[CONTRIBUTION FROM THE GEORGE HERBERT JONES LABORATORY OF THE UNIVERSITY OF CHICAGO]

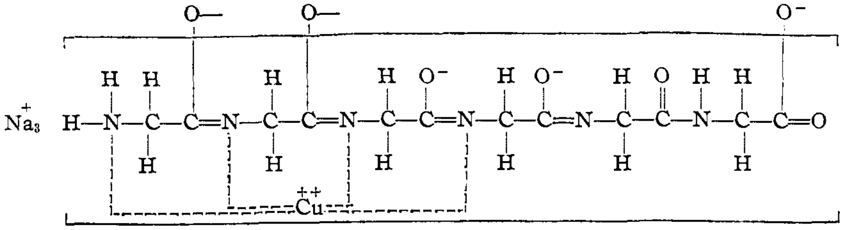
The Biuret Reactions of the Hexapeptide, Pentaglycylglycine, and of the Heptapeptide, Hexaglycylglycine[1]

BY JEAN E. SAURWEIN

In continuation of the study of the biuret reaction of the polypeptides of the glycine series in preparation for a study of the biuret reaction of the proteins,[2] the biuret salts of the hexapeptide, pentaglycylglycine, $NH_3^+(CH_2CONH)_5CH_2CO_2^-$, and of the heptapeptide, hexaglycylglycine, $NH_3^+(CH_2CONH)_6CH_2CO_2^-$, have been isolated and analyzed.

The biuret salts of pentaglycylglycine and of hexaglycylglycine were prepared in the form of hydrates or alcoholates by the saturation with cupric hydroxide of the solutions of the peptides in aqueous sodium hydroxide. Two hydrates of the biuret salt of the hexapeptide were obtained from two separately synthesized hexapeptide samples by precipitation from their aqueous sodium hydroxide solutions, an absolute alcohol–absolute ether mixture being used as precipitating agent. The analytical data for these hydrates agree quite well with the empirical formulas $Na_3[Cu(\text{hexapeptide})]\cdot 5HOH$ and $Na_3[Cu(\text{hexapeptide})]\cdot HOH$, respectively. An alcoholate and a hydrate of the biuret salt of the heptapeptide were obtained from two separately synthesized heptapeptide samples. The alcoholate was obtained by precipitation from the aqueous sodium hydroxide solution of the biuret salt by means of absolute alcohol, the hydrate by precipitation by means of an absolute alcohol–absolute ether mixture. The analytical data for these two compounds agree favorably with the empirical formulas $Na_4[Cu_2(\text{heptapeptide})]\cdot 2C_2H_5OH$ and $Na_4[Cu_2(\text{heptapeptide})]\cdot 3HOH$, respectively. The molecular weights of the salts were not determined.

If its molecular and empirical formulas are identical, the structure of the completely dehydrated biuret salt of the hexapeptide may be



$$\overset{+}{Na_3}\ H-\overset{H}{N}-\overset{H}{\underset{H}{C}}-\overset{O-}{C}=N-\overset{H}{\underset{H}{C}}-\overset{O-}{C}=N-\overset{H}{\underset{H}{C}}-\overset{O^-}{C}=N-\overset{H}{\underset{H}{C}}-\overset{O^-}{C}=N-\overset{H}{\underset{H}{C}}-\overset{O}{\overset{\|}{C}}-\overset{H}{N}-\overset{H}{\underset{H}{C}}-C=O \qquad \overset{++}{Cu} \qquad O^-$$

The structure assigned may be interpreted as follows: the enol tautomer of pentaglycylglycine contains six ionizable hydrogen atoms. Under the conditions of preparation of the hydrates of the biuret salt, only five of these function as acid groups. Three of these are neutralized by the sodium hydroxide used, the amine character of the molecule being thus enhanced. Cupric ion may then enter into a complex of the cupri-ammonium type with four of the strongly basic nitrogen atoms of the peptide enol salt molecule. The two remaining acid groups of the enolate serve to neutralize the two positive charges on the cupric ion. The acid–base balance of the molecule is such that further neutralization of the last group capable of enolization does not take place.

The formula proposed for the biuret salt of the heptapeptide, without either its alcohol or water of crystallization, is $Na_4[Cu_2(\text{heptapeptide})]$ for the case in which the molecular and empirical formulas are identical. This may be interpreted as follows: the enol tautomer of hexaglycylglycine contains seven ionizable hydrogen atoms. The acid–base balance of the molecule is such that only six of them function, under the conditions of preparation of the alcoholate and of the hydrate, four of them being neutralized by the sodium hydroxide present. The amine character of the molecule is thus enhanced, and cupric ion then enters into the molecule. No explanation is offered as to why two cupric ions enter into the molecule, resulting in a ratio of one copper to three and a half nitrogen atoms, rather than the expected one to four ratio. Some new factor must be here involved, and further investigation of the type of complex formed is planned.

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[1] The work here described, conducted first under the direction of Dr. Mary Rising Stieglitz and later of Dr. Julius Stieglitz, constitutes part of the dissertation of the writer, presented in partial fulfilment of the requirements for the doctorate degree at the University of Chicago.

[2] (a) Rising, Parker and Gaston, THIS JOURNAL, **56**, 1178 (1934); (b) Wenaas, *ibid.*, **59**, 1353 (1937).

Experimental

Synthesis of Peptides.—The syntheses of pentaglycylglycine and of hexaglycylglycine were accomplished according to a combination of the procedures of Fischer[3] and of Abderhalden and co-workers.[4] Glycine ethyl ester hydrochloride was obtained from glycine and was converted into glycine anhydride. The hydrolysis and treatment of the anhydride with chloroacetyl chloride produced chloroacetylglycylglycine, which, on ammonolysis, yielded diglycylglycine. Diglycylglycine was converted into its methyl ester hydrochloride, which was neutralized with sodium methylate and then condensed to pentaglycylglycine methyl ester. The ester was saponified with dilute alkali; neutralization produced pentaglycylglycine. Treatment of the hexapeptide successively with chloroacetyl chloride and ammonia gave the heptapeptide, hexaglycylglycine.

Sodium Copper Pentaglycylglycine Pentahydrate, $Na_3CuN_6C_{12}H_{25}O_{12}$.—The salt was prepared as follows: a suspension of 0.05 g. of pentaglycylglycine in 3.65 ml. of water was treated with 1.20 ml. of a 2.084% aqueous solution of carbonate-free sodium hydroxide. The resultant solution was shaken with an excess of cupric hydroxide for fifteen minutes.[5] The unused solid cupric hydroxide was brought upon a filter, and 70 ml. of absolute ethyl alcohol was added to the filtrate. The solution was then filtered to remove traces of a lavender biuret salt formed. Then 50 ml. of absolute ether was added to the filtrate. The pink, flocculent precipitate formed was collected, dried at room temperature over phosphorus pentoxide and analyzed.

Anal. Calcd. for $Na_3CuN_6C_{12}H_{25}O_{12}$: Na, 11.94; Cu, 11.00; N, 14.54; C, 24.93; H, 4.36. Found: Na, 12.10; Cu, 10.91; N, 14.25; C, 24.52; H, 4.30.

Sodium Copper Pentaglycylglycine Monohydrate, $Na_3CuN_6C_{12}H_{17}O_8$.—This salt was prepared from a separately synthesized sample of pentaglycylglycine in exactly the same manner as was the pentahydrate, but was dried over phosphorus pentoxide at 78° before analysis.

Anal. Calcd. for $Na_3CuN_6C_{12}H_{17}O_8$: Na, 13.64; Cu, 12.57; N, 16.64; C, 28.49; H, 3.39. Found: Na, 13.67; Cu, 13.25; N, 16.10; C, 27.80; H, 3.64.

The two hydrates of sodium copper pentaglycylglycine are composed of small, hygroscopic, pink crystals. When heated in a capillary-size melting point tube they both decompose with blackening at 259° (corr.).

Sodium Copper Hexaglycylglycine Dialcoholate, $Na_4Cu_2N_7C_{18}H_{29}O_{10}$.—The salt was prepared as follows: a suspension of 0.05 g. of hexaglycylglycine in 3.65 ml. of water was treated with 1.10 ml. of a 2.30% aqueous solution of carbonate-free sodium hydroxide. The resultant solution was shaken with an excess of cupric hydroxide for fifteen minutes. After removal of the excess cupric hydroxide the biuret salt was precipitated from the reaction mixture by the addition of 70 ml. of absolute ethyl alcohol. The salt was collected, dried at 100° over phosphorus pentoxide and analyzed.

Anal. Calcd. for $Na_4Cu_2N_7C_{18}H_{29}O_{10}$: Na, 12.73; Cu, 17.60; N, 13.57; C, 29.92; H, 4.05. Found: Na, 13.21; Cu, 17.45; N, 13.40; C, 30.04; H, 3.94.

Sodium Copper Hexaglycylglycine Trihydrate, $Na_4Cu_2N_7C_{14}H_{23}O_{11}$.—This salt was prepared from a separately synthesized sample of heptapeptide in exactly the same manner as was the dialcoholate except that it was precipitated by the addition of 70 ml. of absolute alcohol and 5 ml. of absolute ether.

Anal. Calcd. for $Na_4Cu_2N_7C_{14}H_{23}O_{11}$: Na, 13.44; Cu, 18.57; N, 14.33; C, 24.56; H, 3.39. Found: Na, 12.32; Cu, 18.76; N, 14.21; C, 25.58; H, 3.75.

Both the alcoholate and the hydrate of sodium copper hexaglycylglycine are hygroscopic, crystalline, lavender salts, the hydrate being the lighter in color. When heated in a capillary-size melting point tube the alcoholate decomposes with blackening at 271°, at which temperature it also evolves gas; the hydrate decomposes at 275° with blackening, and evolves gas at 279° (all temp. corr.).

Summary

Two hydrates of the sodium biuret salts of the hexapeptide, pentaglycylglycine, and an alcoholate and a hydrate of the heptapeptide, hexaglycylglycine, have been isolated and analyzed. The character of these salts is discussed in terms of the theory of the biuret reaction so far developed.

CHICAGO, ILL.

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[3] E. Fischer, "Untersuchungen über Aminosäuren, Polypeptide und Proteine," Vols. I and II, Verlag von Julius Springer, Berlin, 1906.

[4] (a) Abderhalden and Fodor, *Ber.*, **49**, 561 (1916); (b) Abderhalden and Heumann, *Fermentforschung*, **12**, 42 (1930).

[5] The cupric hydroxide was prepared by the method described by Böttger, *J. prakt. Chem.*, **73**, 49 (1858).