

# THE COPPER COMPLEXES OF AMINO-ACIDS, PEPTIDES AND PEPTONES.<sup>1</sup>

FIRST PAPER.

By P. A. KOBER AND K. SUGIURA.

(From the Harriman Research Laboratory, Roosevelt Hospital, New York City.)

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## INTRODUCTION.

That proteins and their constituents combine with heavy metals to form "complexes" has been known for some time. Among these copper has received the most attention and considerable work has been done on the copper complexes of amino-acids; they have been isolated,<sup>2</sup> analyzed and studied by chemical and physical methods. On the other hand the complexes of peptides and peptones have received only a superficial and qualitative study. This is not due to the unimportance of peptide copper salts, for the behavior of peptide and peptone copper salts on treatment with an excess of alkali has been and is yet the most reliable test known for protein-like substances. The real reason for this lack of investigation is, without doubt, due first to the difficulty of obtaining peptides and peptones pure, and second to the fact that the copper salts of these substances are not easily crystallized. It is our belief that a quantitative study of these copper salts<sup>3</sup> will throw some light on the constitution of proteins and it is therefore our intention to continue from time to time our efforts in this field.

In a previous preliminary communication<sup>4</sup> one of us showed a

<sup>1</sup> Read before the Section on Organic Chemistry, International Congress of Applied Chemistry, New York, Sept., 1912.

<sup>2</sup> Ley: *Zeitschr. f. Electrochem.*, xi, p. 954; Schiff: *Ann. d. Chem.*, xliii, p. 123; Bruin: *Chem. Zentralblatt*, p. 824, 1904; Ley and Krafft: *Ber. d. deutsch. chem. Gesellsch.*, xl, p. 697, 1907; Fischer: *Untersuchungen über Amino-säuren, Polypeptide und Proteine*, 1906.

<sup>3</sup> The term *copper salt*, used here and throughout the paper, has reference to a true salt, *i.e.*, the product of the action of amino-acids and protein-like substances in general on cupric hydroxide, oxide or carbonate.

<sup>4</sup> Kober: this *Journal*, x, p. 9, 1911.

striking difference between the salts of amino-acids on the one hand, and the salts of peptides and peptones on the other. As stated in the previous paper, "the copper salts of the amino-acids in alkaline solutions, ( . . . . . contrary to statements in scientific literature,) particularly on warming or on boiling, precipitate copper as the hydroxide, quantitatively. Peptides and peptones, on the other hand, give very little or no hydroxide under the same conditions."

The explanation of this important difference may possibly be due to one or more of the following reasons: (1) A marked difference in the constitution of these salts; (2) A change of constitution brought about by the excess alkali; (3) A change in the form or condition of the copper due to the presence of the protein-like substances, *e.g.*, "colloidal copper."<sup>5</sup>

The fact that peptides do not form copper salts strictly in accordance with the number of free carboxyl groups,<sup>6</sup> as do most of the amino-acids, favors the theory of a difference in the constitution as the cause of their abnormal behavior on treatment with excess alkali. The fact that most copper salts of peptides and peptones change color on treatment with alkali, giving the so-called "biuret color," indicates that a change of structure in these peptides takes place on adding excess alkali. The fact that a similar phenomenon described by Paal and Leuze<sup>7</sup> was explained as a "colloidal process" gives a certain degree of plausibility to the third reason. As will be seen, the data given in the latter part of this paper support the first two explanations, while not many facts support the third.<sup>8</sup>

As Leuchs,<sup>9</sup> conjunctively with Manasse and La Forge, has considerable experimental basis for considering that carbethoxyl-glycyl-glycine ester and allied compounds have two forms, the lactim and the lactam, we believed at first that the lactim form was the chief cause for the copper not precipitating in alkaline solutions, behaving in this respect like other hydroxy-acids, such

<sup>5</sup> Paal and Leuze: *Ber. d. deutsch. chem. Gesellsch.*, xxxix, p. 1545.

<sup>6</sup> Fischer: *Untersuchungen über Amino-säuren, Polypeptide und Proteine*, 1906, p. 50.

<sup>7</sup> *Loc. cit.*

<sup>8</sup> Copper salts in weak alkaline solutions dialyze, but when the alkali is strong, they combine with the membrane and cause difficulties.

<sup>9</sup> *Ber. d. deutsch. chem. Gesellsch.*, xl, p. 3235; xli, p. 2586.

as lactic, tartaric, etc.; but owing to the lack of spectrographic data we cannot say whether this really plays a part or not.

As part of our results are at variance with some obtained previously by other investigators, our technique and results will be described and discussed before data are weighed in support of any theory.

#### TECHNIQUE.

Fischer,<sup>10</sup> Abderhalden,<sup>11</sup> and others prepared the copper salts of amino-acids, peptides and peptones by boiling with cupric oxide. We found that they were formed best at a low temperature, with cupric hydroxide, preferably in an ice mixture. Since some heat of neutralization makes the process an exothermal one, it is not unexpected that the reaction will be complete at a low temperature. A further objection to boiling lies in the fact that a few of these salts have a slight tendency to hydrolyze. Where a reaction can be brought about at a freezing temperature the danger of decomposing unstable peptides is obviously reduced to a minimum.

The form of the copper hydroxide is very important as on standing it is dehydrated with the formation of cupric oxide. That cupric oxide is slower in its reaction with these peptides can readily be seen from the constitution of the copper salts.<sup>12</sup>

The best conditions for forming these copper salts quantitatively are as follows:

##### *a. For soluble substances.*

For every 0.1 gram of amino-acid or peptide 5 cc. of 5 per cent cupric chloride solution are diluted to 150 or 200 cc. with ice-cold distilled water and neutralized with alkali. This can be done most suitably by mixing 100 grams of fine ice with 5 cc. of 5 per cent cupric chloride solution and 100 cc. of distilled water and neutralizing with  $\frac{N}{5}$  alkali, using phenolphthalein as an indicator. The neutralized mixture is then filtered, the ice being placed on the filter also in order to keep the precipitated cupric hydroxide cool. The hydroxide is washed once or twice with cold distilled water on the filter paper, removed with the ice to the cooled 10–20 cc. solution of the amino-acid or peptide and stirred for from five to ten minutes.

On filtering off the excess cupric hydroxide and washing thoroughly, the solution will contain the copper salts of all the substances. As a rule, the solutions require boiling, to decompose the very appreciable amount

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<sup>10</sup> Fischer: *loc. cit.*

<sup>11</sup> Abderhalden: *Ber. d. deutsch. chem. Gesellsch.*, xxxx, p. 2746.

<sup>12</sup> *Amer. Chem. Journ.*, Nov., 1912.

of carbamino salts that are formed with  $\text{CO}_2$  of the air, and this may be done before filtering. To ascertain the amount of copper hydroxide dissolved, the copper salt may be titrated directly (with 0.04 N  $\text{Na}_2\text{S}_2\text{O}_3$ ) by adding 5–10 cc. of glacial acetic acid and 2–4 grams KI. The amount of carbamino salts formed is increased by the presence of sodium chloride, whereas alcohol, on the contrary, hinders their formation.

*b. For insoluble substances with slightly soluble copper salts.*

If the amino-acid or peptide is insoluble, 10–20 cc. of  $\frac{N}{10}$  ammonia are added to the sample and it is stirred until dissolved. If necessary the mixture may be heated to hasten solution. After cooling the solution in an ice mixture, cold cupric hydroxide, made as above, is added and stirred for from five to ten minutes; 150 cc. of water are then added and the solution filtered after thorough shaking. The precipitate is washed well with hot water and the filtrate and wash waters are concentrated. The amount of copper hydroxide dissolved may be titrated iodimetrically in the usual way. Controls on this method, using copper hydroxide and 20 cc. of  $\frac{N}{10}$  ammonia, gave only 0.0003 to 0.0005 gram of copper oxide in the filtrate.

*c. For insoluble substances with quite insoluble copper salts.*

Where the copper salt crystallizes out and is filtered off with the excess copper hydroxide, it is necessary to separate the two precipitates.

Very satisfactory reagents for this purpose are the bicarbonates of sodium and potassium. Using 10 and 20 per cent solutions of  $\text{KHCO}_3$  we have obtained the results given below on leucine, tryptophane, cystine, amino-*n*-caproic acid and phenylglycine.

The amino substance is treated as in *b*, but the excess copper hydroxide, mixed with the insoluble copper salt, is then treated with 20 cc. of 20 per cent  $\text{KHCO}_3$  and washed with small lots of 10 per cent  $\text{KHCO}_3$  until the filtrate shows only traces of copper. To determine directly the amount of copper hydroxide dissolved, the final residue of copper salt is then transferred with the filter paper to the first filtrate and after being dissolved in dilute hydrochloric<sup>13</sup> acid is titrated as before.

On page 13 under experimental notes we give a few experiments on the solubility of these "insoluble" copper salts in various concentrations of potassium bicarbonate. In the near future we hope to give the details of more experiments along these lines, especially on the determination of amino-acids in the presence of polypeptides.

*Boiling directly with copper hydroxide or oxide will result without doubt in the incomplete formation of copper salts; this fact helps to explain the unexpected results obtained by Abderhalden and Hirsch on *d*-alanyl-*l*-leucyl-isoleucine and its glyceryl derivatives (see page 7).*

<sup>13</sup> As large amounts of potassium acetate retard the iodimetric titrations of copper, we neutralize with pure HCl.

## SUMMARY OF DATA.

*Amino-acid copper salts.*

The results with our technique on  $\alpha$ -amino-acids confirm the analyses made by previous investigators of isolated copper salts, which have without exception the general formula  $\text{CuA}_2$ , where A represents one molecule of monobasic  $\alpha$ -amino-acid.

A few examples will suffice:

*Monobasic amino-acids.*

SUBSTANCES	METHOD OF PREPARATION	WEIGHT OF SAMPLE	CuO IN FILTRATE AFTER ADDING 3 CC. N NaOH (CO <sub>2</sub> FREE) AND BOIL- ING	CuO IN PRECIPITATE AF- TER ADDING 3 CC. N NaOH (CO <sub>2</sub> FREE) AND BOILING	THEORETICAL WEIGHT CuO CALCULATED FOR THIS SAMPLE	TOTAL CuO AS PERCENT- AGE OF THEORETICAL
		grams	grams	grams	grams	per cent
Glycine.....	a	0.1015	0.0000	0.0536	0.0538	99.6
Alanine.....	a	0.1019	0.0000	0.0453	0.0455	99.6
Aminobutyric acid.....	b	0.1022	0.0004	0.0403	0.0395	102.0
Active valine.....	a	0.0944	0.0015	0.0301	0.0321	93.8
Leucine.....	c	0.1010	0.0054	0.0361	0.0307	102.6
Normal aminocaproic acid	c	0.1003	0.0002	0.0315	0.0305	105.7
Isoleucine.....	a	0.1014	0.0011	0.0307	0.0308	103.2
Active proline.....	a	0.1018	0.0078	0.0269	0.0352	98.6
Phenylalanine.....	b	0.1001	0.0000	0.0244	0.0242	100.8
Phenylglycine.....	c	0.1011	0.0000	0.0280	0.0266	105.3
Tyrosine.....	b	0.1027	0.0007	0.0263	0.0226	116.4
Tryptophane*.....	b	0.1022	0.0000	0.0183	0.0199	92.0
Asparagine.....	b	0.1005	0.0005	0.0308	0.0303	101.7
Sarcosine hydrochloride†.	a	0.1002	0.0021	0.0299	0.0315	101.6
Lysine picrate‡.....	a	0.1006	0.0006	0.0097	0.0107	96.3
Arginine dinitrate.....	a	0.1010	0.0011	0.0121	0.0134	98.5
Histidine dihydrochlor- ide§. ....	a	0.1000	0.0161	0.0005	0.0175	94.9

\*If more than 0.1 gram tryptophane is used, method c must be added to this technique.

†All acid salts, such as hydrochlorides, nitrates, etc., are neutralized with  $\frac{N}{10}$  alkali, using phenolphthalein as an indicator, before treating with copper hydroxide as in method a.

‡Our thanks are due to Dr. Levene of the Rockefeller Institute for furnishing us with isoleucine, arginine and lysine.

§Histidine forms a complex salt, as do the other monobasic amino-acids, and on treatment with excess alkali, changes its color but little. Only on boiling the color changes towards a biuret. It is not a clear color, but smoky, and makes the solution look very dark. Characteristic is the deep red color to which the alkaline solution turns on the addition of acid. We expect to make this a basis for the colorimetric qualitative and quantitative estimation of histidine.

With other amino-acids, whose  $\text{NH}_2$  group is in the  $\beta$ -position, Fischer found the formula to be  $\text{CuA}_2$ , where A is monobasic amino-acid, except (as in the case of isoserine) when an oxy group is in the  $\alpha$ -position, where the formula is  $\text{CuA}$ .

We have obtained the same results with isoserine,<sup>14</sup> using the technique  $\alpha$ :

SUBSTANCE	WEIGHT OF SAMPLE	CuO IN FILTRATE AFTER ADDING 3 CC. N NaOH ( $\text{CO}_2$ FREE) AND BOIL- ING	CuO IN PRECIPITATE AF- TER ADDING 3 CC. N NaOH ( $\text{CO}_2$ FREE) AND BOILING	TOTAL CuO	THEORETICAL WEIGHT CuO CALCULATED FOR THIS SAMPLE	TOTAL CuO AS PERCENT- AGE OF THEORETICAL
Iso-serine .....	grams 0.1010	grams	grams	grams 0.0807	grams 0.0765	per cent 105.5
Iso-serine .....	0.1009	0.0299	0.0499	0.0798	0.0764	104.5

According to Fischer,<sup>15</sup> when the amino group is in the  $\gamma$ -,  $\delta$ -, or  $\epsilon$ -position (regardless of an hydroxy group in the  $\alpha$  position) no copper salts are formed.

Our results confirm the previous figures on dibasic salts having a general formula  $\text{CuA}$ , where A is a dibasic acid, such as aspartic, glutaminic, cystinic, etc.

*Dibasic amino-acids.*

SUBSTANCE	METHOD OF PREPARATION	WEIGHT OF SAMPLE	CuO IN FILTRATE AFTER ADDING 3 CC. N NaOH ( $\text{CO}_2$ FREE) AND BOIL- ING	CuO IN PRECIPITATE AF- TER ADDING 3 CC. N NaOH ( $\text{CO}_2$ FREE) AND BOILING	THEORETICAL WEIGHT CuO CALCULATED FOR THIS SAMPLE	TOTAL CuO AS PERCENT- AGE OF THEORETICAL
		grams	grams	grams	grams	per cent
Cystine.....	c	0.1008	0.0000	0.0331	0.0334	99.1
Aspartic acid*.....	a	0.0997	0.0009	0.0579	0.0596	98.6
Glutaminic acid*.....	a	0.1025	0.0004	0.0518	0.0554	94.9

\*Before boiling the solutions of copper salts are diluted to 150 cc. with water.

<sup>14</sup> Made according to Fischer's directions from epichlorhydrin. *Ber. d. deutsch. chem. Gesellsch.*, xxxv, p. 3787.

<sup>15</sup> E. Fischer and L. Zemplen: *Ber. d. deutsch. chem. Gesellsch.*, xlii, p. 4883, 1909.

*Polypeptide copper salts.*

Out of a hundred or more polypeptides, Fischer, Abderhalden and their collaborators have analyzed only about eight of the polypeptide copper salts; and these, with three exceptions (nos. VI, VII and VIII) were insoluble and crystallized out. In such cases there can be no doubt as to the purity of the product and the results are confirmed by our work on the more soluble copper peptides. These cases are as follows:

I. (Leucyl-glycine copper)<sub>2</sub>O + H<sub>2</sub>O<sup>16</sup> or (C<sub>8</sub>H<sub>15</sub>O<sub>3</sub>N<sub>2</sub>Cu)<sub>2</sub>O + H<sub>2</sub>O.

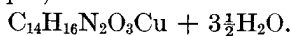
Fischer concluded that one molecule of copper hydroxide combined with one molecule of leucyl-glycine and that two molecules of the copper-leucyl-glycine are connected by an oxygen atom. Although we have found one molecule of copper hydroxide to one of leucyl-glycine, we do not believe that an oxygen atom connects two molecules of copper-leucyl-glycine. We have determined the molecular weight of this salt by the cryoscopic method, and find it to have a molecular weight consistent with the formula leucyl-glycine-Cu;

II. (Phenyl-glycine-glycine)<sub>1</sub>copper,<sup>17</sup> C<sub>10</sub>H<sub>10</sub>N<sub>2</sub>O<sub>3</sub>Cu.

III. (*l*-Leucyl-*l*-histidine)<sub>1</sub>copper,<sup>18</sup> C<sub>12</sub>H<sub>18</sub>O<sub>3</sub>N<sub>4</sub>Cu.

IV. (Alanyl-*l*-tryptophane)<sub>1</sub>copper,<sup>19</sup> C<sub>14</sub>H<sub>15</sub>N<sub>3</sub>O<sub>3</sub>Cu.

V. (*l*-Prolyl-*l*-phenylalanine)<sub>1</sub>copper,<sup>20</sup>



VI. (*d*-Alanyl-*l*-leucyl-isoleucine)<sub>2</sub>copper,<sup>21</sup> (C<sub>15</sub>H<sub>27</sub>O<sub>4</sub>N<sub>3</sub>)<sub>2</sub>Cu.

VII. (Glycyl-*d*-alanyl-*l*-leucyl-isoleucine)<sub>2</sub>copper,<sup>22</sup>  
(peptide)<sub>2</sub>Cu.

VIII. (Tri-glycyl-glycine)<sub>2</sub>copper,<sup>23</sup> (tetrapeptide)<sub>2</sub>Cu.

Out of the large number of peptide copper salts examined by Fischer, Abderhalden and ourselves, the last three form an apparent exception to the rule. There are good reasons to believe that

<sup>16</sup> Fischer: *Ann. d. Chem.*, cccxl, p. 145.

<sup>17</sup> Fischer: *ibid.*, p. 195.

<sup>18</sup> Fischer and L. H. Cone: *ibid.*, ccclxiii, p. 107.

<sup>19</sup> Abderhalden and M. Kempe: *Ber. d. deutsch. chem. Gesellsch.*, xl, p. 2737.

<sup>20</sup> Fischer: *ibid.*, xlii, p. 4752.

<sup>21</sup> Abderhalden and Hirsch: *ibid.*, xliii, p. 2439.

<sup>22</sup> *Ibid.*

<sup>23</sup> Curtius: *ibid.*, xxxvii, p. 1294.

the formulas given for VI, VII and VIII do not represent true copper salts, as the methods used in each case are no guarantee of the complete formation of the salts.

Using the technique described above, we have formed the copper salts of the following peptides, and have found the results to be consistent with the formula, (peptide)<sub>1</sub>Cu<sub>1</sub>.

*Dipeptides.*

SUBSTANCE	WEIGHT OF SAMPLE	CuO IN FILTRATE AFTER ADDING 3 CC. N. NaOH (CO <sub>2</sub> FREE) AND BOIL- ING	CuO IN PRECIPITATE AF- TER ADDING 3 CC. N. NaOH (CO <sub>2</sub> FREE) AND BOILING	TOTAL CuO	THEORETICAL WEIGHT CuO CALCULATED FOR THIS SAMPLE	TOTAL CuO AS PERCENT- AGE OF THEORETICAL.
	grams	grams	grams	grams	grams	per cent
Glycyl-glycine.....	0.1123	0.0536	0.0070	0.0606	0.0616	98.4
Glycyl-alanine.....	0.1006	0.0501	0.0049	0.0550	0.0548	100.4
Glycyl- <i>d</i> -alanine.....	0.1011	0.0524	0.0006	0.0530	0.0550	96.5
Glycyl-aminobutyric acid.....	0.1007	0.0437	0.0030	0.0467	0.0500	93.4
Glycyl-valine.....	0.1012	0.0400	0.0062	0.0462	0.0462	100.0
Glycyl- <i>d</i> -valine.....	0.1015	0.0390	0.0059	0.0449	0.0464	96.8
Glycyl-leucine.....	0.1005	0.0380	0.0022	0.0402	0.0425	94.6
Glycyl- <i>l</i> -leucine.....	0.1006	0.0370	0.0036	0.0406	0.0425	95.5
Glycyl-amino - <i>n</i> - cap- roic acid.....	0.0963	0.0377	0.0014	0.0391	0.0407	96.1
Glycyl-asparagine.....	0.1028	0.0397	0.0031	0.0428	0.0433	98.8
Glycyl-phenylglycine..	0.1021	0.0355	0.0030	0.0385	0.0390	98.7
Glycyl- <i>d</i> -phenylglycine	0.1022	0.0358	0.0010	0.0368	0.0391	94.1
Glycyl-tyrosine.....	0.0545	0.0142	0.0043	0.0185	0.0181	102.2
Glycyl-tryptophane....	0.1016	0.0257	0.0010	0.0267	0.0310	86.1
Alanyl-glycine.....	0.1007	0.0516	0.0032	0.0548	0.0548	100.0
<i>l</i> -Alanyl-glycine.....	0.1005	0.0496	0.0054	0.0550	0.0547	100.5
<i>d</i> -Alanyl- <i>d</i> -alanine....	0.1001	0.0465	0.0041	0.0506	0.0497	101.8
Aminobuteryl-glycine..	0.1010	0.0467	0.0045	0.0512	0.0502	102.0
Valyl-glycine.....	0.1005	0.0410	0.0059	0.0469	0.0459	102.2
Leucyl-glycine.....	0.0901	0.0344	0.0041	0.0385	0.0381	101.0
Leucyl-leucine.....	0.1006	0.0288	0.0014	0.0302	0.0328	92.1
<i>l</i> -Leucyl- <i>d</i> -leucine....	0.1004	0.0266	0.0021	0.0287	0.0327	87.8
<i>d</i> -Leucyl- <i>l</i> -leucine....	0.1011	0.0268	0.0003	0.0271	0.0330	82.1
<i>d</i> -Leucyl- <i>d</i> -leucine....	0.1007	0.0294	0.0013	0.3007	0.0328	93.6
Amino- <i>n</i> -caproic - gly- cine.....	0.0664	0.0257	0.0015	0.0272	0.0281	96.8
Leucyl-asparagine.....	0.1004	0.0205	0.0010	0.0215	0.0326	66.0



*Tripeptides.*<sup>24</sup>

SUBSTANCE	WEIGHT OF SAMPLE	CuO IN FILTRATE AFTER ADDING 3 CC. N. NaOH (CO <sub>2</sub> FREE) AND BOIL- ING		CuO IN PRECIPITATE AF- TER ADDING 3 CC. N. NaOH (CO <sub>2</sub> FREE) AND BOILING		TOTAL CuO	THEORETICAL WEIGHT CuO CALCULATED FOR THIS SAMPLE	CuO IN FILTRATE AS PERCENTAGE OF THEO- RETICAL	TOTAL CuO AS PERCENT- AGE OF THEORETICAL
	grams	grams	grams	grams	grams	grams	per cent	per cent	
Glycyl-glycyl-glycine.....	0.1003	0.0370	0.0006	0.0376	0.0422	0.0422	87.7	89.1	
Glycyl-glycyl-alanine.....	0.1021	0.0356	0.0064	0.0420	0.0400	0.0400	89.0	105.0	
Glycyl-glycyl-aminobutyric acid.....	0.1011	0.0382	0.0006	0.0388	0.0371	0.0371	103.0	104.6	
Glycyl-glycyl-valine.....	0.0516	0.0155	0.0005	0.0160	0.0178	0.0178	87.1	89.9	
Glycyl-glycyl-leucine.....	0.1010	0.0279	0.0044	0.0323	0.0328	0.0328	86.4	98.5	
Glycyl-glycyl- <i>l</i> -leucine.....	0.1020	0.0287	0.0024	0.0311	0.0331	0.0331	86.7	94.0	
Glycyl- <i>d</i> -alanyl- <i>d</i> -alanine.....	0.0536	0.0144	0.0001	0.0145	0.0192	0.0192	75.0	75.5	
Glycyl-leucyl-glycine.....	0.1013	0.0293	0.0004	0.0297	0.0329	0.0329	88.5	90.3	
Alanyl-glycyl-glycine.....	0.0987	0.0254	0.0031	0.0285	0.0387	0.0387	65.6	73.6	
<i>d</i> -Alanyl-glycyl-glycine.....	0.0492	0.0168	0.0014	0.0182	0.0193	0.0193	87.0	94.3	
Alanyl-leucyl-glycine.....	0.1012	0.0274	0.0037	0.0311	0.0311	0.0311	88.1	100.0	
Amino-butyryl-glycyl-glycine.....	0.1011	0.0350	0.0028	0.0378	0.0371	0.0371	94.3	101.9	
Valyl-glycyl-glycine.....	0.1023	0.0288	0.0027	0.0315	0.0352	0.0352	81.8	89.5	
Leucyl-glycyl-glycine.....	0.1013	0.0283	0.0057	0.0340	0.0329	0.0329	86.1	103.3	
<i>l</i> -Leucyl-glycyl-glycine.....	0.1011	0.0284	0.0043	0.0327	0.0328	0.0328	86.6	99.7	
<i>l</i> -Leucyl-glycyl- <i>d</i> -alanine.....	0.0569	0.0161	0.0008	0.0169	0.0175	0.0175	92.0	96.6	
Leucyl-alanyl-glycine.....	0.1002	0.0289	0.0040	0.0329	0.0308	0.0308	93.8	106.8	
Leucyl-alanyl-alanine.....	0.1017	0.0272	0.0050	0.0322	0.0296	0.0296	91.9	108.8	
<i>l</i> -Leucyl- <i>d</i> -alanyl- <i>d</i> -alanine.....	0.1014	0.0262	0.0033	0.0295	0.0295	0.0295	88.8	100.0	
Amino- <i>n</i> -caproyl-glycyl- glycine.....	0.1003	0.0304	0.0035	0.0339	0.0325	0.0325	93.5	104.3	

<sup>24</sup> The majority of the peptides used in this study were prepared or collected by the late Dr. Arthur H. Koelker. A small number (four dipeptides and three tripeptides) were made according to Fischer's directions, by Dr. H. Hager and ourselves, in this laboratory. Glycyl-tryptophane was obtained in very pure crystalline form through the kindness of Kalle and Co.

*Tetrapeptides.*

SUBSTANCE	WEIGHT OF SAMPLE		CuO IN FILTRATE AFTER ADDING 3 CC. N NaOH (CO <sub>2</sub> FREE) AND BOIL- ING	CuO IN PRECIPITATE AF- TER ADDING 3 CC. N NaOH (CO <sub>2</sub> FREE) AND BOILING	TOTAL CuO	THEORETICAL WEIGHT CuO CALCULATED FOR THIS SAMPLE	CuO IN FILTRATE AS PERCENTAGE OF THEO- RETICAL	TOTAL CuO AS PERCENT- AGE OF THEORETICAL
	grams	grams	grams	grams	grams	per cent	per cent	
Alanyl-diglycyl-glycine.....	0.1008	0.0267	0.0017	0.0284	0.0308	86.7	92.2	
Aminobuteryl-diglycyl-glyc- ine.....	0.1050	0.0260	0.0037	0.0297	0.0305	85.2	94.1	
Leucyl-diglycyl-glycine*.....	0.1008	0.0239	0.0030	0.0269	0.0265	90.2	101.5	
<i>n</i> -Amino-caproyl-diglycyl- glycine.....	0.1017	0.0202	0.0005	0.0207	0.0268	75.4	77.2	

\* Filtered without boiling to decompose carbamino salts.

Using our same technique it was of interest to see the amount of copper hydroxide dissolved by peptones.

*Peptones, etc.*

SUBSTANCE	WEIGHT OF SAMPLE		CuO IN FILTRATE AFTER ADDING 3 CC. N NAOH (CO <sub>2</sub> FREE) AND BOIL- ING	CuO IN PRECIPITATE AF- TER ADDING 3 CC. N NAOH (CO <sub>2</sub> FREE) AND BOILING	MOLECULAR WEIGHT* CAL- CULATED FROM TOTAL CuO
	grams	grams	grams	grams	
"Roche" peptone.....	0.1110	0.0302	0.0173		186
"Ereptone".....	0.1005	0.0064	0.0133		406
"Merck" peptone.....	0.1020	0.0044	0.0002		1765
"Witte's" peptone.....	0.1025	0.0041	0.0000		1990
"Witte's" peptone.....	0.1046	0.0042	0.0000		1982

\*This calculation is based on the assumption that one molecule of peptone, as in the case of the peptides, combined with only one molecule of copper hydroxide.

The results on the peptides show unmistakably that one molecule of peptide, whatever number of amino-acids it may contain, combines with only one molecule of copper hydroxide. This interest-

ing fact will no doubt give us an easy method of determining the molecular weights of peptides, provided the copper salts can be separated from the excess copper hydroxide.

The questions that remain to be solved, namely, how are these copper salts formed and what is their structure, will be taken up in a separate paper,<sup>25</sup> in conjunction with the biuret configuration.

#### SUMMARY.

1. We have developed a technique for making, quantitatively, copper salts of (a) soluble amino-acids and peptides; (b) insoluble amino-acids having soluble copper salts; (c) insoluble amino-acids having insoluble copper salts.

2. We have formed copper salts of twenty-six dipeptides, twenty tripeptides and four tetrapeptides, quantitatively, in solution, and have found the results to be consistent with the formula, (peptide)<sub>1</sub>Cu. This supports the work of Fischer and Abderhalden on five isolated salts.

3. We have found: (a) That on an average 99 per cent of the copper of all amino-acid salts (except that of histidine) is precipitated as oxide when treated with a certain excess of alkali.<sup>26</sup>

(b) That on an average 6.4 per cent of the copper of dipeptide salts is precipitated as oxide with the same excess of alkali.

(c) That on an average 6.3 per cent of the copper of tripeptide<sup>27</sup> salts is precipitated as oxide with the same excess of alkali.

(d) That on an average 7.3 per cent of the copper of tetrapeptide salts is precipitated as oxide with the same excess of alkali.

Our thanks are due to Dr. Wm. G. Lyle for his encouragement in this work, and for placing at our disposal the excellent collection of peptides and amino-acids belonging to the late Dr. A. H. Koelker, and to Miss Calm M. Hoke for much assistance in preparing this article for publication.

<sup>25</sup> *Amer. Chem. Journ.*, Nov., 1912.

<sup>26</sup> Using 3-5 cc. N CO<sub>2</sub>-free NaOH for every 0.1 gram substance and boiling.

<sup>27</sup> Assuming all the substances to be perfectly pure, which of course they are not. It is probable that perfectly pure tri- and tetra-peptides will not precipitate any of the copper of their salts under the conditions given above. We are convinced that impurities will account for most of the precipitation.

## Peptide Copper Complexes

## EXPERIMENTAL NOTES.

*a. Copper oxide as a reagent.*

In our first work on copper salts we used black copper oxide obtained in the open market as a reagent, but found that with peptides it reacted too slowly. Then we tried the commercial basic carbonate, and found it more useful, but not quite satisfactory. *Freshly prepared copper hydroxide, however, was found not only efficient but almost instantaneous in its reaction at 0°C.* The amount of surface is, of course, a factor in the reaction, and therefore a gelatinous precipitate is, other things being equal, preferable to a coarse granular precipitate.

As Fischer and Abderhalden used suspended copper oxide in making their salts, it was necessary to investigate the relative efficiency of this reagent on various amino-acids and peptides *when boiled*.

The following table gives the results with *freshly made copper hydroxide* and *freshly made copper oxide suspensions on boiling*. The latter were made by allowing a suspension of the hydroxide to stand several days in the laboratory, during which time it changed its color from blue to almost black.

SUBSTANCE	WEIGHT OF SAMPLE	CuO DISSOLVED AFTER BOILING 10 MINUTES WITH Cu (OH) <sub>2</sub>	CuO DISSOLVED AFTER BOILING 10 MINUTES WITH CuO	THEORETICAL WEIGHT OF CuO	CuO AS FOUND TO THEO- RETICAL
	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>per cent</i>
Glycine.....	0.1011	0.0564		0.0536	105.2
Glycine.....	0.1016		0.0558	0.0539	103.5
Alanine.....	0.1034	0.0482		0.0462	104.3
Alanine.....	0.1021		0.0472	0.0456	103.5
Alanyl-glycine.....	0.1013	0.0562		0.0551	103.0
Alanyl-glycine.....	0.1001		0.0515	0.0547	94.5
Leucyl-glycine.....	0.1023	0.0435		0.0433	100.5
Leucyl-glycine.....	0.1015		0.0435	0.0429	101.4
Alanyl-leucyl-glycine.....	0.1004	0.0210		0.0308	68.2
Alanyl-leucyl-glycine.....	0.1010		0.0137	0.0310	44.2
Glycyl-leucyl-glycine.....	0.1011	0.0242		0.0328	73.8
Glycyl-leucyl-glycine.....	0.1003		0.0150	0.0325	46.2
Alanyl-di-glycyl-glycine..	0.1003		0.0195	0.0307	63.2

These results show that boiling, while suitable for amino-acids and dipeptides, cannot be used with tri- and tetra-peptides, and will easily account for the abnormal results obtained by Abderhalden and Hirsch.<sup>28</sup>

*b. Bicarbonates as solvents for copper hydroxide.*

When cystine, tryptophane, leucine and other amino-acids, whose copper salts are insoluble, are treated as in method *c* (see p. 4), the copper salts are found almost wholly<sup>29</sup> in the precipitate, mixed with the excess copper hydroxide.

In order to show the effect of different concentrations of the bicarbonate on the solubility of copper hydroxide and the "insoluble" copper salts, the following preliminary experiments were made.

Pure copper salts of the amino-acids were made as described under *c* and a small portion (sufficient to leave a slight excess undissolved) was stirred constantly for five minutes in 25 cc. of 5 per cent, 10 per cent and 20 per cent potassium bicarbonate; controls were made on 25 cc. of distilled water.<sup>30</sup>

SUBSTANCE	CuO DISSOLVED IN 25 CC. H <sub>2</sub> O	CuO DISSOLVED IN 25 CC. 5 PER CENT KHCO <sub>3</sub>	CuO DISSOLVED IN 25 CC. 10 PER CENT KHCO <sub>3</sub>	CuO DISSOLVED IN 25 CC. 10 PER CENT NAHCO <sub>3</sub>	CuO DISSOLVED IN 25 CC. 20 PER CENT KHCO <sub>3</sub>
	grams	grams	grams	grams	grams
Copper hydroxide.....	0.00000	0.0283	0.0580	0.0565	0.1680
Leucine, copper.....	0.00020	0.0006	0.0008		0.0015
<i>n</i> -Aminocaproic acid, copper.....	0.00003	0.0007	0.0016	0.0014	0.0060
Phenylglycine, copper.....	0.00003	0.0006	0.0012	0.0013	0.0056
Tryptophane, copper.....	0.00020	0.0004	0.0004	0.0005	0.0007
Cystine, copper.....	0.00000	0.0000	0.0000	0.0000	0.0001

<sup>28</sup> *Loc. cit.*

<sup>29</sup> When less than 0.1 gram of tryptophane is used, the copper salt remains in a supersaturated condition in the filtrate and later crystallizes out.

<sup>30</sup> Only one concentration of sodium bicarbonate was used; the potassium bicarbonate, on account of its greater solubility, is preferable.