116–119° (8 mm.) and contained 3.7% active bromine. It seems likely that this product before fractional distillation may represent an equilibrium mixture of α- and γ-bromo esters rather than pure γ-ester.

Vacuum runs were made essentially according to a procedure which already has been described.[9] Two bomb tubes were connected to a vacuum line by means of a T-tube. One contained the appropriate ester (usually about 1 millimole) dissolved in acetic acid, the other, hydrogen bromide or bromine in acetic acid. Each solution was of twice the concentration desired after mixing. It was important to use fresh, colorless hydrogen bromide solution to eliminate any effects due to bromine molecules. Each tube was cooled sufficiently so that the system could be evacuated to 10^{-4} mm. (or better). The system was then sealed off and the acetic acid and bromine or hydrogen bromide were distilled *in vacuo* into the other bomb tube, which was sealed off while strongly cooled. The reaction mixture was then allowed to stand at room temperature for the desired time, after which it was opened under potassium iodide solution (80% water and 20% alcohol to facilitate solution and titration), acidified with a little acetic acid. After shaking for a minute or two, the solution was titrated with standard thiosulfate solution, using starch as an indicator.

For air runs, similar solutions in Pyrex Erlenmeyer flasks with ground glass stoppers usually were used, preliminary experiments having shown that this method gave the same results as the method described above when air was admitted before sealing off the bomb tubes.

For illumination, a 200-watt incandescent lamp with a silvered reflector, and at a distance of 30 cm. from the reaction vessel, was employed. An electric fan was used to prevent overheating.

Summary

1. Ethyl α-bromoacetoacetate and α-bromo-α-methylacetoacetate are rearranged slowly by hydrogen bromide in darkness, and in evacuated tubes, to the corresponding γ-bromo esters.

2. The above rearrangements are accelerated by either added peroxides, air (oxygen), or illumination.

3. Preparation of the α-bromo esters in the absence of air gives products which are usually stable toward rearrangement by hydrogen bromide in the absence of illumination and air.

4. It is concluded that the observed peroxide effect is due to bromine atoms formed by the action of air or peroxides on hydrogen bromide.

CHICAGO, ILLINOIS RECEIVED JUNE 25, 1937

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(9) Kharasch and Mayo, THIS JOURNAL, **55**, 2468 (1933).

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

The Biuret Reaction of Sarcosyldiglycine and Glycylsarcosyldiglycine[1]

BY JULIUS FELDMAN

The investigation of the biuret reaction by Schiff,[2] Tschugaeff,[3] Ley and Werner,[4,5] Tomita,[6] and Rising and co-workers[7] has led to the postulation of certain hypotheses concerning the atomic groups requisite for occurrence of the biuret reaction. Briefly, these criteria are (1) an ionizable hydrogen atom and (2) a nitrogen atom capable of forming a cupri-ammonium complex. For the fulfilment of these conditions, it is necessary, in most cases, to postulate a tautomeric form in which a hydrogen atom has migrated from an amide nitrogen atom to a carbonyl oxygen atom. Further, the relative strength of the acidic and basic groups seems to play an important role in determining whether or not a given substance will exhibit the biuret reaction. Thus, glycine anhydride forms a complex copper salt, yet glycylglycine does not. Again, N-monoethylmalonamide, monoethyloxamide, and symmetrical diethyloxamide form complex copper salts, yet unsymmetrical diethyloxamide and triethyloxamide do not show the biuret reaction under any known experimental conditions.

The object of the present investigation was (1) to adduce further evidence for the tautomeric structure of the complex copper salts, and (2) to study the effect of increasing the basicity of a polypeptide molecule upon the ease of biuret formation. For this purpose, the complex cop-

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(1) Abstract of a dissertation presented by the author in partial fulfilment for the degree of Doctor of Philosophy at the University of Chicago. The investigation was carried out under the supervision of the late Dr. Julius Stieglitz.

(2) H. Schiff, *Ber.*, **29**, 298 (1896); *Ann.*, **299**, 236 (1898); **318**, 287 (1901); **352**, 73 (1907).

(3) L. Tschugaeff, *Ber.*, **38**, 2899 (1905); **39**, 3190 (1906); **40**, 1973 (1907).

(4) H. Ley, *Z. Elektrochem.*, **10**, 954 (1904).

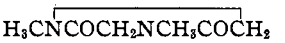
(5) H. Ley and F. Werner, *Ber.*, **40**, 705 (1907).

(6) M. Tomita, *Z. physiol. Chem.*, **201**, 38 (1931).

(7) M. M. Rising and C. Johnson, *J. Biol. Chem.*, **80**, 709 (1928); M. M. Rising, J. Hicks and G. Moerke, *ibid.*, **89**, 1 (1930); M. M. Rising and P. S. Yang, *ibid.*, **99**, 755 (1933); M. M. Rising, F. M. Parker and D. Gaston, THIS JOURNAL, **56**, 1178 (1934); P. S. Yang, *J. Chinese Chem. Soc.*, **4**, 27 (1936); C. Li, Master's Thesis, University of Chicago, 1930; L. Jeffries, Thesis, 1930; P. Wenaas, THIS JOURNAL, **59**, 1353 (1937); J. E. Saurwein, unpublished data.

per salts of sarcosyldiglycine and glycylsarcosyldiglycine were isolated and analyzed. An unsuccessful attempt was made to prepare a complex copper salt of sarcosine anhydride.

Since in sarcosine anhydride,



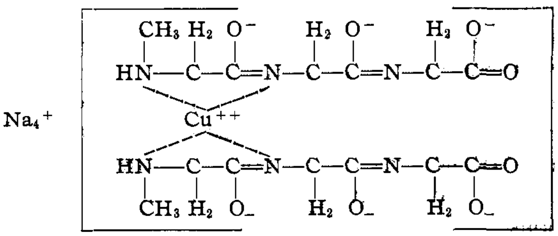
$H_3CNCOCH_2NCH_3COCH_2$

the replacement of the amide hydrogen atoms by less labile methyl groups would leave no acid forming groups, one would predict that this compound would not show the biuret reaction. We were unable to obtain any evidence of a reaction under any conditions which we used. This tends to confirm the enolate structure of complex copper salts proposed by Rising.[7]

Fischer[8] and Parker[7] state that diglycylglycine does not show a positive biuret reaction. Kober and Haw,[9] however, report that diglycylglycine in a dilute solution of sodium hydroxide exhibits a faint reddish coloration when treated with copper hydroxide, this color changing progressively to a blue violet upon increase in the concentration of alkali. Wenaas[7] confirmed these results, finding the color change a reversible phenomenon, but was unable to isolate the colored salt.

Since sarcosyldiglycine should, in our opinion, be somewhat more basic in character than diglycylglycine because of the secondary amino group in the former, we thought that it might be possible to isolate a complex copper salt of the sarcosyl tripeptide. This was accomplished with little difficulty. From the analytical data for the salt the empirical formula was calculated to be $Na_4[Cu(C_{14}H_{20}N_6O_8)]$, or $Na_4[Cu(tripeptide)_2]$.

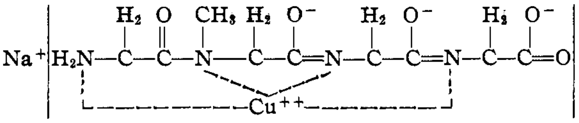
The following structural formula is proposed for the sodium copper salt.



In formulating this structure, it was assumed that two molecules of the enol form of the tripeptide, containing in all six ionizable hydrogen atoms, react with four molecules of base. This so enhances the basic character of the nitrogen atoms that cupric ion is able to enter a complex of the cupri-ammonium type with four of the nitrogen atoms. The two remaining acid groups of the enolate serve to neutralize the two positive charges on the cupric ion. These results are in good accord with those of Wenaas,[7] who obtained a ratio of Cu:Na:N::1:3:5 for the complex copper salt of tetraglycylglycine.

The complex copper salt of glycylsarcosyldiglycine was prepared in the customary manner. The empirical formula of the salt was found to be $Na[Cu(C_9H_{13}N_4O_5)]$, or Na[Cu(tetrapeptide)].

These facts are in excellent agreement with the biuret reaction theory. The substitution of a methyl group for a labile hydrogen atom in the tetrapeptide leaves the complex copper ion uninegative rather than bi-negative, as was the complex copper ion of triglycylglycine.[7] The following structure is postulated for the resulting salt.



Experimental Part

Preparation of Sarcosine Anhydride.—Sarcosine nitrile was prepared by the interaction of formaldehyde, methylamine, and potassium cyanide, and was converted to the ethyl ester hydrochloride by the method of Staudt.[10] The free ester, obtained by the method of Levene and Van Slyke,[11] yielded sarcosine anhydride when heated in a sealed tube at 170° for twenty to twenty-four hours.[12]

Attempts to Prepare a Complex Copper Salt of Sarcosine Anhydride.—To each of five 0.1-g. samples of sarcosine anhydride in 10 cc. of carbon dioxide-free water were added an excess of moist cupric hydroxide[13] and the following quantities, respectively, of carbonate-free sodium hydroxide (0.466 *N*): 0.0 cc., 1.5 cc. (1 mole), 3 cc., 4.5 cc., and 6 cc. The suspensions were shaken for one hour, the cupric hydroxide allowed to settle, and the supernatant liquid examined in a strong light. The solutions were colorless, indicating that no biuret reaction had occurred.

Preparation of Sarcosyldiglycine.—Chloroacetylglycylglycine was prepared by the method of Fischer.[14] From this, sarcosyldiglycine was prepared by a slight modification of the method of Abderhalden, Rindtorff and Schmitz.[15] Chloracetylglycylglycine (20.8 g.) was dis-

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(8) E. Fischer, "Untersuchungen über Amino-säuren, Polypeptide und Proteine," Vol. I, Verlag von Julius Springer, Berlin, 1906, p. 50.

(9) P. A. Kober and A. B. Haw, This Journal, **38**, 469 (1916).

(10) W. Staudt, *Z. physiol. Chem.*, **146**, 286 (1925).

(11) P. A. Levene and D. D. Van Slyke, *J. Biol. Chem.*, **6**, 419 (1909).

(12) F. Sigmund and E. Liedl, *Z. physiol. Chem.*, **202**, 268 (1931).

(13) Böttger, *J. prakt. Chem.*, **73**, 491 (1858).

(14) E. Fischer, *Ber.*, **39**, 2933 (1906).

(15) E. Abderhalden, E. Rindtorff and A. Schmitz, *Fermentforschung*, **10**, 213 (1928).

solved in 100 cc. of 35% methylamine solution and allowed to stand at 40–45° for forty-eight hours. The solution was then evaporated to dryness at reduced pressure, the residue dissolved in the minimum volume of hot water, and the tripeptide precipitated from this solution by the addition of approximately 10 volumes of absolute alcohol. Two more recrystallizations from water–alcohol yielded a product, free from halides, which melted with decomposition at 237–239°. The tripeptide contained one molecule of water of crystallization. This was lost at 78° and 15 mm. pressure. The yield was 15.5 g., or 70% of the theoretical.

Anal. Calcd. for $C_7H_{13}N_3O_5$: C, 41.38; H, 6.40; N, 20.69. Found: C, 41.09; H, 6.73; N, 20.57.

Preparation of Sodium Copper Sarcosyldiglycine.—Sarcosyldiglycine (0.5 g.) dissolved in 20 cc. of carbon dioxide-free water and 10 cc. of carbonate-free sodium hydroxide was shaken for fifteen minutes with an excess of cupric hydroxide. A deep blue-purple color became apparent almost immediately upon the addition of the cupric hydroxide. The excess cupric hydroxide was brought upon a sintered glass filter. To the filtrate was added 700 cc. of absolute ethyl alcohol and an equal volume of absolute ether, the minimum quantity required for the appearance of a permanent cloudiness. The mixture was shaken vigorously, whereupon a flocculent blue-lavender precipitate settled out. This was brought on a sintered glass funnel, washed with 10–20 cc. of a 1:1 alcohol–ether mixture, and dried in a vacuum desiccator over phosphorus pentoxide. The complex copper salt contained 2 molecules of water of crystallization. The formula indicated in the introduction to this paper was calculated on the basis of analyses conducted on a portion of the sample which had been dried at 78° and 1 mm. pressure.

Anal. Calcd. for $Na_4CuC_{14}H_{20}N_6O_8$: Na, 16.55; Cu, 11.44; N, 15.12; C, 30.23; H, 3.60. Found: Na, 16.02; Cu, 11.04; N, 15.28; C, 29.87; H, 3.56.

Preparation of Glycylsarcosyldiglycine.—Carbobenzoxyglycylsarcosyldiglycine was prepared by the method of Bergmann.[16] It was then dissolved in the minimum volume of 2:1 methyl alcohol–water solution, made weakly acid with acetic acid, and hydrogenated under ordinary pressure in the presence of palladium black.[17] After the palladium had been brought on a filter, the filtrate was concentrated under reduced pressure, and the residual sirup was dissolved in hot water. Upon the addition of a 1:1 alcohol–ether mixture to this solution, a sirup was obtained which crystallized on standing. The yield obtained was 2.2 g., or 81% of the theoretical.

Anal. Calcd. for $C_9H_{16}N_4O_5$: C, 41.54; H, 6.15; N, 21.54. Found: C, 41.23; H, 6.31; N, 21.47.

Preparation of Sodium Copper Glycylsarcosyldiglycine.—A solution of glycylsarcosyldiglycine (0.5 g.) in 16 cc. of carbon dioxide-free water and 4 cc. of carbonate-free sodium hydroxide was shaken with an excess of moist cupric hydroxide for twenty minutes. The excess cupric hydroxide was brought on a filter, and 500 cc. of absolute alcohol was added to the filtrate. Absolute ether was added to this solution until the first permanent cloudiness appeared, 400 cc. being required. The mixture was shaken vigorously, whereupon a blue-lavender flocculent precipitate settled out. This was brought on a sintered glass filter, washed with a 5:4 alcohol–ether mixture, and dried over phosphorus pentoxide. The complex copper salt contained four molecules of water of crystallization. The formula indicated in the introduction to this paper was calculated on the basis of analyses conducted on a sample dried at 78° and 1 mm. pressure.

Anal. Calcd. for $NaCuC_9H_{13}N_4O_5$: Na, 6.69; Cu, 18.50; N, 16.30; C, 31.06; H, 3.81. Found: Na, 6.87; Cu, 18.05; N, 16.12; C, 31.12; H, 4.36.

Summary

1. Attempts to prepare a complex copper salt of sarcosine anhydride were unsuccessful. This fact is in accord with previously formulated hypotheses concerning the biuret reaction.

2. The complex copper salt of sarcosyldiglycine was isolated and analyzed. On the basis of these data, the empirical formula $Na_4[Cu(\text{tripeptide})_2]$ was assigned to this substance, and a structural formula conforming well in its essential nature to those assigned to similar salts by Rising and her collaborators was postulated.

3. The tetrapeptide glycylsarcosyldiglycine was prepared, and its complex copper salt isolated and analyzed. The formula obtained, Na[Cu(tetrapeptide)], is in perfect agreement with that predicted by the biuret reaction theory.

CHICAGO, ILLINOIS RECEIVED JUNE 21, 1937

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[16] M. Bergmann, L. Zervas and J. S. Fruton, *J. Biol. Chem.*, **111**, 238 (1935).

[17] J. Tausz and N. von Putnoky, *Ber.*, **52**, 1576 (1918).