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Cross-Reactivity of Amino Acids and Other Compounds in the Biuret Reaction: Interference with Urinary Peptide Measurements

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Background: **Biuret assays for total protein measurement are considered to react with all peptides longer than 2 residues. Some studies using biuret assays of urine suggest that small peptides generally are more abundant than proteins in urine, but it is not clear whether this is a problem of assay specificity.**

Methods: **We analyzed the specificity and kinetics of a biuret reaction for solutions of amino acids, organic compounds, peptides, proteins, and ultrafiltered urine specimens and compared the results with standard clinical assays for protein measurement.**

Results: **The biuret assay cross-reacted with several amino acids, dipeptides, and other organic compounds able to form 5- or 6-member ring chelation complexes with copper. Reactions with amino acids and dipeptides had higher absorbance maxima (blue color) than with larger peptides and proteins (purple). Compounds forming potential 4-, 7-, 8-, or 9-member ring complexes with copper had low reactivity. Amino acid amides, dipeptides, and longer peptides had substantial reactivity, except those containing proline. Proteins and polypeptides had similar biuret reactivities per peptide bond, but reaction kinetics were slower for proteins than peptides. Urine specimens ultrafiltered through 3-kDa–cutoff membranes had substantial biuret reactivity, but absorbance maxima were consistent with cross-reactive amino acids rather than peptides.**

Conclusions: **Many compounds, including amino acids, amino acid derivatives, and dipeptides, cross-react in biuret assays. Our studies improve understanding of the specificity of endpoint and kinetic biuret assays widely used in clinical laboratories. Amino acids, urea, and creatinine contribute to overestimation of urinary peptide content by biuret assays.**

It has been recognized for more than 100 years that a variety of compounds, including proteins, can complex with copper(II) (cupric) ions under alkaline conditions *(1)*. Formation of this complex between protein and cupric ions shifts the color of solutions from blue to purple and commonly has been termed the "biuret reaction" after one of the first compounds that was recognized to yield this color reaction. Subsequently, many variations of this reaction were developed for the measurement of total protein in serum or other specimens *(2, 3)*. One of the major advantages of this method has been considered to be the relatively equivalent reactivity of all proteins, leading to a proposal for a biuret method to serve as a method for standardization of the measurement of total serum protein *(4)*. Because the reaction relies on binding of copper to the peptide backbone, biuret assays primarily reflect the peptide content of proteins; the biuret reactivity of individual serum proteins on a weight basis generally is reduced in proportion to their carbohydrate content *(5)*. Reactivity has been considered by clinical chemists for many years to require a minimum peptide chain length of 3 amino acids. As stated in the 1970 edition of *Fundamentals of Clinical Chemistry (3)*, "Amino acids and dipeptides cannot give the reaction, but tri- and polypeptides and proteins react to give pink to reddish violet colored products." The view that a tripeptide is the minimally sized reactant continues to be expressed in more recent editions of this prominent textbook in clinical chemistry, and this probably represents the common view of clinical laboratorians, although the minimally reactive size of peptides rarely has been a practical concern in the analysis of total protein concentrations.

Because of the relatively constant reactivity of biuret assays with peptides down to very small sizes, these assays are of potential interest for determining the total peptide content of peptide mixtures in biological fluids.

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Recent studies applying mass spectrometric methods found that fluids such as serum and urine contain highly complex mixtures of peptide components that may yield valuable diagnostic or physiologic information *(6–18)*. Although these analyses provide extensive qualitative information about peptide components, they yield little information about the overall abundance of these many components. Recent application of a biuret assay to measure total peptide abundance in urine specimens deproteinized by ultrafiltration through a membrane with a molecular cutoff of 10 000 Da suggested that, in urine specimens from healthy persons, small peptides are up to 50-fold more abundant on a mass basis than proteins, 1.5–4.5 g/day of peptides excreted vs <100 mg/day of protein *(19)*. Another report applying measurements with a Lowry protein assay provides even higher estimates of urinary peptide excretion *(20)*. These results potentially provide a revised view of the physiology of protein and peptide handling by the urinary tract *(21)* vs traditional perspectives *(22)* as well as having implications for diagnostic approaches for evaluation of proteinuria/peptiduria. However, there are doubts about the validity of peptide measurements by biuret and Lowry assays for urinary ultrafiltrates because of potential interferences *(23)*. Earlier studies of the total urinary content of amino acids released by acid hydrolysis *(24)* indicated an upper limit for peptide excretion of 1 g/day, a large proportion of which might be accounted for by abundant amino acid derivatives such as hippuric acid, 5-oxoproline, and phenylacetylglutamine *(25–27)*. Analyses of amino acids released by hydrolysis therefore suggest that the biuret method substantially overestimates urinary peptide excretion. Analyses of peptide components in urine have shown a substantial diversity of dipeptides and other small peptides, but the total quantity of small peptide components has appeared to be small relative to the content of free amino acids *(28, 29)*.

Although the biuret reaction has been considered in the clinical laboratory to be relatively specific for measurement of proteins and peptides, there are studies extending back to the initial discovery of the reaction that describe reactions with a variety of other compounds, such as biuret, for which the reaction is named *(1)*, amino acids and amino acid amides *(30, 31)*, dipeptides *(32)*, and a variety of other compounds *(33)*. The nature of some of these complexes differs from the classic biuret complex of copper(II) with proteins and tends to have a blue color with an absorbance maximum above 600 nm, but the absorbance peak is broad and overlaps with the wavelength, typically ~540 nm, at which absorbance is monitored for protein measurement in the biuret reaction. The present study examined the cross-reactivity of amino acids, short peptides, and a variety of other compounds in the biuret reaction in an attempt to develop a greater understanding of the specificity of the biuret reaction and to examine the suitability of the biuret reaction for quantifying low–molecular-weight peptide components in urine.

Materials and Methods

MATERIALS

Glycine, alanine, serine, threonine, asparagine, proline, methionine, glutamic acid, tyrosine, aspartic acid, tryptophan, lysine hydrochloride, arginine hydrochloride, ethylene glycol, glycylglycine (GlyGly), glycylglycylglycine (GlyGlyGly), hexaglycine [$(Gly)_6$], serinamide, tyrosylalanine (TyrAla), hippurylarginine (benzoyl-GlyArg), hippurylglycylglycine (benzoyl-GlyGlyGly), phenylalanylglycylglycine (PheGlyGly), bovine pancreatic ribonuclease A, aprotinin from bovine lung, chicken egg lysozyme, poly-L-glutamic acid sodium salt (M_r 1500–5000), poly-L-lysine hydrobromide (M_r 1000–4000), imidazole, and creatinine hydrochloride were from Sigma-Aldrich. Malonamide, biuret, ethylenediamine dihydrochloride, 1,4-diaminobutane dihydrochloride, 1,3-diaminopropane, and uric acid were from Acros Chemical. Taurine was from ICN Biomedicals. 1-Methylhistidine and carnosine were from MP Biomedicals. Glycinamide, glycylglycylamide, glycylproline (GlyPro), and valylalanine (ValAla) were from Bachem Bioscience. Urea was from Spectrum Chemicals. Concentrations of stock solutions of salts of amino acids and other compounds were corrected to the concentration of the parent compound.

METHODS

Analyses on a Roche Cobas FARA analyzer were performed at 37 °C with a reagent volume of 280 μL (6 mmol/L $CuSO_4 \cdot 5\ H_2O$, 21 mmol/L sodium potassium tartrate, 0.75 mol/L NaOH), 60 μL of specimen, and 10 μL of water. The reagent was as originally described by Gornall et al. *(2)* and as used in a previous report suggesting a high content of urinary peptides *(19)*. Initial baseline readings at 545 nm were taken after addition of the reagent. After addition of specimen with mixing for 2.5 s, an initial reading was made at 4.5 s, and then absorbance readings were made every 5 s for 3 min. Endpoint measurements determined the absorbance difference between the reagent and final reading. Calibration was with human albumin. No correction for dilution was necessary because absorbance measurements on this instrument are through the length of the cuvettes. Blanking for urine color was performed by subtracting the absorbance of parallel reactions of specimens added to biuret reagent without copper.

Standard clinical laboratory methods on the LX-20 analyzer (Beckman-Coulter) for urine creatinine, microalbumin, and urine protein (pyrogallol red dye-binding method) were used. The kinetic biuret method on the LX-20 was run with the cerebrospinal fluid application; it measures the absorbance change at 545 nm in a biuret reaction between 4.0 and 7.7 s.

Absorbance spectra of biuret reaction products were collected with a Cary 50 spectrophotometer (Varian) in cuvettes with a 1-cm pathlength.

Urine specimens were from 24-h urine collections submitted to the clinical laboratory for clinical analysis.

Use of remnant clinical specimens was according to a protocol approved by an Institutional Review Board. Ultrafiltration of urine was performed with Microcon YM-3 centrifugal filter devices. Before ultrafiltration of specimens, devices were rinsed with water and an aliquot of urine to remove surfactants.

Results

BIURET REACTIVITY OF AMINO ACIDS

Amino acids are relatively abundant components in urine with a normal daily excretion ~20-fold higher than protein excretion on a mass basis *(24, 34)*. We therefore considered amino acids as prime candidates for interference with analyses of urinary protein and peptide content by biuret assays. The reactivity in the biuret reaction of several amino acids at equal concentrations of 2.5 g/L is compared in Table 1. Several amino acids, including histidine, asparagine, threonine, and serine, had substantial reactivity in the biuret reaction. Other amino acids had very weak reactivity that was barely detectable at this concentration. Methylation of the imidazole ring of histidine appeared to substantially decrease its reactivity; this is relevant to biuret reactivity of urine in that methylhistidines are relatively abundant urinary components. Multiple concentrations of each amino acid were analyzed, and all concentrations gave similar relative orders of reactivity (data not shown). Higher concentrations gave clearer evidence of reactivity of weakly reactive amino acids, but linearity of reactivity was lost for some compounds above 2.5 g/L (data not shown).

BIURET REACTIVITY OF SIMPLE ORGANIC COMPOUNDS

We examined various other compounds to gain a better understanding of reactivity in the biuret assay (Table 2). These could be grouped according to whether copper complexes potentially could form variously sized chelation rings. Compounds forming 4- or 5-atom ring complexes had the highest reactivity, where the atoms interacting with copper could be an amino-nitrogen, amide-nitrogen, or hydroxyl-oxygen. Carboxy groups probably are not significant elements in chelation for the biuret reaction; copper in the biuret reaction is stabilized by chelation with carboxyl groups of tartrate, and the absorbance of the reagent with no added biuret-reactive components probably reflects copper interacting with carboxyl groups. Hydroxyl-oxygens appeared to be less effective than amino-nitrogens or amide-nitrogens. As an example, ethanolamine had more than 100-fold higher reactivity than ethylene glycol, although both compounds have the potential to form approximately equivalently sized 5-member ring complexes. Ethylene diamine had substantially higher reactivity than did ethanolamine; therefore, stepwise replacement of 1 and 2 of the hydroxyls of ethylene glycol with amino groups led to greatly increased reactivity.

Ethanolamine serves as a model for the chelate-forming portions of threonine and serine, and its reactivity in the biuret reaction was of similar magnitude. 1,3-Diamino-

Table 1. Color yield in the biuret reaction of amino acids compared with albumin.[a]

Compound	Relative reactivity by weight	M_r	Relative reactivity per mole of amino acid[b]
Histidine	0.65 (0.01)	155	0.89 (0.01)
Asparagine	0.33 (0.01)	132	0.38 (0.01)
Threonine	0.31 (0.01)	119	0.32 (0.01)
Serine	0.27 (0.01)	105	0.25 (0.01)
Proline	0.07 (0.01)	115	0.07 (0.01)
Cystine	0.06 (0.01)	240	0.12 (0.02)
Lysine	0.05 (0.01)	146	0.06 (0.01)
Arginine	0.05 (0.02)	174	0.08 (0.03)
Hydroxyproline	0.05 (0.00)	131	0.06 (0.00)
Glycine	0.03 (0.01)	75	0.02 (0.01)
Alanine	0.03 (0.01)	89	0.02 (0.01)
Glutamic acid	0.03 (0.01)	147	0.04 (0.01)
Tyrosine	0.03 (0.01)	181	0.05 (0.02)
Aspartic acid	0.03 (0.01)	133	0.03 (0.01)
Methionine	0.02 (0.01)	179	0.03 (0.02)
Glutamine	0.02 (0.01)	146	0.03 (0.01)
1-Methylhistidine	0.02 (0.01)	169	0.03 (0.01)
3-Methylhistidine	0.02 (0.01)	169	0.03 (0.01)
Tryptophan	0.02 (0.02)	204	0.04 (0.04)
Taurine	0.00 (0.00)	125	0.00

[a] Each compound was analyzed at a concentration of ~2.5 g/L. Values for reactivity are shown as the mean (SD) of analyses in 3 separate runs.

[b] Versus moles of amino acid in albumin. An average mass of 113.6 per amino acid in albumin was used for calculations.

Table 2. Color yield in the biuret reaction of various compounds compared with albumin.

Compound	Relative reactivity by weight	M_r	Relative reactivity per mole[b]
Four-atom ring complexes			
Urea (20 g/L)	0.003 (0.001)	60	0.002 (0.001)
Creatinine	0.06 (0.00)	113	0.06 (0.00)
Uric acid	0.00 (0.00)	168	0.00 (0.00)
Five-atom ring complexes			
Ethylenediamine	1.09 (0.04)	60	0.58 (0.02)
Ethanolamine	0.28 (0.01)	61	0.15 (0.01)
Tris(hydroxy)aminomethane	0.27 (0.01)	121	0.29 (0.01)
Imidazole	0.09 (0.01)	68	0.05 (0.01)
Glycerol (10 g/L)	0.01 (0.00)	92	0.01 (0.00)
Ethylene glycol (10 g/L)	0.006 (0.001)	62	0.003 (0.001)
Six-atom ring complexes			
1,3-Diaminopropane	0.69 (0.03)	74	0.45 (0.02)
Biuret	0.62 (0.03)	103	0.56 (0.03)
Malonamide	0.40 (0.02)	102	0.36 (0.02)
Seven-atom ring complexes			
1,4-Diaminobutane	0.05 (0.01)	160	0.07 (0.01)

[a] Each compound was analyzed at a concentration of ~2.5 g/L unless noted. Values for reactivity are shown as the mean (SD) of analyses in 3 separate runs.

[b] Versus moles of amino acid in albumin. An average mass of 113.6 per amino acid in albumin was used for calculations.

propane (with 2 amino-nitrogens) and malonamide (with 2 amide-nitrogens) serve as approximate models for the potential 6-atom ring complex formed by asparagine (1 amino-nitrogen and 1 amide-nitrogen). Histidine had substantially higher reactivity than imidazole alone, indicating contributions both from its amino-nitrogen and imidazole ring.

Progressively increasing the size of chelation rings to compounds with the potential to form 7-atom ring complexes with copper, such as 1,4-diaminobutane, led to much lower reactivity. Glutamine is a compound with the potential to form 7-member ring complexes, and this is consistent with its much lower reactivity than asparagine, the structural homolog of glutamine with 1 fewer methylene group in its sidechain and the potential to form 6-member rings. Lysine represents an amino acid potentially forming an 8-member ring complex, and it showed low reactivity. Arginine potentially could form 4-, 7-, or 9-member ring complexes between its nitrogen atoms and copper, and it was observed to have low biuret reactivity.

BIURET REACTIVITY OF SHORT PEPTIDES

The biuret reactivities of a variety of short peptides and amino acid derivatives are presented in Table 3. Amino acid amides such as glycinamide and most dipeptides and dipeptide derivatives had high reactivity. Amino acid amides have the potential for their 2 nitrogen atoms to participate in 5-member ring complexes with copper, so that their reactivity is not surprising. *N*-Benzoylglycine (hippuric acid) has a single nitrogen and does not have the potential to form such chelation complexes; it had low reactivity as expected. Dipeptides have the potential to form 5-member ring complexes similar to amino acid amides. N-Terminal acetylation or benzoylation lowered reactivity, suggesting either steric effects or preference for a free amine over an amide. Tripeptides had higher reactivities than dipeptides per weight or mole of amino acids, and the reactivities started to approach that of albumin. Proline appeared to disrupt the biuret reactivity of dipeptides or tripeptides. Peptides longer than tripeptides had reactivities similar to albumin. On a weight basis, hexaglycine had higher reactivity, but this is explained by a lower mass per amino acid residue, and per mole of peptide bond, it had a reactivity similar to that of albumin.

Table 3. Relative biuret reactivities of peptides compared with albumin.

	Relative reactivity by weight	M_r	Relative reactivity per mole amino acid[b]
Monopeptide derivatives			
Glycinamide	1.00 (0.02)	74	0.65 (0.02)
N-Benzoylglycine (5 g/L)	0.004 (0.004)	194	0.01 (0.01)
Dipeptides			
Glycylglycine	0.55 (0.02)	132	0.32 (0.01)
Alanylalanine	0.52 (0.02)	160	0.37 (0.01)
Valylalanine	0.58 (0.02)	188	0.48 (0.02)
Glycylproline (5 g/L)	0.01 (0.01)	179	0.01 (0.01)
Carnosine	0.63 (0.01)	226	0.63 (0.01)
Dipeptide derivatives			
Glycylglycinamide	1.64 (0.00)	132	0.95 (0.00)
N-Acetyl-GlyGly	0.10 (0.01)	174	0.08 (0.01)
N-Benzoyl-GlyArg	0.14 (0.01)	335	0.21 (0.01)
Tripeptides			
GlyGlyGly	1.33 (0.01)	189	0.74 (0.01)
GlyProGly	0.02 (0.01)	229	0.01 (0.01)
PheGlyGly	0.77 (0.01)	279	0.63 (0.01)
Tripeptide derivative and longer peptide			
Benzoyl-GlyGlyGly	1.30 (0.01)	293	1.12 (0.01)
$(Gly)_6$	1.51 (0.02)	360	0.80 (0.01)

[a] Each compound was analyzed at a concentration of ~2.5 g/L of peptide unless indicated. Values for reactivity are shown as the mean (SD) of analyses in 3 separate runs.

[b] An average mass of 113.6 per amino acid in albumin was used to calculate the relative reactivity per mole of amino acid.

BIURET REACTIVITY OF URINE SPECIMENS

The biuret reactivity of urine specimens with either low or high protein excretion was examined before and after ultrafiltration through membranes with a 3-kDa cutoff to remove all proteins (Table 4). These analyses required correction for endogenous urine color as described in the *Materials and Methods*. For all specimens, there was a large amount of biuret-reactive material that passed through the ultrafiltration membrane, consistent with a previous report *(19)*. The amount of ultrafilterable biuret-reactive material corresponded to a reactivity of ~200–4000 mg albumin/g of creatinine. This was substantially more than the total protein content of the urine specimens measured by a dye-binding method and manyfold higher than albumin measured by an immunoassay, except for specimens that were severely proteinuric. For urine specimens with low protein content, the majority of the biuret-

Table 4. Biuret reactivity of urine before and after ultrafiltration through 3000-Da–cutoff membranes.

	Biuret reactivity,[a] mg/g Cr[b]				
Specimen	Unfiltered	Filtered	% Filterable	Microalbumin, mg/g Cr	Total protein,[c] mg/g Cr
1	570	410	72	6	<70[d]
2	390	230	59	5	80
3	4200	4100	98	5	<120[d]
4	1200	1500	125	<5[d]	<150[d]
5	740	850	115	13	<210[d]
6	1500	960	64	<9	<260[d]
7	4800	2000	42	1120	1380
8	2100	670	32	1340	1650

[a] Biuret reactivity is expressed as albumin equivalent reactivity.

[b] Cr, creatinine.

[c] Total protein was determined by a pyrogallol method.

[d] Below the measurement range.

reactive material passed through the 3-kDa–cutoff membrane.

The high concentrations of urea and creatinine in urine may account for some of this reactivity, although both of these compounds are relatively weakly biuret-reactive. A synthetic urine matrix containing 20 g/L urea, 1 g/L creatinine, and 50 mmol/L ammonium chloride in saline yielded a mean (SD) biuret reactivity of 170 (20) mg protein/g of creatinine. Normal rates of excretion of free amino acids of ~500 mg/g of creatinine *(23, 34)* would account for biuret reactivity of ~40–50 mg protein/g of creatinine based on the reactivities listed in Table 1. Therefore, normal urinary content of amino acids, urea, creatinine, and ammonia would be expected to contribute more than 200 mg peptide/g of creatinine to estimates by biuret methods of the peptide content of urinary ultrafiltrates. This magnitude of interference accounts for virtually all of the ultrafilterable peptide content of specimen 2 in Table 4 but accounts for only ~50% or less of the ultrafilterable peptide content of other specimens. This analysis does not rule out the possibility that many specimens contain substantial ultrafilterable peptide content, but our estimate of the impact of interfering material on peptide measurements by biuret methods represents a minimal value because urinary outputs of amino acids may increase in pathologic states and we do not account for the interference from the many other endogenous constituents of urine or medications. The basic structural requirements for biuret reactivity outlined by the reactivity of compounds in Tables 1 and 2 suggest that many endogenous organic compounds other than peptides, as well as many common drugs such as penicillins, cephalosporins, and aminoglycosides, may react and yield falsely increased estimates of peptide content.

ABSORBANCE SPECTRA OF BIURET REACTIONS

Visual examination of the reaction products of biuret reagent with amino acids, peptides, and proteins showed a variation in color. Reactions with amino acids and dipeptides tended to have a blue color similar to the initial reagent. Longer peptides and proteins gave a purple color more representative of traditional descriptions of the biuret reaction. The spectral differences in reaction products were analyzed by spectrophotometry. The absorbance spectra of the reagent alone showed a broad absorbance peak with a maximum at ~665 nm. The absorbance peak is so wide that it overlaps significantly with the wavelength at which the reaction was read (545 nm). When amino acids, peptides, or proteins at equal concentrations were added to the biuret reagent, the magnitude of the absorbance peak was increased and shifted to lower wavelengths. In Fig. 1A, the spectra for the reaction products of asparagines and histidine are shifted to lower wavelengths, with a shift about half as great as for albumin. The absorbance maxima for biuret complexes with hexaglycine and glycylglycylglycine (GlyGlyGly) were greater and were shifted to lower wavelengths than for albumin (Fig. 1B). The absorbance maxima of different compounds and representative urine ultrafiltrates are listed in Table 5. The absorbance maximum shifted from ~665 nm for reagent alone to 640–650 nm for dipeptides such as glycylglycine, 620–640 nm for amino acids such as threonine, asparagines, histidine, and glycinamide, ~600–610 nm for tripeptides, and 560–610 nm for longer peptides. The absorbance peak for a protein such as albumin was broader than for a simple peptide such as hexaglycine, and the maximum was at a longer wavelength. This suggests that absorbance for the biuret reaction product of albumin was the sum of peptide backbone

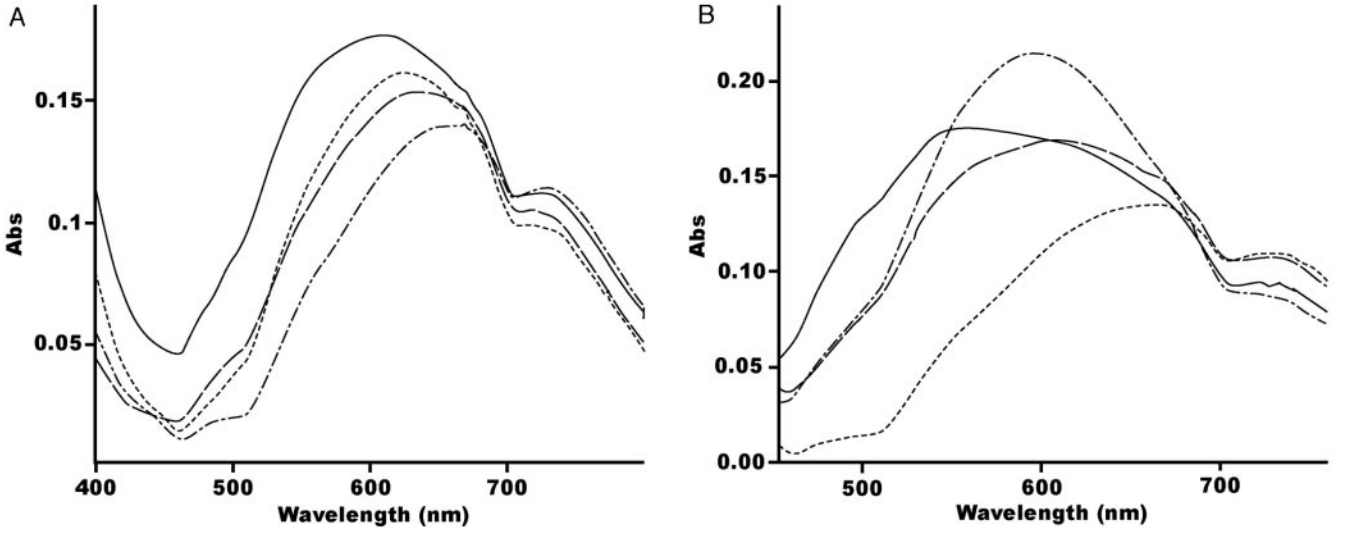

Fig. 1. Absorbance spectra of biuret reaction products.

(*A*), absorbance spectra for reaction products of albumin (———), histidine (- - - -), asparagines (— — —); and reagent blank (water addition; — - — - —). Each protein or amino acid was at 2.5 g/L. (*B*), absorbance spectra for reaction products of GlyGlyGly (— - — - —), $(Gly)_6$ (———), albumin (— — —), and reagent blank (- - - -). Each peptide or protein was at 2.5 g/L.

Table 5. Absorbance maxima of different compounds or urine ultrafiltrates in the biuret reaction.[a]

Compound/Specimen	Mean (SD) absorbance maximum, nm
Reagent alone	665 (3)
Creatinine	666 (2)
Glycine	665 (5)
Acetylglycylglycine	653 (1)
Glycylglycine	647 (1)
Threonine	640 (4)
Asparagine	629 (3)
Histidine	622 (2)
Glycinamide	620 (1)
PheGlyGly	610 (4)
Albumin	609 (5)
GlyGlyGly	601 (1)
Benzoyl-GlyGlyGly	600 (2)
$(Gly)_6$	562 (3)
Urine ultrafiltrate 1	642 (3)
Urine ultrafiltrate 3	619 (2)

[a] Results are the means from 3 or more scans.

and sidechain interactions with copper. The spectra and absorbance maxima of urine ultrafiltrates were consistent with amino acids, simple amino acid derivatives, or dipeptides, and not with longer peptides or proteins.

Reactivity of different proteins and polypeptides in endpoint and kinetic biuret methods and a pyrogallol red method

Biuret methods have been considered to yield relatively equivalent reactivities for different proteins. We examined this assumption for several purified proteins and polypeptides (Table 6). The biuret endpoint method yielded values within ~10% of values expected from the weighed protein content for several proteins, including albumin, lysozyme, aprotinin, RNase A, and α_1-acid glycoprotein after correction for the carbohydrate content of ~45% for the latter protein. Values for the endpoint biuret method were ~30% lower than expected for gelatin, possibly reflecting decreased reactivity of a proline-rich protein, and were ~30%–40% lower than the expected value for weighed peptide content for polylysine and polyglutamic acid (both poly amino acids ~10–30 residues in length). Lysine and glutamic acid have a higher mass by 10%–15% than an average amino acid residue in albumin, and this accounts for part, but not all, of the decreased reactivity. In addition, these polyamino acid salts are particularly hygroscopic so that associated water may comprise part of the weighed values.

The kinetic biuret method had variable reactivity with different proteins. Lysozyme yielded a value 30% higher than the weighed protein content, whereas aprotinin yielded a value ~75% lower than expected. The polypeptides polylysine and polyglutamic acid yielded measured values severalfold below their peptide content. These results indicated that the reactivity of the kinetic biuret method varied substantially for different proteins and peptides and that endpoint and kinetic biuret methods had markedly different specificities.

A method based on the binding of the anionic dye pyrogallol red to protein varied by ~20% from weighed values for several proteins, including albumin, lysozyme, aprotinin, RNase A, and gelatin. However, it was ~40% lower than expected based on the weighed content of α_1-acid glycoprotein, 4-fold higher than expected for polylysine, and >10-fold lower than expected for polyglutamic acid. This probably reflects some charge selectivity in the binding of the negatively charged pyrogallol red: decreased binding to proteins and polypeptide with a high negative charge, such as α_1-acid glycoprotein and polyglutamic acid, and increased binding to polypeptides with a high positive charge, such as polylysine.

Although the kinetic biuret method and pyrogallol red methods yielded measurements of a small protein such as

Table 6. Comparison of different methods for protein analysis.[a]

Polypeptide	Concentration by weight, g/L	Protein, g/L		
		Endpoint biuret	Kinetic biuret[b]	Pyrogallol red[b]
Albumin	1.25	1.25 (0.01)	1.18 (0.01)	1.17 (0.01)
Lysozyme	1.25	1.09 (0.02)	1.61 (0.01)	1.05 (0.01)
Aprotinin	1.25	1.30 (0.01)	0.35 (0.00)	1.49 (0.00)
RNase A	1.25	1.18 (0.01)	0.92 (0.01)	1.47 (0.01)
α_1-Acid glycoprotein	1.25	0.62 (0.01)	0.56 (0.01)	0.36 (0.01)
Gelatin	1.25	0.85 (0.06)	0.89 (0.00)	1.03 (0.02)
Polylysine HBr	1.25[c]	0.48 (0.02)	0.19 (0.01)	2.71 (0.01)
Polyglutamic acid, sodium	1.25[d]	0.58 (0.03)	<0.10[e]	<0.06[e]

[a] Each compound was prepared as a solution containing 1.25 g/L by weight, but polypeptide concentrations were corrected for counterion concentrations. Values for reactivity are shown as the mean (SD) of analyses in 3 separate analyses.
[b] Performed on Beckman LX-20 analyzer.
[c] Peptide content corrected for counterion, 0.76 g/L.
[d] Peptide content corrected for counterion, 0.90 g/L.
[e] Below indicated measurement limits.

aprotinin (M_r 6500), they did not provide measurements of small peptides such as tripeptides or hexaglycine (data not shown). The kinetic biuret method yielded either very low values for small peptides or error codes for high baseline absorbance. Thus, it appeared that the kinetic biuret and pyrogallol red methods were not suitable for quantifying small peptides.

KINETICS OF THE BIURET REACTION

We examined the substantial differences in the reactivities of endpoint and kinetic biuret methods with respect to small peptides and proteins by a more detailed analysis of reaction curves. It was apparent that amino acids and small peptides reacted with biuret reagent very rapidly (Fig. 2A). Approximately 2.5 s were required to complete mixing of specimen and reagent, followed by absorbance readings 2 s later and then at 5-s intervals. The biuret reaction of amino acids and small peptides was completed by the time of the first postbaseline reading: within ~2–4 s depending on the speed of mixing. A high rate might be expected considering that reactants are at high concentrations and it is a simple complexation rather than chemical reaction.

Proteins also had an initial rapid reaction with the biuret reagent, but this was followed by a slow reaction phase over minutes (Fig. 2B). The rates of reactivity differed and the proportion of total absorbance represented by the fast and slow phases differed substantially for different proteins. The slow phase of reactivity may represent the denaturation of protein in the strongly basic biuret reagent, making additional segments of the peptide chain accessible for interaction with copper. Variable rates of reaction may represent differing rates of denaturation for different proteins. All peptide bonds in short peptides would be available for immediate interaction with copper, as none of the peptide bonds will be buried within a globular structure; no denaturation would be required before interaction with copper. These experiments helped explain the major difference in specificity of endpoint and kinetic biuret methods and the apparent lack of reactivity of small peptides in a kinetic method on the LX-20 analyzer. The reaction with small peptides would be completed before the first reading is taken.

Discussion

The biuret reaction commonly has been considered to selectively measure peptides 3 amino acid residues in length or longer *(3)*, although there have been reports extending back many years indicating that amino acids, dipeptides, and other compounds are reactive *(30–33)*. The present study shows that amino acids, dipeptides, and many other compounds indeed react in biuret assays, and our data provide approximate measures of the reactivities of a variety of amino acids, amino acid derivatives, and dipeptides. The exact magnitude of cross-reactivity of amino acids and other compounds may vary slightly depending on the sample matrix, composition of the biuret reagent, and instrumentation, but however these conditions are varied, there is likely to be substantial cross-reactivity of these compounds in endpoint biuret assays designed to measure polypeptides and proteins. The biuret complex of amino acids, peptides, or other compounds with copper is not a simple bimolecular interaction. Copper probably is in coordination with 4 or more interacting atoms *(29–33)*. This may lead to some complexity in the interactions of mixtures of components.

The cross-reactivity of low–molecular-weight components generally should exert a negligible effect on the most common clinical application of the biuret reaction—measurement of total serum and plasma protein—because of the high concentration of serum proteins relative

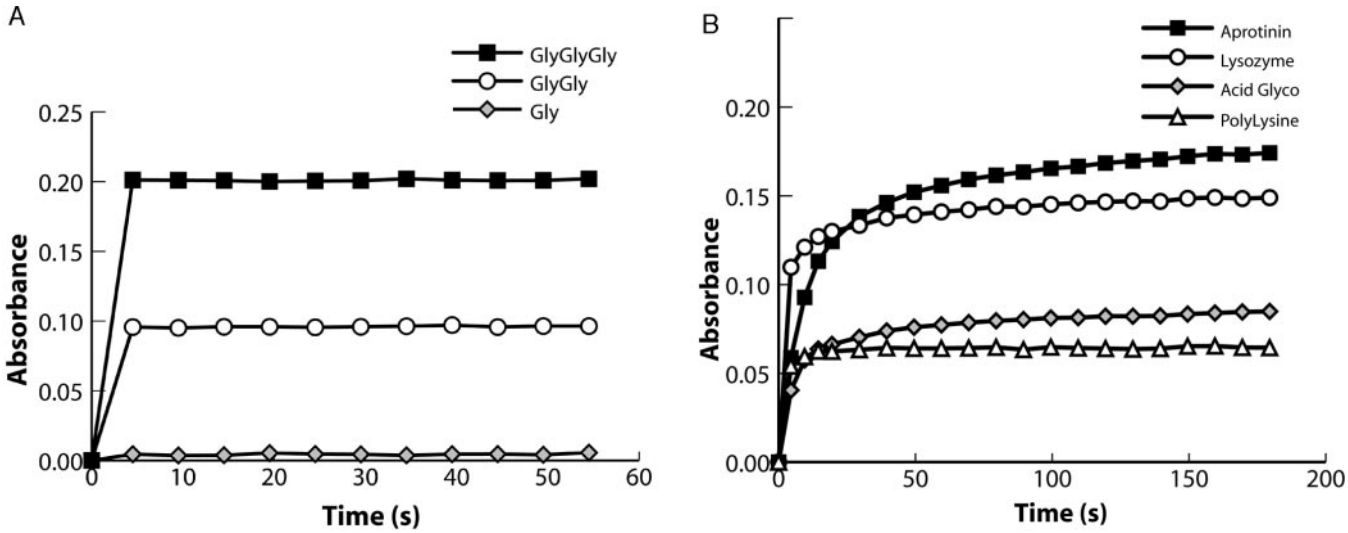

Fig. 2. Timecourse of biuret reactions.

(*A*), reactions with GlyGlyGly (■), GlyGly (○), and glycine (◆), each at 2.5 g/L. (*B*), reactions with aprotinin (■), lysozyme (○), α_1-acid glycoprotein (◆), and polylysine (△), each at 2.5 g/L.

to low–molecular-weight reactive components. Endpoint biuret assays appear to measure different proteins with approximate equivalency per peptide bond as commonly accepted *(4, 5)*. The present study suggests that there may be some effect of amino acid composition in that proline residues appear to diminish reactivity, and the absorbance spectrum for the biuret complex of a protein such as albumin suggests contributions both from peptide backbone and sidechain complexes with copper. Our results also point out some practical differences between endpoint and kinetic biuret methods, both of which are in widespread use in clinical laboratories. Kinetic methods provide higher specificity for the measurement of proteins vs peptides and low–molecular-weight biuret-reactive components, but they have more variable reactivity with individual proteins than do endpoint methods. Depending on the particular application, these characteristics could be either advantageous or disadvantageous.

For biological fluids such as cerebrospinal fluid and urine, with usual protein concentrations 2–3 orders of magnitude lower than serum, the biuret reactivity of low–molecular-weight components becomes significant. For many years, variations of the biuret reaction were applied to measure urine protein, but it was considered necessary to separate protein from other low–molecule-weight components by methods such as acid precipitation or gel filtration *(35–38)*. The nature of the low–molecular-weight components reacting in biuret assays was not identified; some recent reports raised the possibility that there is an abundance of small peptide components derived from protein degradation along the urinary tract *(19–21)*. The present analysis provides evidence that urea, creatinine, amino acids, and other low–molecular-weight compounds contribute to substantial overestimation of the peptide content of low–molecular-weight fractions of urine by biuret assays. Analysis of the absorbance maxima of biuret reaction products of urinary ultrafiltrates suggests that the primary biuret reactants are not peptides. This conclusion is supported further by a recent report that appeared during the course of our study, which estimated the normal excretion of peptides in the size range from 750 to 10 000 Da to be only 22 mg/day *(39)*. This analysis relied on amino acid analysis after acid hydrolysis and should provide quantitative measurement with few interferences. Low peptide excretion was also observed for the size range of 250–750 Da, and high recovery of amino acids was achieved only for the fraction containing molecules under 250 Da, which would include free amino acids and amino acid derivatives such as hippuric acid and 5-oxoproline.

One of our aims in investigating the peptide reactivity of the biuret assay was to determine whether it is suitable for quantification of the total peptide content of complex mixtures of short peptides. Endpoint biuret methods appear to have the potential for quantification of complex mixtures within limitations of the method: the need for an absence of free amino acids and interfering buffers such as tris(hydroxymethylamino)methane and ethanolamine, relatively low sensitivity such that peptide concentrations should approach the gram per liter range, and possible decreased reactivity of proline-rich peptides. Size fractionation of urinary peptides *(39)* or solid-phase extraction *(16)* serve as potential tools for separation of peptide components from substances that interfere with the biuret assay and for severalfold concentration of peptide components. Biuret reactions may be suitable for analyzing peptide mixtures isolated and concentrated by such methods. Modification of reaction conditions, reading times, or wavelengths for absorbance readings do not appear to provide a means of differentiating between the reactivity of amino acids and other small organic compounds from small peptides so that a physical means of separation is necessary. The very broad absorbance peaks of the biuret reagent and its many reactants suggest that use of alternative wavelengths for detection offer little opportunity for solving problems of cross-reactivity.

It is apparent that even urine specimens from healthy individuals contain a complex mixture of peptides *(16, 18, 36, 39)*. Although the mixture of peptides probably has a concentration of only a few milligrams per liter, the peptides are in a low-protein matrix that is well suited for simple methods of sample concentration and analysis, and mass spectrometric technologies require only subnanogram quantities of individual peptide components for analysis. The high information content of urinary peptide mixtures *(18)* and the large changes in peptide concentrations that can occur physiologically *(39)* offer exciting potential for many diagnostic applications, such as detection of urinary tract cancers *(15)* or detection of kidney transplant rejection *(40)*. Accurate quantitative analysis of the urinary excretion of selected components as well as the total peptide mixture represent challenges for clinical applications. Quantitative measures of total peptide content may also be useful in assisting with differentiation of tubular and glomerular injury, as tubular injury has been considered to have greater increases in low–molecular-weight polypeptides *(19)*.

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