On the Biuret Reaction of Peptides

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A biuret reaction is the most general of color tests for proteins and their hydrolytic products, and even many simple molecules which contain two or more peptide linkages are positive to the reaction as well as to biuret itself. In the biuret reaction of the peptides, a cupric ion give too faint a color for a quantitative determination of photometric differences. Nielson[1] has studied the spectrophotometric measurement of the color produced by the reaction of the copper salts of diethyldithiocarbamic acid with proteins or peptides. In this way proteins and peptides were determined in micrograms with the same degree of accuracy as they were determined in milligrams by the Kjeldahl method.

The present paper will describe the spectrophotometric observation of the colored materials in the biuret reaction, and the relationship between the biuret reaction of peptides and the amount of copper in the colored materials.

All the peptides used in this study were of an analytical grade. Triglycine (GGG), tetraglycine (GGGG), and L-leucyldiglycine (LGG) were obtained from the Nutritional Biochemical Corporation, Ohio. Biuret was prepared from urea according to the usual method. Polymyxin B sulfate was obtained from the Pfeizer Pharmaceutical Co., New York. Crystalline whale insulin was kindly given us by the Taiyo Fishery Co., Osaka.

Biuret reactions were carried out by the method of Baldwin and Bell.[2] The concentration of each peptide was 3×10^{-3} M, except for insulin 3×10^{-4} M, polymyxin B 7.5×10^{-4} M, and biuret 9×10^{-3} M. After the biuret reaction and the centrifugation of the reaction mixture, a spectrum of the color layer of supernatant was observed with a Beckman DU spectrophotometer at room temperature. The copper in the color layer of the supernatant was determined by a modification of the Dithizone method.[3] The supernatant was digested using a hydrochlorite nitric acid reagent. The digest was then diluted with 15 m*l* of water and a drop of bromothymol blue. Then the resultant was neutralized with ammonia, adjusted to 25 m*l* in volume, and transferred into a funnel into which 0.4 m*l* of concentrated hydrochloric acid, 1.0 m*l* of a 10% (w/v) hydroxylamine solution, and 5.0 m*l* of a Dithizone reagent were then added with shaking. The optical density of the color of the carbon tetrachloride layer was read at 503 mμ. A standard curve for the copper solution was obtained by treating the standard solutions by the same procedure.

Figure 1 shows the absorption spectra of the color compounds in biuret reactions in the region between 480 and 610 mμ. Differences in the absorption maxima (λ_{max}) of the color of the treated compounds are shown.

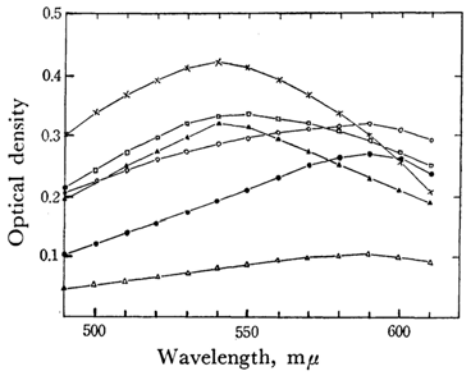

Fig. 1. Absorption spectra of the color compounds in the biuret reaction of several peptides and proteins.

●—● GGG; ×—× GGGG
○—○ LGG; △—△ Biuret
▲—▲ Insulin; □—□ Polymyxin
Concentration of each peptide was 3×10^{-3} M except to insulin and polymyxin B. That of insulin, 3×10^{-4} M, of polymyxin B, 7.5×10^{-4} M and of biuret, 9×10^{-3} M.

Their λ_{max} values were as follows: GGG, 590 mμ; GGGG, 540 mμ; LGG, 590 mμ; biuret, 590 mμ; polymyxin B, 550 mμ, and insulin, 540 mμ. Although Plekhan[4] reported the absorption maximum of insulin biuret at 535 mμ, the experimental conditions used in obtaining this value were not made clear. The λ_{max} of the bluish tint in the biuret reaction was observed at a longer wavelength than that of the red-bluish color in the reaction. Table 1 shows the relationship between

1) H. Nielson, *Acta. Chim. Scand.*, **12**, 38 (1958).
2) E. Baldwin and D. J. Bell, "Cole's Practical Physiological Chemistry," W. Heffer & Sons, Cambridge, England (1955), p. 77.
3) R. Ballentine and D. D. Burford, "Method in Enzymology," Academic Press, New York, Vol. III (1957), p. 1024.
4) M. I. Plekhan, *Zhur. Obshchei. Khim.*, **28**, 3133 (1958); *Chem. Abstr.*, **53**, 6865 (1959).

the molar extinction coefficient (ε) in the absorbance at λ_{max} for each peptide specimen and the number of peptide linkages.

TABLE 1. THE RELATIONSHIP OF THE MOLAR EXTINCTION COEFFICIENTS (ε), THE NUMBER OF PEPTIDE LINKAGES AND THE AMOUNTS OF COPPER IN THE COLORED MATERIALS IN THE BIURET REACTION

Substance	Molecular weight	ε	Number of peptide linkages	Amount of copper** (γ/ml)
Triglycine	189	33.5	2	1.3
Tetraglycine	246	48	3	1.7
L-Leucyldiglycine	245	32.5	2	1.3
Biuret	103	10.5	—	1.0
Polymxin B Calcd	1100*	150	11	0.6
Insulin	6000	670	49	0.6

* Calculated
** Determined against a control value of water.

As a result, the linear relationship between the molar extinction coefficient and the number of peptide linkages of these compounds shown in Fig. 2 was observed. Therefore, the number of peptide linkages in the peptide molecules may be calculated from the ε value in the biuret reaction of these peptides. It is expected that this relation will be examined with other peptides as well.

The amounts of copper in the colored materials in the biuret reactions is shown in Table 1 unexpectedly, a small amount of copper was observed in the color layer of insulin and polymyxin B. The procedures of Rising and her associates were repeated by Manyak *et al.*[5-7] and the copper complexes of diglycine and of triglycine were isolated and analysed for Cu and N. These complexes were found to possess N : Cu ratios of 2 : 1 and 3 : 1 respectively; for the complex with tetraglycine this ratio was found to be 4 : 1[5] and with pentaglycine it was 5 : 1[6]. However, the ratios of Cu and N are not clear in the large molecules of peptides and proteins. Although the color development was not observed with some natural proteins, the reactions were observed in denatured proteins.[8] Thus, it is probable that the relation of the number of peptide linkages in the large molecules of peptides to the amounts of conjugated copper is not simple.

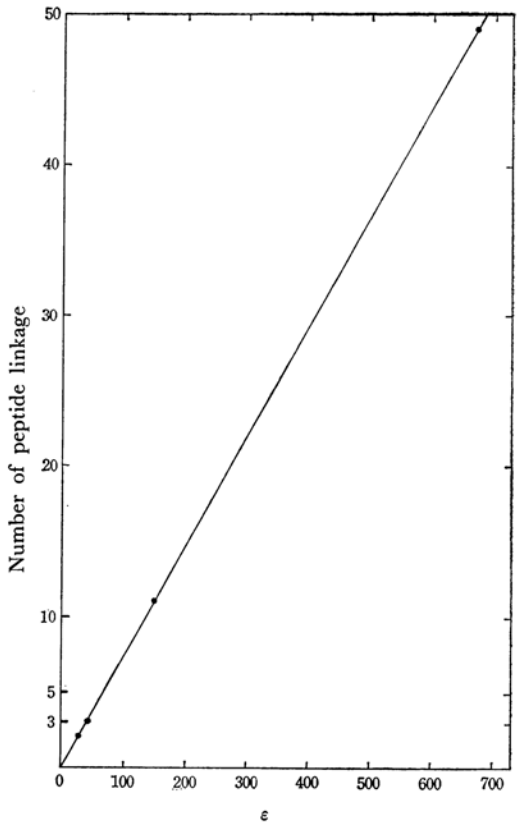

Fig. 2. Relation between the molar extinction coefficients (ε) and the number of peptide linkages.

Polymyxin B has a smaller ring structure than gramicidin S.[9,10] It is a very strongly basic cyclic polypeptide, with a terminal aliphatic carboxylic acid. Although polymyxin B showed a positive biuret reaction, this reaction was not observed on gramicidin S in the range of concentration from 7.5×10^{-4} M to 3×10^{-3} M. The reason for this is not clear. The formation of copper complex of gramicidin has been reported by Akimova *et al.*[11] and the absorption maximum of the copper complex has been observed to be at 515 mμ, though the experimental conditions were not the same as have been described above.

5) M. M. Rising, F. M. Parker and D. P. Gaston, *J. Am. Chem. Soc.*, **56**, 1178 (1934).
6) P. C. Wenaas, *ibid.*, **59**, 1353 (1937).
7) A. R. Manyak, C. B. Murphy and A. E. Martell, *Arch. Biochem. Biophys.*, **59**, 373 (1955).
8) F. R. Senti, *J. Phys. Chem.*, **49**, 192 (1945).
9) N. Hausemann, *J. Am. Chem. Soc.*, **78**, 3663 (1956).
10) G. F. Gause and M. G. Brazhimikova, *Lancet*. **11**, 715 (1944).
11) L. N. Akimova and N. I. Gavrilov, *Chem. Abstr.*, **49**, 8809, 8117 (1955).