	101.2	104.0	106.9	101.4	106.8
Hyland	99.4	102.4	105.9	99.2	104.4
BSA (Cohn Fraction V)					
Metrix Lot 50404	99.8	99.4	99.4	99.5	99.9
Sera					
1	—	53.2	53.7	54.0	53.8
2	—	57.5	57.7	58.0	57.8
3	—	67.6	68.3	68.2	68.2
4	—	78.2	78.3	79.0	78.8
5	—	71.9	72.3	72.8	72.6
6	—	68.0	68.7	68.6	72.0
7	—	77.5	86.0	78.4	83.0
8	—	70.0	77.5	71.0	76.4
9 (Pool)	—	64.5	65.0	64.6	64.8
10 (Pool)	—	59.4	59.3	60.2	59.2

Specified protein concn: Miles Lot 25, 97 g/L; Miles Lot 24, 101 g/L; Kabi 103 g/L; Hyland, 100 g/L; BSA 100 g/L.

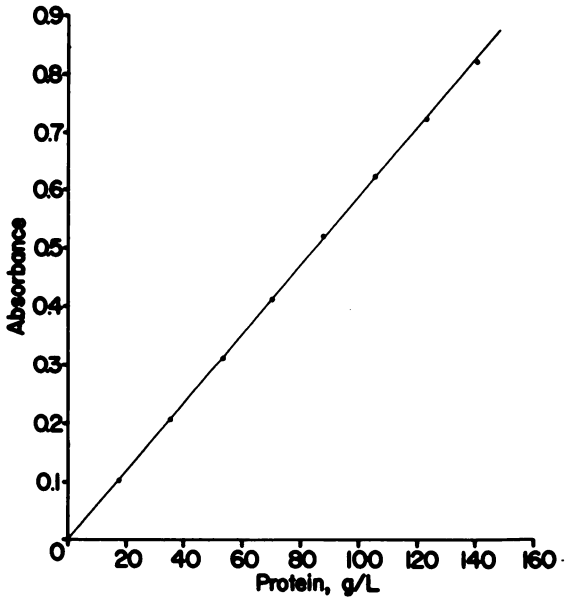

Fig. 2. Absorbance–concentration relationship for SRM 927 in the biuret reaction

$y_{abs.} = 5.86 \times 10^{-3} (\pm 2.7 \times 10^{-5})\, x_{concn} + 1.8 \times 10^{-4} (\pm 2.4 \times 10^{-3})$; $r^2 = 0.9999$. In Figs. 2–6, first ± expression is SE of slope, second is SE of intercept

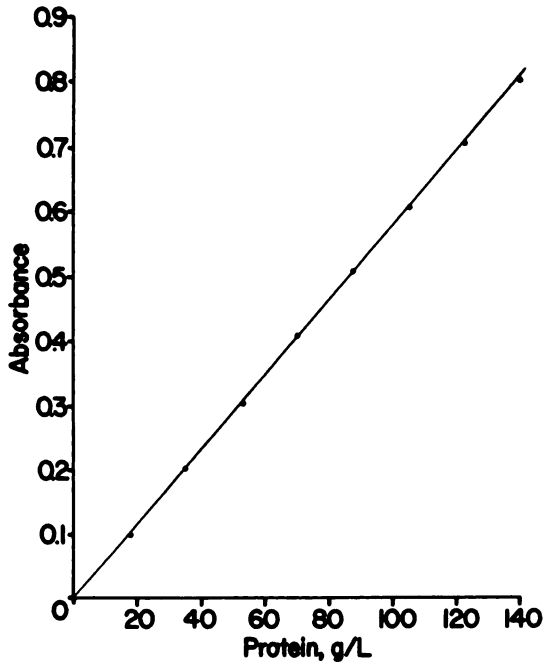

Fig. 3. Absorbance–concentration relationship for BSA in the biuret reaction

$y_{abs.} = 5.81 \times 10^{-3} (\pm 2.0 \times 10^{-5})\, x_{concn} + 8.4 \times 10^{-4} (\pm 1.8 \times 10^{-3})$; $r^2 = 0.9999$

acting compounds in serum is very low (*15*, *25*), we conducted an experiment to examine the nature of the substances, in addition to high-molecular-mass proteins ($M_r > 10\,000$), being measured by the biuret reaction. A fresh serum pool and two lyophilized control sera were filtered through a membrane (UM 10 Diaflo membrane; Amicon Corp., Lexington, MA 02173) that retains particles with $M_r > 10\,000$.

The concentration of biuret-reacting substances in the filtrates ranged from 0.004 to 0.7 g/L, but it is doubtful that the reacting material was only small peptides; electrophoresis of the filtrate revealed that some serum protein fractions had leaked through the filter.

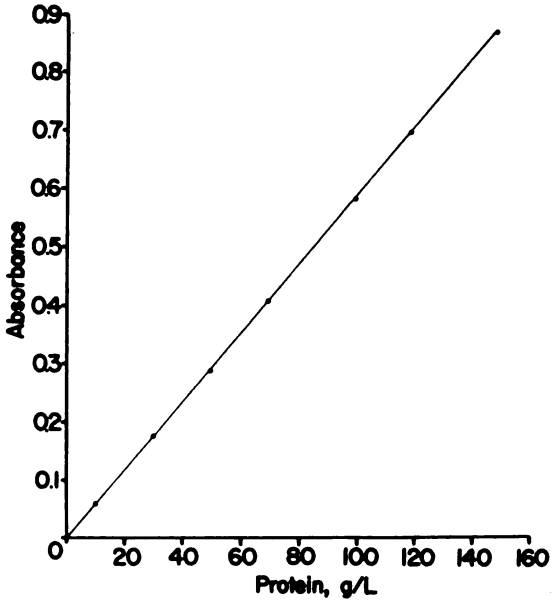

Fig. 4. Absorbance–concentration relationship for serum pool in the biuret reaction

$y_{abs.} = 5.94 \times 10^{-3} (\pm 1.2 \times 10^{-5})\, x_{concn} - 1.7 \times 10^{-3} (\pm 1.0 \times 10^{-3})$; $r^2 = 0.9999$

Effect of spectral bandpass on the absorbance of the copper–protein complex. The absorption spectrum of the copper–protein complex exhibits a maximum at 540 nm (with the instrument zeroed on the biuret reagent) and has a natural bandpass of 140 nm. Absorbance measurements on the reaction mixtures were made by varying the bandpass of the spectrophotometer from 1.1 to 8 nm. Compared to 1.1 nm, use of a bandpass of 8 nm decreased the absorbance by 0.4%, a negligible difference. Estimated protein values were independent of the bandpass used.

Alternative procedures. Absorbance values of sample blanks on fresh sera remain essentially constant throughout the incubation interval (60 min). However, with some freeze-dried control sera and (on two occasions) BSA preparations (*1*), we have observed an increase in the absorbance of sample blanks during the incubation. The increase in absorbance, due to development of turbidity, results in erroneously low protein values because the turbidity occurs mainly in the sample blank. This was established by measuring the absorbance of blanks and Tests before and after they were centrifuged at 18 000 × *g* at 4 °C; the net absorbance was considerably higher after centrifugation than before. In such a case, because turbidity always developed during the last 30 min of the incubation, we evaluated the effect on serum protein values when the color was developed for 30 min at 25 °C or for 10 min at 37 °C. We analyzed 20 fresh sera in duplicate and measured the absorbance at 30 ± 1 and 60 ± 1 min. Mean protein values were 58.58 g/L at 30 min and 58.67 g/L at 60 min, the largest difference between mean values of pairs being 0.4 g/L. The difference between mean protein values, although clinically negligible, was statistically significant by the paired *t*-test.

Nineteen fresh sera were analyzed by the proposed method and by a modified procedure that includes a temperature of 37 °C and a 10-min incubation; in the latter case, absorbances were measured after the solutions were equilibrated to 25 °C. Mean protein values were 74.6 g/L at 25 °C and 75.0 g/L at 37 °C, the largest difference among pairs being 2.5 g/L. The difference between mean protein values was not statistically significant by the paired *t*-test. C. Garber (University of Wisconsin-Madison) confirmed these findings.

Interferences. Experiments with possible interfering substances were conducted in vitro. The substances tested are shown in Table 2. Known amounts of these substances were added to pooled serum specimens or to BSA solutions. The original protein solutions and those containing the interfering substances were analyzed by the proposed method; the protein concentrations were kept constant in both.

Hemoglobin solutions were prepared from whole blood according to the method of Sunderman (*26*), and bilirubin was dissolved in 0.1 mol/L Na_2CO_3; all other substances were dissolved in water.

As shown in Table 3, the interferences by hemoglobin and bilirubin are small and perhaps negligible. Sample blanks overcorrect for the presence of hemoglobin in such a way that

Table 2. Substances Tested for Interference in the Proposed Total Serum Protein Reference Method

Hemoglobin	1 to 3 g/L
Bilirubin	50 to 300 mg/L
Dextran	5 to 20 g/L
Glucose	1 to 10 g/L
Antibiotics	0.5 to 2.0 g/L
Hydroxyethyl starch	20 g/L
Lipids (lipemia)	

Table 3. Interference by Hemoglobin and Bilirubin in the Proposed Total Serum Protein Assay[a]

	Pool			
	1	2	3	4[b]
Hemoglobin, g/L				
1.0	0.1	−0.3	−0.2	−0.3
2.0	0.3	0.2	0.1	−0.5
3.0	0.7	0.3	0.1	−0.7
Bilirubin, mg/L				
50	−0.5	−0.1	−0.4	−0.5
100	−0.3	0.0	−0.3	−0.7
200	−0.2	0.1	−0.6	−0.8
300	−0.4	0.2	−0.6	−0.2

[a] Values represent changes in apparent protein concentration (g/L) as a result of the presence of the interferent. [b] Data from Dr. K. Borner, Free University of Berlin, West Berlin, F.R.G.

the added protein is not all accounted for. Without the blank correction, hemoglobin causes a positive interference. Our data confirm a previous observation that a 1 g/L concentration of hemoglobin, if uncorrected by blanking, is equivalent to about 1.8 of serum protein per liter (*8*). The presence of 300 mg of bilirubin per liter causes an increase in protein of 2 g/L if the blank correction is not applied.

It has been reported that carbenicillin interferes in total protein assays performed in the Technicon SMAC Analyzer (*27*). Carbenicillin and seven other antibiotics (see *Materials*) that may be administered to patients at high doses (10–40 g/day) were tested for interference. At a concentration of 0.5 g/L, a positive bias ranging from 0.1 to 1.0 g/L protein was observed. Since therapeutic concentrations of these antibiotics in serum (*28*) are much lower than 0.5 g/L, they are not expected to cause significant interference.

The results of earlier experiments (*1*) indicated that glucose interfered in the total protein assay. Our conclusion from subsequent experiments is that glucose, even at a concentration of 10 g/L, causes no interference.

Dextran, if present in serum, combines with copper from the biuret reagent to form a precipitate. In the absence of copper (i.e., in the alkaline tartrate) dextran does not precipitate. It is easily detected in serum by the appearance of turbidity in the reaction mixture. This interference can be eliminated by centrifuging the reaction mixture and measuring the absorbance of the clear supernate (*18*). We recommend that all Test solutions be inspected before absorbance is measured; if a precipitate is present, or the specimen was drawn from a patient treated with dextran, centrifuge and measure the absorbance of the supernatant fluid.

Hydroxyethyl starch (Hetastarch; McGaw Laboratories, Division of American Hospital Supply Corp., Irvine, CA 92705), a plasma extender, did not cause any interference when added in concentrations up to 20 g/L to serum.

Turbidity in serum may be due to the presence of chylomicrons or insoluble lipid–protein complexes. The latter are commonly seen in control sera that have been lyophilized, even at low triglyceride concentrations (<1 g/L). The most common approach used to overcome the interference of lipemia was to extract the final reaction mixture with diethyl ether (*8*). Our attempts to eliminate turbidity by filtering the final mixture through 0.22-μm Millipore filters, incorporating surfactants (Triton X-100 or Brij-35) in the biuret reagent, or extracting with diethyl ether or hexane proved tedious and unsuccessful.

To circumvent the problem of turbidity, Chromy and Fischer (*29*) recommended that acetone be added to lipemic sera. Acetone precipitates the serum proteins and extracts the

Table 4. Differences between Mean Total Serum Protein Values in Clear and Lipemic Sera by the Reference and Chromy–Fischer (*29*) Methods (Reference minus Chromy–Fischer Method)

	Protein, g/L		
	Lab 1	Lab 2[a]	Lab 3[b]
Clear sera	0.1	−0.3	−0.3
Lipemic sera[c]	0.9	−0.4	−0.9
SRM 927	0.001[d]	0.002[d]	0.000[d]

[a] Data from Dr. C. C. Garber, University of Wisconsin, Madison, WI. [b] Data from Dr. K. Borner, Free University of Berlin, F.R.G. [c] Range of triglyceride concn, 1.25–14.5 g/L. [d] Difference between mean absorbance values.

serum lipids. After the mixture is centrifuged and the acetone layer is decanted, biuret reagent is added to dissolve the protein precipitate. We evaluated this procedure for the analysis of samples with excessive turbidity and found it reliable. To improve the precision of the assay, we increased the volumes of sample, water, and acetone to 0.1, 0.5, and 10 mL, respectively. We performed the analysis in quadruplicate: two sample blanks and two Tests. To rapidly dissolve the precipitated protein, we added the biuret reagent and alkaline tartrate immediately after decanting and draining the supernate. Clear and lipemic serum samples were analyzed by both the reference method and the Chromy–Fischer method in three laboratories (Medical College of Wisconsin, University of Wisconsin-Madison, and Free University of Berlin, F.R.G.). Fourteen to 21 samples in each category were analyzed in each laboratory. Results from these experiments are summarized in Table 4 and Figures 5 and 6. From the data for clear sera, there was no statistically significant difference between the mean protein values found in two out of three laboratories; for lipemic sera the situation was reversed. The absence of any difference in the SRM 927 absorbance values indicates that analytical recovery of protein by the Chromy–Fischer method is complete.

Results were inconsistent for eight grossly lipemic sera with triglyceride concentrations from 10 to 97 g/L. For two samples, protein values by the Chromy–Fischer procedure were 15.6 and 19 g/L higher than those obtained with the present

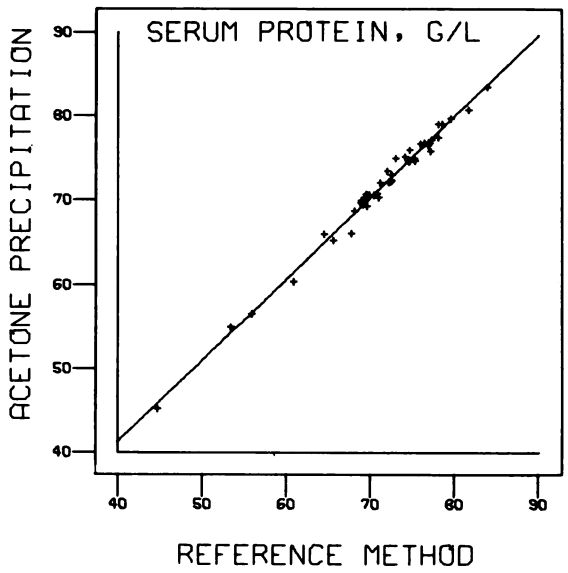

Fig. 5. Protein concentrations in clear sera by the reference and acetone-precipitation methods

$y_{acet.} = 0.967\ (\pm 0.016)\ x_{ref.} + 2.5\ (\pm 1.2)$; $r = 0.994$; $\bar{y} = 71.4$ g/L; $\bar{x} = 71.6$ g/L; n = 45. Combined data from three laboratories

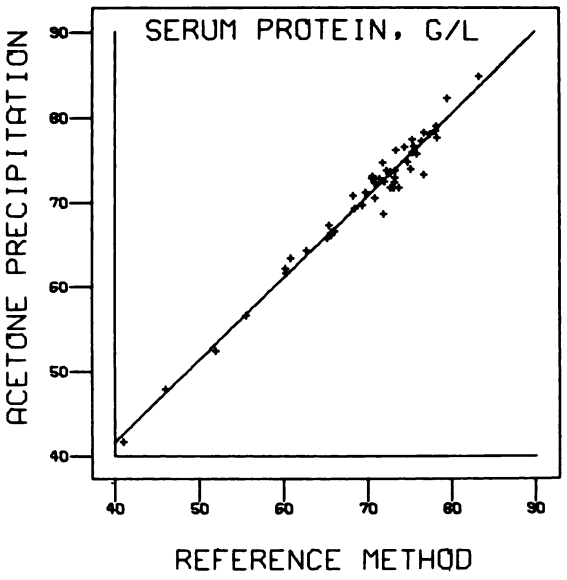

Fig. 6. Protein concentrations in lipemic sera by the reference and acetone precipitation methods

$y_{acet.} = 0.970 (\pm 0.025)\ x_{ref.} + 2.9 (\pm 1.7)$; $r = 0.985$ $\bar{y} = 70.70$ g/L; $\bar{x} = 69.9$ g/L; n = 50. Combined data from three laboratories

method; differences in the other six samples ranged from −4.1 to 3.2 g/L. In view of the results shown in Table 4 and the fact that there is no protein loss in the Chromy and Fischer procedure, this method should provide more reliable data with grossly lipemic sera.

Reference Values

Table 5 lists some of the reference values (normal values) for total serum protein in healthy adults reported in the past (*30*). The substantial differences among mean values and intervals are not unexpected, in view of the variables involved in establishing such intervals for serum protein. Perhaps the most important of these was the use of a variety of protein standards (*4*), all of uncertain accuracy. Other factors possibly contributing to the differences among the reported reference intervals are: (*a*) vertical vs horizontal position of the subjects during sampling (*31–33*); (*b*) type of specimens (serum vs plasma), and nature of the anticoagulant when plasma was used (*34*); (*c*) exercise (*32, 33*); (*d*) age; and (*e*) correction for background absorbance of specimens (of exceptional importance in case of hemolysis and turbidity). Since a well-characterized protein standard (SRM 927) has been made available, we decided to obtain data for establishing a reference interval for total serum protein.

Table 5. Reference Values for Total Serum Protein, g/L

A. (Ref. 30) No. subjects	Method	$\bar{x}$	Range
40	Biuret	75.0	66.0–84.0
87	Kjeldahl	69.0	56.0–82.0
80	Kjeldahl	72.3	61.0–83.0
42	Biuret	71.4	66.0–76.0
24	Biuret	74.0	63.0–85.0
400	Biuret (Automated)	—	60.0–74.0
2400	Biuret (SMA 12/60)	69.1	60.0–79.0
1419	Biuret (SMA 12/60)	74.0	66.0–83.0

B. This study No. subjects	$\bar{x}$	SD	$\bar{x} \pm 2SD$
116 male students	74.7	3.37	68.0–81.4
35 female students	73.0	3.76	65.5–80.5
18 male lab. staff	74.0	3.72	65.6–81.4
62 female lab. staff	73.1	4.00	65.1–81.1
134 all males	74.6	3.41	67.8–81.4
97 all females	73.1	3.89	65.3–80.9
231 all combined	74.0	3.69	66.6–81.4

Blood specimens from medical students and laboratory personnel were collected without anticoagulant. The subjects had fasted for 10–12 h and had been in the upright position for at least 2 h before blood was collected. The specimens were allowed to clot for 1 h, and then were centrifuged (1500 × *g*, 15 min). The sera were kept stoppered and analyzed on the day the samples were collected; only clear sera without hemolysis were analyzed. The data are summarized in Table 5. The upper level of protein concentration is similar for all groups tested in this study. Mean values for women are significantly lower ($p < 0.05$) than those for men, because of the wider range of protein values obtained with sera from women. This is clearly demonstrated in the probability plot (Figure 7). The combined mean and range agree well with those reported by Reed et al. (*35*), who considered the difference between the two populations negligible and, for practical purposes, suggested one set of reference values for both groups.

Discussion

The Reference Method for total serum protein we describe here is simple and precise, and almost free of interference from substances that occur naturally in serum or are administered during treatment of disease.

Standardization

The use of different concentrations of standard for calibrating analytical methods is usually the recommended practice in chemical analysis. We deviated from this approach because the certified reference material is available at a single concentration (70.45 g/L) and the packaging (2.2 mL per ampoule) does not permit dilutions to be prepared accurately with volumetric pipets. However, in the present method, we demonstrated that single-point calibration does not compromise the accuracy to a significant extent. This is the case because of the excellent adherence of the Cu(II)–protein complex to Beer's law and the reproducibility of the color yield (*24*).

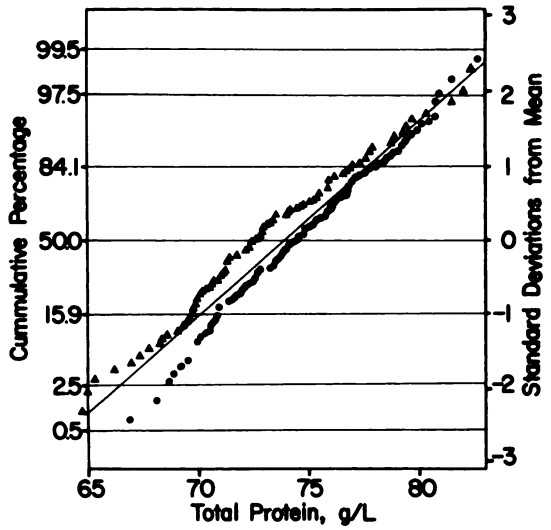

Fig. 7. Total-protein concentrations in serum of healthy women (▲) and men (●), plotted on probability paper

The diagonal line is defined by the mean and the standard deviation of the composite population

For a potential user, we recommend that the linearity of the method be checked periodically as described above in the *Linearity* section.

Time for Color Development

We found that complete color development requires an incubation of 60 min at 25 ± 1 °C or 10 min at 37 ± 1 °C. When the incubation was shortened to 30 min at 25 °C, the color yield was about 1.5% less than at 60 min in both standards and serum specimens. The two alternative procedures provide essentially the same serum protein values as does the Reference Method.

Short incubation intervals, from a few seconds to a few minutes, may result in significant errors in the estimation of serum protein. This is due to the substantial differences in rates at which color develops for different proteins in the early stage of the reaction. With short incubation times (10–55 s) and the SRM (or another BSA solution) as the standard, protein in sera is underestimated because human albumin, the predominant serum protein, reacts slower than BSA. We find that accuracy and precision are optimal only when the reaction is allowed to go to completion.

Composition of the Biuret Reagent

Identical serum protein values were obtained with the proposed reagent and the reagent used over a number of years at the Centers for Disease Control. We examined the effect of increased cupric sulfate and NaOH concentrations on serum protein values. A 67% increase in $CuSO_4$ had little effect (less than 1 g/L) on the protein values (Table 1). A similar increase in NaOH may result in higher protein values because of turbidity, but this was never encountered with the proposed reagent.

Accuracy and Precision

Any claim about the accuracy of the proposed method must await the availability of reference preparations for the main serum-protein fractions and the establishment of their absorptivities with the biuret reagent. Although absorptivities for the major serum protein fractions have been reported to be similar (*4*), it is uncertain whether the purity and peptide content of the preparations used fulfill present criteria for reference materials. We examined a purified preparation of gamma-globulin (Hyland Laboratories) consisting of 99.1% IgG and 0.7% IgM. The absorptivity of this preparation with the proposed biuret reagent was identical to that of human albumin. This finding was confirmed by T. Peters.

The precision of the test exceeds the criterion recommended by Barnett (*36*) and is comparable to that obtained with the glucose Reference Method (*22*). We recommended the use of high-precision mechanical pipettors and of flow-through cuvets for absorbance measurements.

Linearity

Linear regression analysis of the data shown in Figures 2–4 confirmed previous observations (*1*) that there is no evidence of lack of linearity or non-adherence to Beer's law. This statement is based on the small standard errors of the slopes (ranging from 0.2 to 0.5% of the slope values) and on intercepts that are not significantly different from zero (*37*). Pearson's coefficients (r^2) are near 1.0 but, in our experience, the use of this statistic is of questionable value in assessing adherence to Beer's law.

Interferences

The interferences by bilirubin and hemoglobin are small, almost negligible. Blanking errors due to gross lipemia can be overcome by the acetone-precipitation technique. Dextran is the only substance found to interfere, but inspection of the reaction mixture for turbidity will reveal the presence of dextran. We recommend that the solutions be visually inspected for turbidity before absorbance is measured.

There was interference by carbenicillin (*27*) because of the short incubation and the high concentrations of the antibiotic used in that study. Carbenicillin produces a transient color with the biuret reagent, which disappears during the 60-min incubation.

Reference Values

The establishment of reference values cannot be a requirement in the development of reference methods, because such values depend upon many variables, such as geography, nutritional status, age, and socio-economic factors. The data reported here were obtained from ambulatory individuals and, therefore, are not valid for recumbent subjects. Furthermore, the data may not apply to locations other than the Midwestern United States. The data can, however, serve as an approximation of expected serum protein values in the Midwest.

We wish to express our appreciation to Drs. Klaus Borner and Carl C. Garber for their help in confirming certain parts of this work, to Dr. Alan Mather for his advice in the preparation of this manuscript, and to Miss Bernadine Jendrzejczak for her outstanding technical assistance.

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