Quantitative Micro-Determination of Proteins and Peptides

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The spectrophotometric measurement of the colour, produced by the biuret reaction of proteins and peptides, can be replaced by measurement of the colour, produced by the copper salt of diethyldithiocarbamic acid. In this way microgram of proteins and peptides can be determined with the same accuracy as milligram by Kjeldahl. The method is easy and rapid and suitable for hospital laboratories.

The proteins and peptides in biological fluids are continuously built up and broken down, and they never maintain the same pattern. Nevertheless, their quantitative estimation is of practical importance. We have to resort to indirect analyses, and by determination of the amount of nitrogen some idea of the quantity of proteins or peptides can be obtained when other nitrogen-containing sources are removed beforehand. Native proteins can be precipitated by trichloroacetic acid or sulfosalicylic acid. Amino-acids can be removed from polypeptides by dialysis. For more than half a century the Kjeldahl method has been and still is the classical procedure for determination of proteins by multiplying the nitrogen found by an empirical factor, most often 6.25. That does not tell us anything about the structural distribution of the nitrogen atoms in the protein molecules. As the number of N in one amino-acid may be different from those of another and because of the inconstant distribution of the individual amino-acids in proteins and peptides it is impossible to calculate the number of amino-acids from the number of N-atoms, found by the Kjeldahl analysis.

Other difficulties arise when small amounts of proteins have to be examined, *e.g.* in the cerebrospinal fluid (CSF) where often less than 10 mg/100 ml has to be measured. A reliable micro-Kjeldahl analysis cannot be performed as routine in the clinical laboratories unless many precautions are taken. That is why the analytical accuracy is often replaced by some easy but very inexact method.

The extensive literature about the Kjeldahl method and its numerous modifications shows us the difficulties, and it is the scope of the present publication to find a way out of the uncertainty and try to obtain some more accurate results and propose a new method which is easy to perform and suitable for application in the clinical laboratories of the hospitals.

THE BIURET REACTION AND ITS MODIFICATION

This new method is based upon the biuret reaction in which a red-violet colour appears when a cupric salt is mixed with substances containing more than one —CONH_2 or —CONH— radical in their molecules. Consequently, the reaction is also given by proteins and peptides.

We are dealing here with the formation of a copper complex, established by chelation and ring closure with the metallic ion. The development of the chemistry of complex formation and the importance of ligands as donors of a lone pair of electrons for coordinative bonds is thoroughly explained in two excellent monographs by American authors^{1, 2} that may be consulted for proper understanding of the structural explication to follow. Schwarzenbach^{3, 4} has described the practical utilization of this knowledge for many purposes.

Fig. 1 shows a schematic structural design of the biuret reaction and the way in which peptide chains combine with cupric ions by chelation to form a multinuclear complex. The figure represents the glycyl backbone of the molecular structure of a protein, and it shows how four nitrogen atoms from the peptide bonds, —CONH— , can establish four coordinative bonds to the cupric ion. R represents the non-glycyl moiety of the individual amino-acids. All the nitrogen atoms in R are too far away from the metallic ion to produce an electrostatic field. The size of the cupric ion is small enough to be wedged between four glycyl-N, and a strong electrostatic field is established hereby stabilizing the compound.

In a strong alkaline solution uncoiling of peptide chains is effected in such a way that two peptide chains with four nitrogen atoms can be placed in coplanar position. An electronic explanation is put forward by Rây and Sen⁵ and cited in the monograph by Martell and Calvin¹. It is evident that there exists a correlation of colour and bond type, and when $3d^9$ in the lower orbit is the unpaired electron a blue colour is produced, but when $4p^5$ is occupied a red-violet colour prevails. The 4 nitrogen ligands with their 8 electrons approximate the electron configuration of Cu to that of Kr (36). They give a purely covalent coordination and 2 five-rings are established when 4 peptide bonds take part in the formation of the complex. In this way a great stability is obtained. In glycine and glycylglycine the ionic bond from Cu^{++} to the carboxyl group produces a salt-like compound with blue colour because the $4p$ -orbit is not occupied.

Cox, Wardlaw and Webster⁶ have pointed out that the 4 nitrogen atoms from the ligands must be coplanar with the copper ion and have proved it by X-ray analysis. The continuity of the peptide chain is not broken by the chelation and a multinuclear complex is formed. The length of the chain decides the numbers of copper ions.

From these stoichiometric considerations it appears that the ratio $4\text{N}/\text{Cu}^{++}$ or $4(\text{—CONH—})/\text{Cu}^{++}$ can be calculated from the amount of complexed copper. The picture of a vertebral column of glycyl radicals with R representing the ribs, attached to the backbone, and the row of cupric ions, placed as a *medulla spinalis*, gives us an idea of the structural edification of the biuret compound.

Fig. 1

Fig. 2.

The colour[†] of the biuret reaction compound is too faint for quantitative determination of small differences by measurement of the extinction. Moreover,[‡] there is another inconvenience which is often overlooked. A small part of the precipitated cupric hydroxide is dissolved in the strong alkaline solution of NaOH and imparts to the solution a bluish tint which moves the maximum of spectrophotometric absorbancy to lower wave-length.

PROCEDURE

- Reagents.** Trichloroacetic acid, 10 % aqueous sol.
 Trisodiumphosphate ($\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$), 5 % aqueous sol.
 Cupric sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), 4 % aqueous sol.
 Sodium diethyldithiocarbamate, solid. (DDTC).
- Equipment.** Conical centrifuge tubes
 Sintered glass disc filters (3G5, Jena)
 Spectrophotometer, Beckman B.

Procedure for plasma and serum. Dilute 1 ml of the sample with 49 ml of normal saline sol. Mix 1 ml of the diluted sample with at least 2 ml of the trichloroacetic reagent in a conical centrifuge tube. After centrifugation for 10 min at 3 000 r.p.m. the supernatant fluid is discarded and drained off the inside of the tube. Dissolve the precipitate in 5 ml of the phosphate reagent and add 1 drop of the copper reagent. Place the tube in a mechanical device for gentle shaking for 30 min. The excess of copper is precipitated as cupric phosphate and brought down by centrifugation for 10 min. The supernatant fluid is poured upon a sintered glass filter and by suction the complexed copper compound is collected. Mix 1 ml of the filtrate with 5 ml of the phosphate reagent and add a few crystals of DDTC. The yellow colour is fully developed in 15 min and the extinction is measured in the Beckman B against the phosphate reagent at 440 mμ.

Fig. 2 shows the absorbancy, plotted against the amount of copper, as a linear function from 1 to 6 μg of Cu^{++} . Taking account of the dilution, here 1 500 times, and by introduction of an experimental factor of 9.80 for the ratio protein/ Cu^{++} the percentage of protein is found directly. For instance, an extinction of 0.72 corresponds to 4.87 μg of Cu^{++} and 7.15 % of protein ($4.87 \times 1\,500 \times 9.80$).

Procedure for CSF. 1 ml of CSF is mixed with 1 ml of the trichloroacetic reagent in a centrifuge tube and treated as above. The filtrate from the sintered glass filter is diluted 1:1 with the phosphate reagent. Now the dilution is only 10 times, and by multiplication by the factor 9.80 the content of protein is found directly in mg/100 ml.

Since a reagent blank, carefully performed, never shows a transmission less than 98 % it can be replaced by the phosphate reagent. Cleaning of the sintered glass filters must be made by suction through the filters in the inverted position, first with 0.85 % NaCl, next with 2 N HCl and finally twice with water.

DISCUSSION

In order to separate the native proteins from peptides which also form copper complexes, we have preferred the use of trichloroacetic acid instead of sulphosalicylic acid. The latter has the inconvenience that the precipitate may adhere to the inside of the tubes. Stiff⁷ has introduced cupric phosphate for reaction with the protein, but we consider cupric sulphate to be more suitable, for the reactivity of cupric ions with protein proceeds easier and more completely when the precipitation of cupric phosphate occurs at the same time. Mehl, Pacowska and Winzler⁸ have found it feasible to calculate the amount of protein, bound by 1 g of copper atom in the complex. The most consistent investigations are perhaps made by Plekhan⁹ who has tried to depict the structural relationship between copper and proteins. Beauchene *et al.*¹⁰ determine the copper by flame photometry. Recently Kingsley and Gatchell¹¹ have developed a method for quantitative estimation of small amounts of proteins in serum and in CSF by use of tetrabromophenolphthalein ethylester which in form of the blue potassium salt is absorbed to protein. They find, however, a marked difference in the binding capacity of the dye by proteins in CSF and by those of serum. With the present copper chelation method no such dissimilarity of the proteins exists.

THE ACCURACY OF THE METHOD

As already mentioned not only the native proteins but also polypeptides and oligopeptides down to tripeptides can be measured by this copper test, and the accuracy of the method can be demonstrated by the following example.

Glycylglycylglycine (GGG) was examined *. The sample was accompanied by the statement that the GGG had 2H₂O, but had lost part of the water. 4 mg is calculated to contain 0.746 mg of N, but 0.773 mg was found. In my laboratory we determined the water content by the Karl Fischer method, according to the procedures described in detail in the monograph by Mitchell and Smith¹². We used a micro-method where an end-point-stop device, equipped with a double triode and a magic eye as indicator, permits exact measurement. We have also examined the GGG sample by the copper chelation method and found complete accordance.

Found by Kjeldahl	0.193 mg	of N	per	mg	of GGG
» » Karl Fischer	0.194	»	»	»	»
» » Cu-Chelation	0.194	»	»	»	»

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The amount of N in the water analysis is computed as follows. There is

0.222 mg of N in 1 mg of GGG, (mol.-weight 189)
0.186 » » » » 1 » » » 2H₂O (mol.-weight 225)

We soon found that the copper chelation method yields more accurate results than the Kjeldahl procedure when we made both kind of tests as a routine *in duplo*. Yet, by painstaking performance of the Kjeldahl analyses the deviations are in mg almost the same as are in μ g those of the copper chelation method.

REFERENCES

1. Martell, A. E. and Calvin, M. *Chemistry of the Metal Chelate Compounds*. Prentice-Hall, Inc., New York 1952.
2. Bailar, J. C. jr. (Ed.). *The Chemistry of the Coordination Compounds*. Reinhold, New York 1956.
3. Schwarzenbach, G. *Analyst*. **80** (1955) 713.
4. Schwarzenbach, G. *Die Komplexometrische Titration*. Ferdinand Enke, Stuttgart 1955.
5. Rây, P. and Sen, D. N. *J. Indian Chem. Soc.* **25** (1948) 473.
6. Cox, E. G., Wardlaw, W. and Webster, K. C. *J. Chem. Soc.* **1935** 1475.
7. Stiff, H. *J. Biol. Chem.* **177** (1949) 1798.
8. Mehl, J. W., Pacowska, E. and Winzler, R. J. *J. Biol. Chem.* **177** (1949) 13.
9. Plekhan, M. I. *J. gen. Chem.* (in Russian). **21** (1952) 347, 635; **22** (1952) 1633; **23** (1953) 512.
10. Beauchene, R. E. et al. *J. Biol. Chem.* **214** (1955) 731.
11. Kingsley, G. R. and Getchell, G. *J. Biol. Chem.* **225** (1956) 545.
12. Mitchell, J. jr. and Smith, D. M. *Aquametry*. Interscience, New York 1948.

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