Evaluation of the Coomassie Brilliant Blue G-250 Method for Urinary Protein


The Coomassie Brilliant Blue G-250 method for protein in urine has been evaluated for analytical accuracy and clinical applicability. Extremely simple to perform, the test exhibits good precision and sensitivity. The color developed per gram of protein is protein-type dependent, so no single protein standard is completely satisfactory. Color intensity is linearly related to concentration up to 1500 mg/L when used with a manual bichromatic method. Accuracy was clinically acceptable for patients with a variety of protein-losing diseases, and for patients having received renal transplants; however, the method underestimates urinary light-chain proteins. In athletes and premature neonates, we observed increased protein excretion during periods of stress. The upper reference limit for protein excretion in healthy adults is about 120 mg/24 h.

Additional Keyphrases: reference interval, proteinuria, renal transplantation, stress, effects of light-chain proteins, 24-h urine collections in premature neonates

The "best" method for protein in urine continues to be debated in the literature (1, 2). Methods involving acid precipitation and the biuret reaction (3) appear to give the same amount of color per gram of protein, regardless of the type of protein present; disadvantages of these methods are tediousness of performance, poor sensitivity below about 100 mg/L, and reliance on analytical skill for acceptable results. Turbidimetric methods, e.g., precipitation with sulfosalicylic acid or trichloroacetic acid, are easy to perform, but tend to be imprecise. In a 1982 College of American Pathologists' interlaboratory survey of urinary protein determinations (4), the sulfosalicylic and trichloroacetic acid methods had CVs of 50% and 43%, respectively, for a survey specimen containing about 1000 mg of protein per liter.

Dye-binding methods have the disadvantage that they give different amounts of color per gram of protein with different proteins. With Coomassie Brilliant Blue G-250 (CBB) for example, albumin gives more color per gram of protein than globulins do (5), which creates a problem in standardization. The absorbptivity of other proteins after reaction with CBB has been described by Bradford (6).

A commonly performed test such as the determination of protein in urine should be simple to carry out. In an earlier study involving CBB to estimate the concentration of cerebrospinal fluid proteins, sensitivity was excellent, and precision and accuracy were acceptable. The method was simple, consisting only of adding the specimen or standard to the reagent and then measuring the absorbance at two wavelengths (5). The utter simplicity of the method and our good experience with the cerebrospinal fluid specimens prompted the present study to learn whether this method could be applied to urine.

In evaluating the CBB method for urinary protein, we had these questions in mind: Can it detect clinically important proteinuria of various kinds, and can it detect clinically important changes in proteinuria? Is the method free of interferences from commonly prescribed drugs, metabolites, and preservatives?

Tests for urinary protein need only identify those patients who need further testing to establish the cause and type of proteinuria. We describe the CBB method here in detail, its advantages and disadvantages, the patient populations to whom it can be applied, its adaptability to automation, reference values, etc.

Materials and Methods

Materials

CBB reagent. Coomassie Brilliant Blue G-250 (CBB; CI no. 42655), 100 mg/L. (Do not confuse with R-250.) To 100 mg of CBB (e.g., no. B1131, Sigma Chemical Co., St. Louis, MO 63178), add 50 mL of 95% ethanol, mix to dissolve, and warm to about 50 °C if necessary to hasten dissolution. Add 100 mL of 15 mol/L H3PO4 to about 500 mL of distilled H2O, then add the diluted H3PO4 to the CBB in ethanol. Bring to 1 L with distilled H2O and mix well. Add 8 to 10 g of siliceous earth (Cellite, no. 545; Sargent-Welch Co., Skokie, IL 60076) and stir for 6 h. Store at 4 °C for 24 h, then filter the cold solution twice through Whatman no. 1 filter paper (Whatman Lab Products, Inc., Clifton, NJ 07014). The reagent may develop a very fine, dark precipitate on standing, but this does not interfere with the analysis. Decant the reagent from the precipitate or centrifuge at 800 × g for 10 min before using.

The absorbance of the CBB reagent at 465 nm vs H2O must be 0.800 ± 0.100 A. Use more or less CBB to adjust the absorbance into this range. Use of the much more expensive purified CBB (e.g., Sigma no. B5133) will require only about 50 mg/L, however, the cheaper reagent and reagents from other sources are satisfactory (see below). The reagent is stable for at least four months at 25 °C. As the reagent ages, there is some loss of sensitivity, owing to the very slow precipitation of CBB; this is marked by a change in color of the reagent to a brownish-blue or green.

Specimens. Urine specimens should be fresh; if analysis is delayed, store them at 4 °C in well-closed containers. Preservatives such as gentamicin, sodium metabisulfite, boric acid, and thymol crystals do not interfere. Do not add HCl to urine, because this invalidates the test.

Standards. Pooled fresh human serum or a mixed protein standard (e.g., 80 g/L, Sigma no. 540-10) can be used after standardization with the biuret method (7). Dilute the protein standard with 154 mmol/L NaCl solution to give working standards of 250, 500, 1000, and 1500 mg/L. Store aliquots of the standards in 1-mL plastic tubes, cap tightly, and freeze at −20 °C. These are stable for at least six months at −20 °C.

Controls. Use commercially prepared lyophilized controls. We found that a fresh normal urine fortified with protein from pooled serum had poor stability even at −20 °C. After reconstitution, aliquot and freeze the controls like the working standards.
Procedures

Manual procedure. Add 50 μL of standard, specimen, or control to 5 mL of the CBB reagent. After 5 min and before 120 min, measure the absorbance at 465 and 595 nm vs a water blank. Select a time between adding the reagent and reading the absorbances, and then use this time (e.g., 10 min) for all the analyses. Plot the ratio of the absorbances, $A_{465}/A_{595}$, vs the concentration of the standards, on linear graph paper. Prepare a standard curve each day, and analyze at least one normal and one abnormal urine control with each batch of specimens. Specimens with protein concentrations exceeding 1500 mg/L should be diluted with 154 mmol/L NaCl. Centrifuge specimens with obvious particulate matter at 800 × g for 10 min. Cloudy urines can be analyzed as such. If the protein concentration is less than 250 mg/L, double the volume of the specimen. Because the dye stains plastic and glass, cuvets cannot be reused without cleaning. We therefore recommend using disposable plastic cuvets. CBB can be removed from plastic and glass by washing with detergent or rinsing with 0.1 mol/L HCl, but all detergent must be removed from the cuvets because it interferes with the analysis. Household bleach diluted 10-fold is also suitable for cleaning cuvets.

We found it very convenient to estimate the urinary protein and pH with a Chemstrip 6 (Bio-Dynamics, Indianapolis, IN 46250) before analysis. In hundreds of analyses, we have found that, with few exceptions, a 2+ qualitative protein test indicates protein above 1000 mg/L; such urines should be analyzed both undiluted and diluted fivefold. In addition, the pH pad on the Chemstrip 6 identifies acidified urines, which cannot be analyzed with CBB because of the serious interference from exogenous strong acids.

Automated procedures. We have also adapted the procedure to the Abbott ABA-100 Analyzer (Abbott Laboratories, North Chicago, IL 60064) and the Flexigem (Electro-Nucleonics Inc., Fairfield, NJ 07006). Details of these procedures are summarized in Table 1.

Results and Discussion

Method Evaluation

Reagent. We evaluated CBB from three sources: cat. no. 20,139-1 (Aldrich Chemical Co., Milwaukee, WI 53201), cat. no. F789 (J. T. Baker Chemical Co., Phillipsburg, NJ 08865); and from Sigma as described above. CBB reagent prepared from the Baker product appeared browner than that made from the Aldrich or Sigma product. The Aldrich CBB was a brownish crystalline product that resembled recrystallized CBB; the other two were dark-brown powders. Freshly prepared CBB reagent from any of the three sources gave the same results on patients' specimens with the ABA-100. CBB reagent prepared from CBB recrystallized from ethanol by us always had an orange-brown color. To recrystallize the dye, saturate 95% ethanol with the dye at 50 °C, cool, and filter off the crystals 24 h later. CBB reagent prepared from recrystallized CBB exhibited more sensitivity, i.e., the slope of the curve of the $A_{350}/A_{465}$ absorbance ratio vs the concentration of the protein was greater with the recrystallized dyes. Using 1-cm cuvets, we determined the slopes of these plots for the fresh reagent prepared from recrystallized Sigma CBB, for the as-received Sigma dye and a four-week-old reagent, and for the as-received Sigma dye and an eight-month-old reagent, as $9.50 \times 10^4$, $9.27 \times 10^4$, and $6.9 \times 10^4$, respectively. If the slope is less than $6.0 \times 10^4$, the reagent should not be used. The CBB need not be extremely pure to obtain satisfactory sensitivity; however, the reagent, regardless of source, ages with time and loses sensitivity. A spectrum of CBB with and without added protein is shown in Figure 1.

Reaction rate and color stability. The reaction of CBB with protein is essentially instantaneous. Solutions of Sigma mixed protein standard, 0 to 1600 mg/L, were analyzed according to the manual method, except that the absorbance was measured at only 595 nm. For concentrations of 0 to 400 mg/L, the reaction is complete in 10 min; for the 800 and 1600 mg/L standards, the reaction at 10 min reached 96% and 97%, respectively, of the absorbance at 2 h. The absorbance of the reagent blank changes slightly with time, the time for absorbance readings should be standardized and controlled; however, the color is stable for at least 2 h. Contrary to our findings, Bradford (6) found that the absorbance at 595 nm peaks at about 10 min and then declines.

Recovery studies. The analytical recovery of the method was examined by using specimens with a constant matrix but increasing concentrations of protein and the manual method. Fresh urine was divided into two equal aliquots, mixed protein standard or saline was added, and admixtures were prepared as follows:

<table>
<thead>
<tr>
<th>Specimen no.</th>
<th>Untreated urine, mL</th>
<th>Protein-supplemented urine, mL</th>
</tr>
</thead>
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<tr>
<td></td>
<td>1 2 3 4 5 6</td>
<td>0 1 2 3 4 5</td>
</tr>
</tbody>
</table>

Fig. 1. Absorption spectrum of CBB reagent with and without added protein.
We performed two recovery studies: A. Protein in untreated urine, 369 mg/L; in supplemented urine, 3180 mg/L; B. Untreated urine, 180 mg/L; in supplemented urine, 1870 mg/L. The recoveries of added protein ranged from 97 to 101% in study A, and from 96 to 112% in study B. The good agreement between the found and expected values indicated that the urine matrix was without effect, and that under the conditions of the experiment, the CBB method had good accuracy. The regression equation for study A was: found (y) = 0.955 expected (x) + 55, r = 0.9992 (n = 6); for B it was y = 0.968 x + 72, r = 0.9968 (n = 6). An advantage of using these sets of specimens is that the matrix is constant and only the analyte of interest is changed.

Linearity. For pooled patients' sera or the Sigma mixed protein standard diluted with 154 mmol/L NaCl, the results by the manual method appeared to be linear with concentration to about 2600 mg/L. We recommend, however, that urine specimens with protein concentrations greater than 1500 mg/L be diluted. For some patients' specimens with protein concentrations exceeding 1500 mg/L, the apparent protein concentration increased upon dilution. Dilution changes the matrix, which may explain this effect.

With the automated methods, linearity extends to at least 1000 mg/L, based on the following found (and expected) data for the diluted Sigma protein standard as measured with the ABA-100: 140(140), 510(460), 840(780), 1120(1090), 1350(1410), 1560(1730) mg/L. The expected values were calculated from the concentrated preparations of the diluted standard. We specify a conservative upper limit of linearity, owing to the increase in apparent protein of some urines after dilution. A linearity study with the Flexigem gave equivalent results.

Precision. We have used this method routinely with the Abbott ABA-100 Analyzer since April 1982. The results for one serum with two different commercially available controls (Quantitative Urine Control; Environmental Chemical Specialties, Anaheim, CA 92806) are: Control 1, mean = 240 mg/L, CV = 5.7%, n = 729; control 2, mean = 840 mg/L, CV = 2.9%, n = 686. For the trichloroacetic acid/buieret method (3), used only during the following study with other controls, the results were: control 3, mean = 218 mg/L, CV = 8.7%, n = 16; control 4, mean = 872 mg/L, CV = 3.5%, n = 16.

Interference studies. Among the large group of substances studied for possible interferences, we chose drugs that are given to patients with kidney diseases or diabetes, drugs used for diagnostic purposes, preservatives, metabolites, and a few miscellaneous substances.

Two concentrations of drugs were evaluated: the maximum daily dose, and twice the maximum dose, in 1 L of urine. In a few cases, the latter exceeded the solubility of the drug, so lower concentrations had to be used. The concentrations of the metabolites tested exceeded those ever seen in urine.

The dyes bind to the NH4+ groups of the protein, causing a shift in the absorption spectrum. Because the CBB is believed not to bind to small molecules, few interferences should be expected (8). We observed positive interferences from tolbutamide at moderate concentrations and from urea at very high concentrations. NaCl at very high concentrations caused falsely-low results (Table 2).

HCl added to urine causes a serious negative interference. Bradford (6) reported interferences from strongly alkaline buffers, Tris, glycerol, succrose, acetone, phenol, Triton X-100, dodecyl sodium sulfate, commercial dishwashing detergents, and Hemosol. McIntosh (9) reported that "salicylates" at 5 g/L and thymol caused a positive interference. We saw neither effect with sodium salicylate at a final concentration

<table>
<thead>
<tr>
<th>Substance added to urine</th>
<th>Conc of substance in urine, mg/L</th>
<th>Protein conc in urine, mg/L</th>
<th>Apparent protein conc, % change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before substance added</td>
<td>After substance added</td>
<td></td>
</tr>
</tbody>
</table>

*HCl (patient 1) to pH 2 140 50 64
| HCl (patient 2) to pH 1 110 60 45
| HCl (patient 3) to pH 1.5 508 330 -35
| HCl (patient 4) to pH 1.5 690 450 -35

*Note: in parentheses indicate average daily dose (mg) of drugs.

1mEq/L

2mL/L

3Diazoxide meglumine and diazoxide-Na injection, 7.4 g of I2 per 20 mL (no. NDC0003-0821-25; E. R. Squibb & Sons, Inc., Princeton, NJ 08540).

4Diazoxide meglumine and diazoxide-Na injection, 293 mg of I2 per 50 mL (no. 54763-79; Mallinckrodt, Inc., St. Louis, MO 63134).

1948 CLINICAL CHEMISTRY, Vol. 29, No. 11, 1983
of 1 g/L. Perhaps McIntosh added to the urine an alcoholic solution of thymol, which produces a very cloudy urine and hence the interference. Others (8) found significant interferences from 0.1 mol/L Na2HPO4, 25 mmol/L EDTA, 100 mg/L Tween 80 polyoxyethylene (20) sorbitan monoleate, and 10 mmol/L Na2SO4.

The effect of turbidity was examined by analyzing slightly cloudy, cloudy, and very cloudy urines before and after centrifugation at 800 × g for 10 min. The centrifugation tended to clear the urines, but was unnecessary because the results with and without centrifugation were the same. Urines with obvious particulate matter should be centrifuged at 800 × g for 10 min.

Hemoglobin gave a positive interference as expected; 1 mg of hemoglobin increased the apparent protein by 1 mg, as shown in Table 2.

Manual vs automated methods. For the manual method we used a Model 200 spectrophotometer with 1-cm cuvets (Gilford Instrument Co., Oberlin, OH 44074). We used the automated CBB method (ABA-100) to determine the reference values and to compare the results for patients’ urines with those by trichloroacetic acid/biuret reagent (3). For 60 patients’ specimens having protein concentrations from 20 to 3800 mg/L, we found that the manual results (y) averaged 8% higher than those obtained with the ABA-100 (x): y = 554 mg/L, x = 508 mg/L, y = 0.880 x + 56 (r = 0.996, n = 60) (10). We also compared the ABA-100 (x) and the Flexigem (y) results for 187 urines in which protein concentration ranged from 0 to 1200 mg/L. The Flexigem results averaged 12% higher than the ABA-100 results (y = 0.990 x + 52, r = 0.977); thus the Flexigem results should be expected to agree better with the manual method.

Clinical Evaluation

We analyzed fresh urines by the CBB (ABA-100) and trichloroacetic acid/biuret methods from patients with a variety of causes for increased protein excretion, patients with light-chain proteinuria, and patients who had received renal transplants. The comparison statistics for these groups are given in Table 3. For groups 1 and 3, the agreement between the CBB and trichloroacetic acid/biuret methods was acceptable. For the patients in group 1, who had proteinuria from various causes, there was good agreement over the entire range of observed values (60–9280 mg/L by the CBB method), and there were no outliers. We did not observe falsely low values for the diabetics in group 1, as has been reported by others (11). For the renal transplant patients, there was good agreement over the range of protein content of 320–4050 mg/L (by CBB) with the exception of one outlier (CBB 1980 mg/L, trichloroacetic acid/biuret 2800 mg/L). The outlier had a disproportionate effect on the slope and intercept owing to the small number of patients in this group.

Myeloma patients. The CBB method underestimates the concentration of kappa and lambda light-chain proteins in urine. The results by the CBB method were, on average, 63% of the trichloroacetic acid/biuret results (range 25–100%, SD 20%). Others found CBB results that were 5–35% of values obtained by an HC1O4-biuret method in six patients (11). Thomas et al. (12) described similar findings in 11 patients with Bence Jones proteinuria. Although we have only 19 results on individuals with light-chain proteinuria, none of these patients would have been left unidentified by the CBB procedure if it alone had been used as the test for proteinuria (Table 4).

In the case of patients with light-chain proteinuria, the urines were concentrated 50-fold with Minicon filters (Amicon Corp., Danvers, MA 01923). The percentage of the total protein that was light-chain protein was estimated by electrophoresis on agarose followed by densitometry (Model 720; Corning Medical, Medfield, MA 02052). In a plot of protein concentration by the CBB method as a percentage of the protein concentration by the trichloroacetic acid/biuret method (column 5, Table 4) vs the percentage of light-chain protein (column 6), we obtained an r of −0.43 (n = 19, p < 0.1, or not significant). In general, the underestimation of the light-chain protein concentration was greatest in those patients where the excreted protein was mostly light chain.

Swimmers. Thirteen well-trained varsity swimmers participated in a study to determine the effects of acute physical stress on certain laboratory test results, including urinary protein. A random (untimed) urine specimen was collected just before the stress test, which consisted of cranking a bicycle ergometer with the arms. The load was increased every 3 min until the subject’s heart rate reached 150 beats/min. Ten minutes later another urine specimen was collected. Figure 2 shows the findings by the manual method, the means of five trials over a five-week period. The basal excretion of protein was nearly the same for all the swimmers; after acute physical stress, protein excretion increased in nine of the 13 subjects, in agreement with many other reports (13). The sensitivity of the CBB method was more

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**Table 3. Comparison Statistics of ABA-100 CBB and Trichloroacetic Acid (TCA)-Biuret Methods for Three Groups of Patients**

<table>
<thead>
<tr>
<th>CBB (x)</th>
<th>TCA (y)</th>
<th>SD</th>
<th>SD</th>
<th>Slope</th>
<th>Intercept</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>1329</td>
<td>1578</td>
<td>1305</td>
<td>1662</td>
<td>1.037</td>
<td>−72</td>
<td>0.984</td>
</tr>
<tr>
<td>1237</td>
<td>838</td>
<td>1958</td>
<td>1234</td>
<td>1.260</td>
<td>399</td>
<td>0.856</td>
</tr>
<tr>
<td>1278</td>
<td>989</td>
<td>1248</td>
<td>1130</td>
<td>1.097</td>
<td>−154</td>
<td>0.960</td>
</tr>
</tbody>
</table>

1. Increased protein excretion (n = 61)
2. Light-chain proteinuria (n = 19)
3. Renal transplant (n = 12)

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**Table 4. Results for 19 Myeloma Patients Excreting Light-Chain Proteins**

<table>
<thead>
<tr>
<th>Light chain</th>
<th>mg/24 h</th>
<th>CBB as % of TCA</th>
<th>% light chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>1066</td>
<td>43</td>
<td>81</td>
</tr>
<tr>
<td>L</td>
<td>712</td>
<td>80</td>
<td>63</td>
</tr>
<tr>
<td>L</td>
<td>1364</td>
<td>51</td>
<td>75</td>
</tr>
<tr>
<td>L</td>
<td>1570</td>
<td>62</td>
<td>92</td>
</tr>
<tr>
<td>L</td>
<td>3222</td>
<td>71</td>
<td>91</td>
</tr>
<tr>
<td>L</td>
<td>1307</td>
<td>(&gt;100)</td>
<td>5</td>
</tr>
<tr>
<td>K</td>
<td>285</td>
<td>71</td>
<td>50</td>
</tr>
<tr>
<td>K</td>
<td>791</td>
<td>74</td>
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</tr>
<tr>
<td>K</td>
<td>306</td>
<td>66</td>
<td>40</td>
</tr>
<tr>
<td>L</td>
<td>1918</td>
<td>(&gt;100)</td>
<td>54</td>
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<tr>
<td>K</td>
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<td>67</td>
<td>82</td>
</tr>
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<td>2802</td>
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<td>K</td>
<td>1618</td>
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<td>25</td>
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</tr>
<tr>
<td>K</td>
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</tr>
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<td>K</td>
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<td>72</td>
</tr>
<tr>
<td>K</td>
<td>1675</td>
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<td>54</td>
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<tr>
<td>L</td>
<td>1087</td>
<td>86</td>
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</tr>
<tr>
<td>Mean</td>
<td>1237</td>
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<td>64</td>
</tr>
<tr>
<td>SD</td>
<td>862</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>n</td>
<td>17</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

*As determined by densitometry of electrophoretic pattern.

Values >100 not included in calculation of mean and SD.

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**CLINICAL CHEMISTRY, Vol. 29, No. 11, 1983**
than adequate to detect these small changes.

Premature neonates. Using the manual CBB method, we measured protein in 24-h urines collected on the first and second postnatal day from pre-term infants, who had been fitted with collection cups for this purpose. The 18 infants were divided into two groups, those with and those without "stress." Stress was defined as the presence of respiratory acidosis, or a low Apgear score at birth, or hyaline membrane disease. Their average protein excretion per 24 h during the first two days of life in shown in Figure 3. Two 24-h collections were obtained from all except patients G, J, O, Q, and R. Infants A through K experienced stress, and their average urinary protein excretion was 21 (SD = 23) mg/24 h; infants L through R did not have stress and had average excretions of 7 (SD = 6) mg/24 h. Except for patient P, the neonates without stress generally excreted less protein. The cause of the higher protein excretion during stress may have been hypoxia and possible kidney injury. Patients A, E, J, and K in the stress group died; the rest of the infants in both groups survived.

Reference values. Twenty-four-hour urine collections were obtained from the laboratory staff, ages 22 to 58 years, and from four male students in their early twenties. The CBB results for protein from 12 women and 13 men are given in Figure 4. The four students provided additional 24-h collection on successive days and the results (mg/24 h) were 1: 35, 38, 35, 37, 46, 49; 2: 48, 37, 28, 31, 32; 3: 31, 24, 34; and 4: 72, 62, 57.

Only one of these 25 individuals had a protein excretion exceeding 120 mg/24 h. Others (12) have reported 31–120 mg/24 h by CBB as the central 90%-region for 49 healthy persons; another report (14) gives a range of 25–75 mg/24 h for a trichloroacetic/turbidimetric method.

We thank Tim McManamon, Richard Strauss, Edward Fox, Robert Bartela, Jose Urrutia, and Jerry Johnson for their help with the study and Christy Anderson for preparing the manuscript.

References

Fig. 2. Protein excretion by swimmers before (bar) and after (heavy line) acute physical stress (see text for details)

Fig. 3. Protein excretion by premature neonates with (A–K) and without (L–R) "stress" (see text for details)

Fig. 4. Protein in 24-h urines from 25 presumably healthy adults
Open bars, men; shaded bars, women

1950 CLINICAL CHEMISTRY, Vol. 29, No. 11, 1983