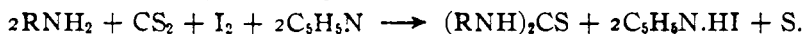
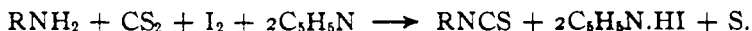


The second method employs calculated quantities of iodine and pyridine in a carbon bisulfide solution of the amine. The reaction has been shown to proceed according to the equation:



This latter method is especially noteworthy since the yields of thiocarbanilides actually obtained run from 75 to 99% of the theoretical yields.

A quantitative method for the determination of aniline (dissolved in carbon bisulfide and pyridine) by titration with a standard solution of iodine is also described. The reaction proceeds according to the equation:



The failure to obtain thiocarbanilides from *o*-nitraniline, *p*-nitraniline and *o*-aminobenzoic acid, and an extension of the methods herein outlined to other amines, are subjects of investigation now in progress.

My grateful acknowledgments are due Mr. Edward M. James for the valuable assistance he has rendered in the experimental part of the present work.

CINCINNATI, OHIO.

[FROM THE HARRIMAN RESEARCH LABORATORY, ROOSEVELT HOSPITAL, NEW YORK.]

A MICRO-CHEMICAL METHOD FOR THE DETERMINATION OF α - AND β -AMINO ACIDS AND CERTAIN DERIVATIVES; IN PROTEOLYSIS, BLOOD AND URIN.

BY PHILIP ADOLPH KOBER AND KANEMATSU SUGIURA.

Received July 7, 1913.

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I. Introduction.

As is well known, the copper compounds of amino acids and their derivatives, in certain cases, have been used for their isolation, but the amount of cupric hydroxide or oxide dissolved has not been used heretofore as an index to the amount of amino acid nitrogen in solution.

E. Fischer¹ and G. Zemplén, in 1909, suggested such a possibility; writing on the failure of γ -, δ - or ϵ -amino acids to dissolve cupric oxide they state: "Es liegt nahe diese Beobachtung in Zukunft als diagnostische Mittel für die stellung der Amino-gruppe zu benutzen." It is also of

¹ Ber., 42, 4883 (1909).

interest, that Levene¹ and Heimrod, in 1907, at the Rockefeller Institute, made an attempt to utilize cupric oxide to estimate the amino carboxyl group but discontinued the work owing to certain difficulties: such as the quantitative formation of the copper complexes and the separation of insoluble amino acid copper complexes from an excess of the reagent, cupric oxide.

In our previous papers² we showed how these difficulties were overcome: (1) By using cold cupric hydroxide instead of boiling with cupric oxide, as was done heretofore, the formation of these complexes was practically quantitative; (2) by using a 20% solution of potassium bicarbonate, any excess of cupric hydroxide was dissolved, leaving the insoluble crystalline complex after filtering as a residue, which could be dissolved in a little acid and titrated directly, iodometrically.

Although the results were practically quantitative, yet the soluble complexes, as a rule, gave slightly higher than the theoretical amounts (from 5 to 15%). Three possible causes suggest themselves: (1) Carbamino salts are formed to a slight extent from carbon dioxide of the air and from reagents; (2) cupric chloride is adsorbed by the hydroxide in precipitating; (3) the combination of the amino copper complex with the excess cupric hydroxide, to form, to a slight extent, an unstable complex.

Boiling, as was suggested in a former paper, reduces this error considerably (1 to 5%) but does not do so entirely. This fact points to the carbamino reaction as the source of error, but on this point we have not arrived at a definite conclusion.

The object of this paper is to show how the "excess copper" can be eliminated and the α - and β -amino acids and certain of their derivatives can be estimated accurately. This can be done in an aqueous neutral solution at a concentration as low as 0.002% without either boiling or using a vigorous reagent. The determination can be made at room temperature or even in an ice mixture by simply adding a "buffer" solution with the cupric hydroxide, shaking, filtering and determining the dissolved cupric complex iodometrically.

2. The Basis of the Method.

The basis of the method is the property of amino acids and such derivatives as are designated below, to dissolve cupric hydroxide quantitatively to form a metallic complex in a neutral or slightly alkaline solution (concentration of H ions from $10^{-7.07}$ to $10^{-8.8}$). *The reaction, which is extremely rapid and sensitive, will give good results with 0.005% solution of amino acid and peptides, and becomes by far the most delicate reaction we have for these substances.*

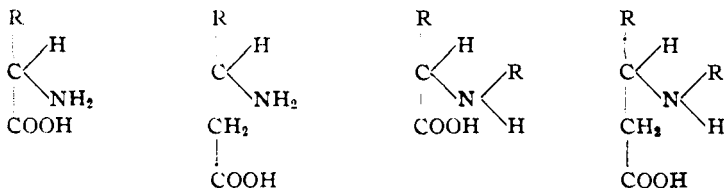
¹ Private communication.

² Kober and Sugiura, *J. Biol. Chem.*, 13, 1 (1912), and *Am. Chem. J.*, 48, 383 (1912).

The only apparent exception¹ (a substance which did not form a copper complex) was "leucyl-prolin," which Fischer² showed was not a peptide, but a hydroxyamide, and, therefore, we may be justified in believing that a substance which does not form a copper complex in neutral solution cannot be an α - or β -amino acid or any polypeptide of these amino acids. Since the iodometric titration of copper is one of the most sensitive and accurate titrations we have, the quantitative estimation of amino acid and polypeptides by means of these copper complexes becomes not only a simple, but also, a fairly accurate method.

3. The Applicability of the Method.

The method proposed in this paper is applicable only to those substances that have one of the following compositions:



where "R" represents any positive radical or group as H, CH₃-, C₂H₅-, an amino acid, or a combination of amino acids. This includes all the amino acids thus far obtained from proteins, all the polypeptides and the peptones.

Under the conditions given for this method, two molecules of all the amino acids thus far isolated from proteins, including α - and β -mono-amino, mono- and di-basic acids, and di-amino-mono-basic acids, dissolve one molecule of cupric hydroxide, while only one molecule of a di-amino di-basic acid reacts with one molecule of cupric hydroxide. The first class includes all the amino acids, except cystin, which is the only representative of the second class. All the peptides studied thus far, also form the "di-basic" complex, (peptide) CuOH (see table of precipitabilities).

Other similar substances like formyl, acetyl and benzoyl derivatives, of which hippuric acid is an example, do not react with the reagent, cupric hydroxide, to form in neutral or slightly alkaline solution the necessary metallic complexes, and therefore cannot interfere. The details of the configurations necessary, for the formation of a copper complex, were discussed in a previous paper³ and need not be repeated here.

4. The Correct Conditions for the Formation of Copper Complexes.

When solutions of amino acids and polypeptides are stirred with cupric hydroxide, the complexes are formed almost instantly, and, as we pointed

¹ E. Fischer and E. Abderhalden. *Ber.*, **37**, 3071 (1904).

² *Ann.*, **363**, 120 (1909).

³ Kober and Sugiura. *Am. Chem. J.*, **48**, 383 (1912).

out in the introduction, the amount is slightly more than the theoretical in each case. This "excess copper" is comparatively unstable and is removable by an excess of alkali or by boiling.

The first experiments to eliminate this "excess copper" were made with a slight excess of ammonia. The results indicate that at room temperature the ammonia forms with cupric hydroxide the well-known complex, thus making the results too high, while at boiling temperature, the ammonia not only throws down this "excess copper" but precipitates a small amount of amino acid copper, thus making the results too low. The right condition for ammonia might have been found, but as a method, it would not have been sufficiently flexible. Therefore we abandoned the work with ammonia.

The next step was the application of a "buffer" solution which would maintain a degree of alkalinity, sufficient to prevent the "excess copper," and which at the same time would not dissolve any cupric hydroxide. A mixture of di-sodium (9.65 parts of $N/15$ Na_2HPO_4) and mono-potassium (7 parts of $N/15$ KH_2PO_4) phosphate was found which gave theoretical results with glycine. But on trying it with other amino acids, the results were too low. These unexpected results seem to have been caused by the precipitation of insoluble phosphates, and while the concentration of the cupric ions of the glycine complex was too small to cause such precipitation, the concentration of cupric ions of the other amino acid complexes was large enough to cause considerable deviation from the theoretical results.

For this reason other "buffer" solutions were considered, it being necessary to find one which would not dissolve, precipitate, or prevent the copper from precipitating. We limited ourselves to those carefully worked out by Sørensen¹ which are: (1) citrate mixtures; (2) glycine mixtures; (3) borate mixtures.

Citrate mixtures were not tried as we thought they would act as alkaline tartrates (Fehling's solution) and would hinder the precipitation of copper with alkali. Moreover, Sørensen's results show that the concentration of hydrogen ions, at which the citrate mixtures act as "buffer" solutions are either too low or too high for our purpose. Glycine mixtures for obvious reasons, could not be used and therefore only the borate mixtures remained to be tested. These met the requirements; the desired alkalinity was easily obtained and the reagent did not interfere in any way, with subsequent operations.

5. The Estimation of Amino Acids in the Presence of their Derivatives, the Polypeptides.

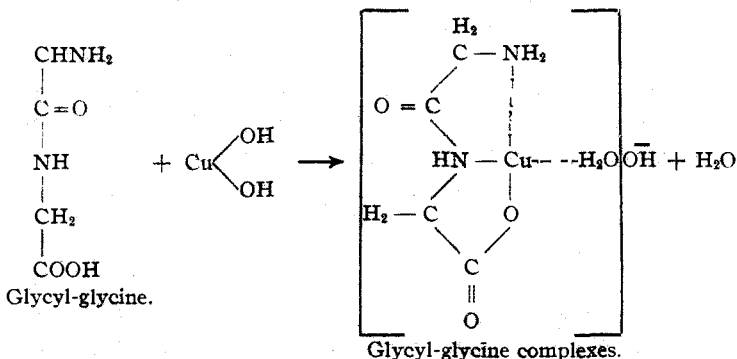
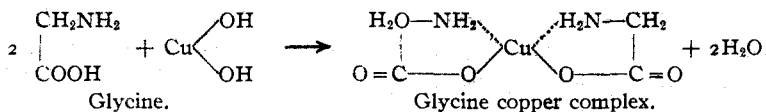
In a preliminary paper² one of us (K.) showed how the amino acids could be distinguished from the polypeptides by the difference in the be-

¹ *Biochem. Z.*, **21**, 175; **22**, 355.

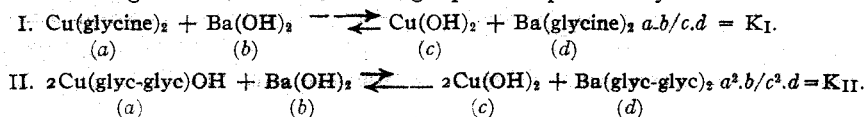
² *J. Biol. Chem.*, **10**, 9 (1911).

havior of their copper complexes on treatment with alkali. At that time the correct procedure for the formation of copper complexes of the peptides, quantitatively, had not been found and, therefore, the results given were slightly low and were, to some extent, accidental. When polypeptides are completely converted into copper complexes, a small amount of copper as hydroxide or oxide (depending on the temperature of the solution) may be precipitated by an excess of alkali, but, since the fraction precipitable is constant for a given concentration of polypeptides and a given amount of alkali, the results will not be appreciably affected. The same holds true in regard to the copper complexes of the amino acids, the reaction with alkali is not complete and a small but a definite fraction remains unprecipitated.

The reactions involved in the formation of the complexes and the precipitation with alkali will be better understood when given in a form of an equation.



Theoretically, these reactions should be reversible, but the reverse action is so small that for practical purposes they may be written as irreversible. On treating with alkali the following equilibria practically exist:



Therefore, in the determination of amino acids in the presence of their derivatives, the polypeptides, a factor must be employed, which we will call the "precipitability" of the substance and which is equivalent to the per cent. of the copper precipitated, as $\text{Cu}(\text{OH})_2$, from a given concentration of complex, when in equilibrium with 0.06 N $\text{Ba}(\text{OH})_2$.

Objections may be raised to the use of such equilibrium reactions in

analytical work but the fact that the polypeptide complexes have practically no, and the amino acid complexes a very small temperature coefficient, shows that these substances are unusually stable and in physiological work, will not be easily affected. Another point, is the fact that the equilibrium is quickly reached and that it is only dependent on the amount of free alkali, which can easily be kept constant. When the estimation is made with mixtures of amino acids and polypeptides, an average factor (amino acid factor = 86.5%, the polypeptide factor = 12.4%) will be necessary, but in studying the hydrolysis of a single peptide the use of a specific precipitability will enable us to make slightly more accurate estimations of the amount of free amino acids.

In the following tables of "precipitabilities" the second column gives the amount of substance taken. This was dissolved in 25 cc. of water to which was then added 25 cc. of our "amino acid reagent." After shaking at intervals for 10 minutes the mixture was filtered through a dry filter into a dry flask and 25 cc. of the filtrate was pipetted into a dry Erlenmeyer flask. Five cc. of 0.360 *N* Ba(OH)₂ were then added to the Erlenmeyer flask and after stoppering and shaking thoroughly the solution was allowed to stand for exactly 15 minutes. As was shown in the experimental part of the paper this is sufficient time for the reaction given above with barium hydroxide to come to an equilibrium. At the end of this time, the amount of cupric hydroxide precipitated by the barium was determined by filtering through a filter paper and titrating the residue, iodometrically. The figures given in the other columns therefore refer to one-half of the amount taken.

AMINO ACID COMPLEXES.

Total vol. = 50 cc. On adding 5 cc. of 0.360 *N* Ba(OH)₂ to 1/2 of filtrate.

Glycine.						
Amt. taken. Gram.	In filtrate found CuO. Gram.	In residue found CuO Gram.	Total CuO found for 1/2 of subst. Gram.	Theor. amt. (amino A) ₂ Cu ₁ .	Per cent. of total to theor.	"Precip- itability" per cent. of res. to total.
0.00104	0.000035	0.000227	0.000262	0.000276	94.8	86.7
0.00104	0.000045	0.000219	0.000264	0.000276	95.7	83.0
0.00517	0.000062	0.001300	0.001362	0.001372	99.4	95.4
0.01035	0.000139	0.00260	0.00274	0.00274	100.0	94.8
0.01563	0.000190	0.00395	0.00414	0.00414	100.0	95.5
0.02605	0.00079	0.00612	0.00691	0.00691	100.0	88.6
Average						91.2
Alanin.						
0.00101	0.000025	0.000193	0.000218	0.000225	96.9	88.4
0.00101	0.000023	0.000200	0.000223	0.000225	99.2	89.6
0.00101	0.000039	0.000185	0.000224	0.000225	99.5	(82.6)
0.00502	0.000060	0.00105	0.00111	0.00112	99.1	94.6
0.01009	0.00011	0.00210	0.00221	0.00224	98.7	94.8
0.01510	0.00013	0.00321	0.00334	0.00337	99.2	96.1
0.02517	0.00037	0.00520	0.00557	0.00562	99.2	93.3
Average						91.2

Amt. taken. Gram.	Per cent. of total to theor.	"Precip- itability" per cent. of res. to total.	Amt. taken. Gram.	Per cent. of total to theor.	"Precip- itability" per cent. of res. to total.
Amino butyric.			Phenylalanine.		
0.00101	94.8	76.7	0.00102	101.7	78.3
0.00514	100.0	95.2	0.00510	100.0	93.4
0.01009	99.0	96.4	0.01010	99.2	95.8
0.02598	100.0	95.7			
			Average 89.1		
Average 91.0			Tyrosine.		
Valine.			0.00101	90.8	90.0
0.000964	101.8	91.0	0.00505	101.8	(80.0)
0.004820	99.8	92.6	0.01010	100.0	91.8
0.009640	99.3	96.2	0.01503	98.2	94.3
0.015600	100.0	96.2	0.02505	97.5	89.6
0.026000	100.0	93.2			
			Average 89.1		
Average 93.8			Aspartic acid.		
Isoleucine.			0.00101	93.3	78.5
0.000394	100.0	83.0	0.00503	100.0	74.7
0.00197	105.0	90.5	0.01007	100.5	80.3
0.00985	100.0	95.2	0.01516	100.9	87.7
			0.02527	101.8	72.4
			Average 78.7		
Average 89.3					

INSOLUBLE COMPLEXES.

Phenyl-glycine.

Amount taken. Gram.	CuO found in residue. Gram.	Solubility in KHCO_3 . Gram.	CuO in filtrate. Gram.	Total CuO found. Gram.	Theor. CuO. Gram.	Ratio of CuO found to theor. Per cent.	Precip- itability.
0.01012	0.00270	0.00001	0.00000	0.00271	0.00266	101.9	100.0
0.01012	0.00264	0.00001	0.00000	0.00265	0.00266	99.6	100.0
0.02530	0.00660	0.00001	0.00000	0.00661	0.00665	99.3	100.0

N-Amino caproic acid.

0.01013	0.00263	0.00038	0.00000	0.00301	0.00308	97.8	100.0
0.01013	0.00277	0.00038	0.00000	0.00315	0.00308	102.3	100.0
0.01013	0.00271	0.00038	0.00000	0.00309	0.00309	100.0	100.0
0.02533	0.00729	0.00038	(0.00057)	0.00767	0.00770	99.6	100.0

Leucine.

0.001010	0.000264	0.000264	0.000309	85.5	93.0
0.001010	0.000269	0.000269	0.000309	87.1	93.0
0.001010	0.000269	0.000269	0.000309	87.1	93.0
0.001010	0.000283	0.000283	0.000309	91.6	93.0
0.001010	0.000267	0.000267	0.000309	87.1	93.0
0.001010	0.000600	0.000600	0.000618	97.0	93.0
0.001010	0.000568	0.000568	0.000618	91.8	93.0

Cystine.

0.01008	0.00192	0.00083	0.00067	0.00342	0.00334	102.4	100.0
0.01009	0.00197	0.00083	0.00074	0.00354	0.00334	106.0	100.0
0.01512	0.00329	0.00083	0.00089	0.00501	0.00500	100.2	100.0
0.01514	0.00330	0.00083	0.00073	0.00486	0.00501	97.0	100.0
0.02523	0.00663	0.00083	0.00076	0.00822	0.00835	98.3	100.0

Amount taken. Gram.	Per cent. of found to theor.	Precipitability. Per cent.	Amount taken. Gram.	Per cent. of found to theor.	Precipitability. Per cent.
Glutamic acid.			Arginine dinitrate.		
0.00102	101.3	77.2	0.00513	(79.3)	88.9
0.00509	102.0	90.1	0.01027	91.0	90.4
0.01027	100.5	97.2			Average 89.6
0.01528	102.0	96.7			
0.02547	100.4	96.3			
		Average 91.5	Asparagine + H ₂ O (Merck's)		
			0.00506	100.0	70.2
			0.01013	100.0	59.6
					Average 64.9
Proline.			Asparagine + H ₂ O (E. & A.)		
0.00078	87.3	92.2	0.00101	90.3	87.5
0.00776	99.5	62.9	0.00506	95.4	70.3
0.01163	99.5	52.7	0.01013	100.6	56.2
		Average 69.2	0.01520	101.8	48.7
			0.02532	99.6	44.3
					Average 61.2
Tryptophane.			Histidine for (Hist) ₃ Cu ₂ Di.HCl.		
0.00082	96.3	100.0	0.00106	40.2	93.8
0.00411	99.4	81.2	0.00529	100.0	57.7
0.00822	0.01058	100.0	42.8
		Average 90.6	0.01588	100.0	37.1
			0.02664	99.0	24.8
					Average 51.2
Lysine picrate.					
0.00100	100.0	60.0			
0.00501	96.0	75.6			
0.01002	(90.0)	77.4			
0.02505	94.5	84.0			
		Average 74.3			

DIPEPTIDES.

Alanyl-glycine.

Amount taken. Gram.	CuO found in filtrate. Gram.	CuO found in residue. Gram.	Total CuO found. Gram.	Theor. amount of CuO. Gram.	Ratio of CuO found to theor. Per cent.	Precip- itability. Per cent.
0.001020	0.000178	0.000096	0.000274	0.000278	98.6	35.0
0.002040	0.000371	0.000173	0.000544	0.000556	97.8	31.8
0.003060	0.000599	0.000219	0.000818	0.000834	98.1	26.8
0.004080	0.000838	0.000252	0.001090	0.001112	98.0	23.1
0.005100	0.001023	0.000345	0.001368	0.001390	98.4	25.2
0.00765	0.00157	0.00048	0.00205	0.00208	98.6	23.4
0.01020	0.00218	0.00055	0.00273	0.00278	98.3	20.1
0.01530	0.00355	0.00058	0.00413	0.00417	99.0	14.1
0.02550	0.00619	0.00064	0.00683	0.00695	98.3	9.4
					Average 23.2	

Amount taken.	Per cent. of found to theor.	Precipitability.	Amount taken.	Per cent. of found to theor.	Precipitability.
<i>l</i> -Alanyl-glycine.			<i>Aminobutyryl</i> -glycine.		
0.001007	100.4	38.5	0.001017	101.2	42.2
0.005035	100.5	14.1	0.005085	100.1	27.9
0.02518	98.1	9.4	0.02543	97.9	11.3
Average 20.6			Average 27.1		
<i>d</i> -Alanyl- <i>d</i> -alanine.			<i>d</i> - <i>l</i> -Amino- <i>n</i> -caproic acid-glycine.		
0.001016	104.8	21.2	0.001007	102.3	21.1
0.005080	99.8	13.3	0.005035	98.5	14.6
0.02540	96.2	7.6	0.02518	97.3	7.5
Average 14.0			Average 14.4		

Glycyl-glycine.

Amount taken. Gram.	CuO found in filtrate. Gram.	CuO found in residue. Gram.	Total CuO found. Gram.	Theor. amount of CuO. Gram.	Ratio of CuO found to theor. Per cent.	Precipitability. Per cent.
0.001076	0.000230	0.000092	0.000322	0.000324	99.4	28.6
0.002152	0.000404	0.000240	0.000644	0.000648	99.4	37.3
0.003228	0.000665	0.000294	0.000959	0.000972	98.7	30.6
0.004304	0.000922	0.000349	0.001271	0.001296	98.1	27.5
0.005380	0.001249	0.000378	0.001627	0.001619	100.5	23.2
0.00807	0.00201	0.00043	0.00244	0.00243	100.4	17.8
0.01076	0.00260	0.00061	0.00321	0.00324	99.1	19.0
0.01614	0.00396	0.00083	0.00479	0.00486	98.6	17.3
0.02690	0.00698	0.0094	0.00792	0.00810	97.8	11.9
Average 23.7						

Amount taken.	Per cent. of found to theor.	Precipitability.	Amount taken.	Per cent. of found to theor.	Precipitability.
<i>Glycyl-d</i> -alanine.			<i>Glycyl-d</i> -valine.		
0.001011	104.7	32.6	0.001011	97.8	18.6
0.005055	97.6	18.6	0.005055	96.4	10.8
0.02528	96.7	7.7	0.02528	93.6	8.3
Average 19.6			Average 12.6		
<i>Glycyl-r</i> -alanine.			<i>Glycyl-l</i> -leucine.		
0.001013	102.5	29.3	0.001015	99.5	27.6
0.005065	98.1	25.8	0.005075	99.9	21.7
0.01013	99.6	20.4	0.02538	96.8	13.8
0.02533	97.2	12.9	Average 21.0		
Average 22.1			<i>Glycyl</i> -leucine.		
0.001028	104.7	20.7	0.001008	103.3	24.5
0.005140	99.2	15.5	0.005040	101.0	24.2
0.02570	96.4	14.1	0.02520	95.5	19.4
Average 16.8			Average 22.7		

Amount taken.	Per cent. of found to theor.	Precipitability.
Glycyl-tyrosine.		
0.001022	107.1	21.4
0.005110	100.2	23.8
0.01022	101.2	21.5
0.01533	100.0	26.0
0.02555	99.8	24.5

Average 23.4

Glycyl- <i>l</i> -tyrosine.		
0.001069	99.4	20.0
0.005345	95.3	19.1
0.02673	92.4	12.9

Average 17.3

Glycyl-tryptophane.		
0.001380	101.4	18.8
0.006900	93.4	15.1
0.003450	91.1	7.3

Average 13.7

Glycyl-phenylglycine.		
0.001025	100.0	26.5
0.005125	99.9	18.9
0.02563	95.0	8.4

Average 17.9

Glycyl-asparagine.		
0.001024	101.4	10.5
0.005120	97.6	9.2
0.02560	95.7	5.0

Average 8.2

Glycyl-aminobutyric acid.		
0.001003	101.2	25.4
0.005015	99.0	20.1
0.02508	94.2	10.1

Average 18.5

Glycyl-amino- <i>n</i> -caproic acid.		
0.001012	104.2	20.2
0.005060	98.4	21.6
0.02530	96.1	10.1

Average 17.3

Amount taken.	Per cent. of found to theor.	Precipitability.
Glycyl- <i>d-l</i> -amino- <i>n</i> -caproic acid.		
0.001038	100.9	23.4
0.005190	97.4	15.4
0.02595	96.9	9.4

Average 16.1

Leucyl-glycine.		
0.001055	102.2	32.0
0.005275	99.4	23.1
0.01055	99.6	25.7
0.02638	99.6	9.9

Average 22.7

<i>d</i> -Leucyl- <i>d</i> -leucine.		
0.001007	103.0	21.3
0.005035	94.5	13.3
0.02518	92.0	5.6

Average 13.4

<i>d</i> -Leucyl- <i>l</i> -leucine.		
0.001011	100.0	19.4
0.005055	93.1	10.6
0.02528	90.8	7.8

Average 12.6

Leucyl-leucine.		
0.001021	99.4	16.4
0.005105	99.8	11.8
0.02553	97.3	6.5

Average 11.6

Leucyl-aspartic acid + H ₂ O		
0.001022	103.9	18.1
0.005110	96.4	11.7
0.02555	96.4	8.9

Average 12.9

Leucyl-asparagine + 2H ₂ O.		
0.001035	78.8	9.6
0.005175	74.2	9.0
0.02588	73.8	6.7

Average 8.4

<i>r</i> -Valyl-glycine.		
0.001018	98.7	33.0
0.005090	98.5	25.7
0.02545	96.6	10.9

Average 23.2

TRI-PEPTIDES.

Amount taken, Gram.	Ratio of CuO found to theor. Per cent.	Precipita- bility, Per cent.	Amount taken, Gram.	Ratio of CuO found to theor. Per cent.	Precipita- bility, Per cent.
<i>r</i> -Alanyl-glycyl-glycine.			Glycyl-glycyl-L-leucine.		
0.001012	108.6	12.6	0.001017	95.2	22.9
0.005060	95.1	5.5	0.005085	94.2	18.5
0.02530	96.2	3.6	0.02543	92.3	8.1
Average 7.2			Average 16.5		
Alanyl-leucyl-glycine.			Glycyl-glycyl-alanine.		
0.001030	98.7	11.5	0.001013	106.0	28.4
0.005150	96.3	7.5	0.005065	98.8	16.9
0.02575	96.2	4.7	0.02533	96.0	5.7
Average 7.9			Average 17.0		
Amino-butyryl-glycylglycine.			Glycyl-glycyl-valine.		
0.001009	108.1	15.5	0.001060	105.5	21.4
0.005045	100.0	9.2	0.005300	99.2	21.0
0.02523	98.1	3.5	0.02650	97.8	9.6
Average 9.4			Average 17.3		
Amino- <i>n</i> -caproyl-glycyl-glycine.			<i>r</i> -Glycyl-amino-butyryl-glycine.		
0.001023	101.8	13.0	0.001018	102.1	17.3
0.005115	100.1	7.8	0.005090	101.6	10.6
0.02558	97.6	4.9	0.02545	98.1	4.6
Average 8.6			Average 10.8		
Glycyl-leucyl-glycine.			<i>r</i> -Glycyl-alanyl-glycine.		
0.001027	104.2	9.2	0.001008	101.5	12.9
0.005135	98.2	8.3	0.005040	101.5	7.4
0.02568	96.2	4.5	0.02520	102.0	3.4
Average 7.3			Average 7.9		
Glycyl-glycyl-leucine.			Glycyl-glycyl-amino-butyric acid.		
0.001036	95.8	25.2	0.001014	109.1	20.2
0.005180	91.8	22.1	0.005070	108.5	19.5
0.02590	90.7	9.9	0.02535	105.4	14.7
Average 19.1			Average 18.1		
Glycyl-glycyl-glycine.			Leucyl-alanyl-glycine.		
0.001010	93.9	17.5	0.001010	98.1	0.0
0.005050	86.0	10.5	0.005050	97.4	5.3
0.02525	86.5	4.8	0.02525	99.7	3.1
Average 10.9			Average 2.8		

Amount taken. Gram.	Ratio of CuO found to theor. Per cent.	Precipita- bility. Per cent.	Amount taken. Gram.	Ratio of CuO found to theor. Per cent.	Precipita- bility. Per cent.
<i>l</i> -Leucyl-glycyl- <i>d</i> -alanine.			<i>l</i> -Leucyl- <i>d</i> -alanyl- <i>d</i> -alanine.		
0.001038	110.1	14.9	0.001006	103.4	13.9
0.005190	101.0	7.7	0.005030	99.9	9.2
0.02595	98.7	4.8	0.02515	97.8	5.3
Average		9.1	Average		9.5
<i>l</i> -Leucyl-glycyl-glycine.			Valyl-glycyl-glycine.		
0.001070	103.4	16.7	0.001007	97.9	7.9
0.005350	99.7	6.7	0.005035	84.3	6.8
0.02675	95.9	4.1	0.02518	81.5	4.2
Average		9.2	Average		6.3

TETRAPEPTIDES.

Amount taken	Per cent. of found to theor.	Precipita- bility.	Amount taken.	Per cent. of found to theor.	Precipita- bility.
<i>r</i> -Alanyl-diglycyl-glycine.			<i>n</i> -Amino-caproyl-diglycyl-glycine.		
0.001023	94.9	13.4	0.001022	76.9	6.8
0.005115	90.5	11.0	0.005110	70.9	9.2
0.02558	89.8	7.7	0.02555	69.3	4.7
Average		10.7	Average		6.9
<i>r</i> -Aminobutyryl-diglycyl-glycine.			Leucyl-diglycyl-glycine.		
0.001024	98.6	14.4	0.001018	97.0	12.3
0.005120	95.8	9.8	0.005090	97.0	9.2
0.02560	92.2	6.7	0.02545	95.6	5.0
Average		10.3	Average		8.8

6. Solutions and Reagents.

(a) To make cupric hydroxide for this method, the following procedure is recommended. Pure cupric chloride is dried to the anhydrous form, by heating it on a steam bath for 24 hours at 100°. Ten grams of anhydrous cupric chloride, dissolved in sufficient water to make 200 cc. gives a 5% stock solution. Ten to twenty-five cc. of the cupric chloride solution, depending upon the number estimations to be made, are then diluted with ten volumes of chopped ice and water and after adding three or four drops of a 0.1% phenolphthalein indicator, the solution is neutralized with normal (CO₂ free) sodium hydroxide or with a saturated solution of barium hydroxide, slowly and with continuous shaking, until the color of the mixture begins to take a purple shade. Then 1 cc. of the same alkali is added in excess. More than this excess of alkali, delays the subsequent washing of the cupric hydroxide. The blue gelatinous cupric hydroxide is filtered, the ice being placed on the filter paper in order to keep the hydroxide cool and after washing two or three times, or until the wash

water shows no alkaline reaction with phenolphthalein, the ice is removed and the cupric hydroxide is ready for use. Barium hydroxide is used, in preference to sodium or potassium hydroxide, owing to the fact that its insoluble carbonate cannot dissolve any cupric hydroxide as a carbonate complex and also because in the subsequent iodometric titration, barium acetate does not retard the reaction nearly as much as sodium or potassium acetate. Merck's blue label reagent (3.3% solution) is well adapted for the purpose but good results can be obtained with the average C. P. material on the market.

(b) *In making the "buffer" solution for this method*, the following procedure is recommended: The sodium hydroxide solution is made free from carbonate, according to the suggestion of Sørensen, as follows: 250 grams of sodium hydroxide are dissolved into 250 cc. of water and placed in a flask furnished with a soda-lime tube. After cooling and allowing the solution to settle, the clear, saturated supernatant solution which contains no carbonate—sodium carbonate being insoluble in saturated sodium hydroxide—is diluted with sufficient boiled distilled water¹ to make a normal solution. 0.2 gram-molecules of boric acid (12.404 grams) are dissolved with distilled water and 100 cc. of normal sodium hydroxide and made up to a liter. This solution is designated as "sodium borate" and, if kept free from carbon dioxide, can be preserved indefinitely. Three volumes of "sodium borate" mixed with one volume of 0.1 *N* hydrochloric acid, we have found thus far to be a suitable "buffer" solution. This has a hydrogen concentration of about $10^{-8.8}$ and has about the same alkalinity as a solution of 0.00001 *N* sodium hydroxide. As a check on these solutions 25 cc. of 0.1 *N* hydrochloric acid solution with two drops of 0.1 sodium sulfo-alizirinate, should require not more than 25.06 of "sodium borate" solution and not more than 50.06 cc. of "buffer" solution for neutralization.

(c) *For Iodometric Solutions*.—The directions for making and for standardizing the iodometric solutions necessary for this work, can be found in any standard text-book on quantitative analysis and it suffices to mention simply the solutions and reagents required:

(1) Glacial acetic acid.

(2) 0.004 *N* sodium thiosulfate solution; 0.632 gram $\text{Na}_2\text{S}_2\text{O}_3$ or 0.993 gram $\text{Na}_2\text{S}_2\text{O}_3 + 5\text{H}_2\text{O}$ in a liter of carbon-dioxide-free water. This solution should be kept in a dark colored bottle to which is attached a soda-lime tube. For very small amount of substances a 0.001 *N* solution, made by diluting the 0.004 *N* solution with three volumes of water should be used. It is best to make this very weak solution from the stronger, just before using.

¹ As a rule ordinary distilled water, in all these solutions is suitable, but boiled distilled water is preferable.

(3) Solution of the starch, made from "soluble starch."

(4) Potassium iodide, crystals, C. P.

Since potassium iodide, as a rule, gives enough iodine with acetic acid to make an appreciable correction necessary, we have found it convenient to make a solution of potassium iodide, sufficient for a given series of titrations, with starch solution and acetic acid; titrating just before using with 0.004 *N* thiosulfate solution until the blue color of the starch is discharged. On standing, a subsequent titration is sometimes necessary, but if the solution is kept free from iodine in this way, no corrections for reagents are necessary in the final results. An example will help to make this clear. For 14 titrations we made the following solution: 43 grams of iodide were dissolved in 70 cc. of water to which 10 cc. of starch solution, and 1 cc. of glacial acetic acid were added. After titrating with 0.004 *N* thiosulfate, the solution had a volume of about 85 cc. On taking 5 cc. for titration we obtained about 2.5 grams of potassium iodide for each titration, which was ample.

7. Directions for making Estimations of the Total Amino Acid Nitrogen.

(a) *Including the Polypeptide Amino-nitrogen.*—All solutions to be tested for amino acids should be neutralized or made slightly alkaline to phenolphthalein. Insoluble substances may be dissolved with the aid of 0.1 *N* NaOH, using not more than 5–6 cc. For the factors given in this paper the solution should not contain more than 0.025 gram of amino acids in 25 cc. The volume after neutralization should be made to about 25 cc. and placed in a 50 cc. graduated flask so that, when stoppered, carbon dioxide from the air can, to some extent, be avoided. Twenty cc. of "buffer" solution are now added and with a small spatula or glass tube, a little freshly made cupric hydroxide is introduced and the mixture vigorously shaken for about a minute. If the cupric hydroxide has not completely dissolved and, therefore, is in excess, the solution, after bringing to room temperature, is made up to the mark¹ and shaken from two to three minutes. The mixture is now filtered through a good dry filter paper (S. & S. No. 590.11 cm. in diameter is satisfactory). The filtrate contains all of the soluble complexes while the residue contains the insoluble complexes and the excess of $\text{Cu}(\text{OH})_2$.

An aliquot portion (25 cc.) of the filtrate is then taken and after acidification with 1 to 2 cc. of glacial acetic acid, 5 cc. of potassium-iodide-starch solution is added and the solution titrated with 0.004 *N* thiosulfate solution. Every cubic centimeter of thiosulfate solution is an equivalent to 0.0003184 gram of cupric oxide or 0.0001120 gram of amino acid nitrogen or 1 cc. of 0.001 *N* thiosulfate solution is equivalent to 0.0000280

¹ The amount of cupric hydroxide, which should not be more than 0.030 gram, will produce an error in the volume of solution of not more than 0.05%, which is in most cases negligible.

gram of amino acid nitrogen. When calculated in terms of peptide amino nitrogen, these values should be halved.

If a long series of estimations are to be made it is advantageous to take the cupric hydroxide obtainable from 20 cc. of 5% cupric chloride and suspend it in 500 cc. of "buffer" solution. Then by taking 25 cc. of this mixture enough cupric hydroxide is obtained for the estimation of 25 mg. of amino acid. The reagent, if it is kept in a cool place, preferably in a refrigerator may be preserved for 12 to 15 hours. It is best, however, to make the cupric hydroxide fresh every day. If the amount of substance is less than 10 mg., 10 cc. of this reagent are sufficient. This mixture of "buffer" solution and cupric hydroxide we shall designate hereafter as the "amino acid reagent."

(b) *Excluding Polypeptide Amino Nitrogen.*—If the free amino acids are to be determined alone, the procedure, for making the copper complexes, is almost identical with that described for the total amino nitrogen. The only change necessary, is to add to 25 cc. of the filtrate, instead of directly titrating iodometrically, 5 cc. of 0.360 N $Ba(OH)_2$, allow mixture to stand for 15 minutes in a stoppered Erlenmeyer flask and then filter. This precipitates a definite fraction (87%) of the copper from the amino acid complexes (see table of precipitabilities). After washing the precipitate of cupric hydroxide, it is transferred with the filter paper to the Erlenmeyer flask and the hydroxide is dissolved in 10 cc. of 10% of acetic acid (warming if necessary). After adding the potassium-iodide-starch solution, the copper is titrated with 0.004 N thiosulfate solution, every cubic centimeter being equivalent to 0.000112 gram amino acid nitrogen, multiplied by the proper precipitability.

On acidifying with acetic acid and concentrating to about 15 cc. the filtrate can then be titrated iodometrically. The amino acid nitrogen of the filtrate plus that of the precipitate will give, if the polypeptides have five or less conjugated amino acids, the total amino acid nitrogen. If polypeptides or peptones of six or more conjugated amino acids are present they will prevent the precipitation of the free amino acid copper. Therefore, the increase in the amount of copper dissolved after total hydrolysis, alone, will give the information as to the amount of polypeptide present. No particular method of hydrolysis has been tried, but is probable that the method used by Levene¹ and Van Slyke will serve the purpose.

(c) *For Substances with Quite Insoluble Copper Salts.*—For substances with slightly soluble copper complexes, the technic just described is suitable. Where the copper complexes crystallize out as is the case when leucine, normal-amino-caproic acid, phenyl glycine, or cystine is present, and are filtered off with the excess of cupric hydroxide, it is necessary to

separate the two precipitates. Very satisfactory reagents for this purpose are the bicarbonates of sodium and potassium, which will dissolve the excess of cupric hydroxide without appreciably disturbing the complexes.¹

The filter paper² containing the insoluble complexes and the cupric hydroxide is transferred to a 100 cc. Erlenmeyer flask, and by means of a stirring rod the paper in the flask is unfolded so that the residue is on top, and so that it can be rubbed with a rod. Five cc. of 0.1 *N* HCl are then put into the graduated flask in which the complexes were originally made, so that the residue clinging to the sides of the flask, is dissolved. This solution is then added to the filter paper in the flask and boiled gently. Another portion (5 cc.) of 0.1 *N* HCl is used in exactly the same way. Finally the graduated flask is washed with 6 to 10 cc. of water and this added to the complexes in the Erlenmeyer flask.

After the residue is dissolved the volume of the solution in the flask is made up to about 20 cc. and two grams of powdered potassium bicarbonate are then added as follows: A little (0.2–0.3 gram) of the bicarbonate is first added with a spatula and the solution shaken. This precipitates the insoluble complexes and the excess of cupric hydroxide or carbonate again. After standing one to two minutes the remainder of the bicarbonate is added. On shaking two to three minutes the excess of cupric hydroxide or carbonate is completely dissolved and insoluble complexes are then filtered through paper (S. & S. No. 590, 7 cm. in diameter) and washed with a little water. On returning the filter paper to the Erlenmeyer (also washed), adding 10 cc. 10% acetic acid³ and heating until the copper of the complexes is dissolved, the solution can then be titrated iodometrically. As a table of precipitabilities shows, a correction for solubility in KHCO_3 is necessary. This technic gives good results with most of the insoluble complexes, but with leucine the technic described in a former paper⁴ is preferable. This technic required the washing of the first residue with small amounts of 10% KHCO_3 until the filtrates gave no appreciable tests for copper. Then the insoluble complexes were dissolved in 10% acetic acid and titrated. It may be possible, by using an amino acid like glycine which gives a soluble complex, to replace this bicarbonate solution and reduce the solubility corrections appreciably. As may be seen from the table, one to two milligrams of leucine and cystine may be determined as soluble complexes.

¹ See Kober and Sugiura, *J. Biol. Chem.*, **13**, 13 (1912).

² S. & S. No. 590, paper, 7 cm. in diameter, is preferable, when it is necessary to determine the insoluble complex, as it is easier to handle than the 11 cm. paper.

³ The cystine complex dissolves only slowly in acetic acid. To expedite matters a few cc. of 10th normal HCl may be added.

⁴ *J. Biol. Chem.*, **13**, 4 (1912).

8. Interfering Substances.

The results given in the tables of precipitabilities, show that cupric hydroxide reacts quantitatively with amino acids and their derivatives, even in very dilute solutions. For practical work it is also of importance to know what substances interfere.

We have studied twenty-two representative and important constituents of physiological material, and can show that the interfering substances can be roughly divided into two classes. One class, contains the dicarboxylic acids, like oxalic, citric and tartaric, which form complexes with cupric hydroxide in neutral or slightly alkaline solution similarly to the amino acids. The other class may be called the "reducing substances" and include uric acid and the sugars. In very weak solutions as in blood, these substances do not interfere (one part in 10,000).

The first class of substances interferes only in neutral solution. The second class, with the exception of uric acid, which also interferes in neutral solutions, dissolves appreciable amounts of copper only in alkaline solution.

To remove these interfering substances without disturbing the amino acids or polypeptides, we have found two special reagents. Ammoniacal lead acetate will precipitate quantitatively the dicarboxylic acid, sugars, and other poly-hydroxy substances,¹ without throwing down any amino

TABLE I.
(In "neutral solution" $C_H = 10^{-8.8}$).

Substance.	0.150 g. in 25 cc.	0.050 g. in 25 cc.	0.020 g. in 25 cc.	0.010 g. in 25 cc.	0.005 g. in 25 cc.	0.0025 g. in 25 cc.
Glucose	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Fructose	0.00034	0.00000	0.00000	0.00000	0.00000	0.00000
Lactose	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Galactose	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Maltose	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Glycerol	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Lactic acid	0.00231	0.00000	0.00000	0.00000	0.00000	0.00000
Taurine	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Urea	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Hippuric acid	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Ammonium sulfate	0.00089	0.00048	0.00000	0.00000	0.00000	0.00000
Uric acid	0.00163	0.00102	0.00187	0.00000	0.00000	0.00000
Oleic acid	0.000181	0.00000	0.00000	0.00000	0.00000	0.00000
Guanine	0.000000	0.000001	0.000000	0.000000	0.000000	0.000000
Creatine	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
Creatinine	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
Hypoxanthine	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
Guanidine carbonate	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
Tartaric acid	0.01376	0.00480	0.00281	0.000526	0.00059	0.00024
Citric acid	0.01154	0.00580	0.00365	0.00204	0.00175	0.00039
Oxalic acid	0.00189	0.00158	0.00033	0.00018	0.00009	0.00000

¹ Glycerol is the only exception we have found thus far. The sugars tested and found precipitable with lead acetate and ammonia are: glucose, fructose, sucrose, maltose and lactose.

TABLE II.
(In "alkaline solution.")
(5 cc. 0.360 *N* Ba(OH)₂ in 25 cc.)

Substance.	0.150 g. in 25 cc.	0.050 g. in 25 cc.	0.020 g. in 25 cc.	0.010 g. in 25 cc.	0.005 g. in 25 cc.	0.0025 g. in 25 cc.
Glucose.....	0.00367	0.00119	0.00029	0.00016	0.00003	0.00000
Fructose.....	0.00370	0.00205	0.00105	0.00072	0.00023	0.00007
Lactose.....	0.00381	0.00016	0.00030	0.00005	0.00000	0.00000
Galactose.....	0.00367	0.00249	0.00061	0.00024	0.00005	0.00000
Maltose.....	0.00346	0.00210	0.00093	0.00061	0.00012	0.00000
Glycerol.....	0.00112	0.00022	0.00000	0.00000	0.00000	0.00000
Tartaric acid.....	0.00052	0.00000	0.00000	0.00000	0.00000	0.00000
Citric acid.....	0.00408	0.00054	0.00009	0.00000	0.00000	0.00000
Lactic acid.....	0.00710	0.00015	0.00000	0.00000	0.00000	0.00000
Taurine.....	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Urea.....	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Hippuric.....	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Ammonium sulfate.....	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Uric acid.....	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Oleic acid.....	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Guanine.....	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Creatine.....	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Creatinine.....	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Hypoxanthine.....	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Guanidine carbonate.....	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Oxalic acid.....	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000

acids or peptides in very dilute solutions. When two cc. of a 5% solution of cupric chloride are added, with 0.5 cc. of saturated magnesium sulfate solution to a solution of uric acid, such as urin, a cuprous compound of uric acid is rendered sufficiently insoluble, so that it does not interfere with our reagent.

The preceding tables give the amount of copper dissolved under experimental conditions when varying amounts of substance were taken. We have attempted to make this a representative list but there are many substances occurring in physiological material in small amounts that have not been investigated. We feel sure, however, that they will have very little influence on the results, as the tables show a substance must be in considerable quantity, to have any interference. The figures given in the column are expressed as cupric oxide and roughly represent the amount dissolved by the concentration of the substance indicated at the top of the column.

9. Applications to Physiological Material.

(a) *In Proteolysis.*—In studying proteases, this method in conjunction with the nephelometric¹ will give considerable information. (1) With the nephelometer we can estimate the amount of undigested protein at any time. (2) The total amino acid nitrogen obtained with this copper

¹ Kober, *J. Biol. Chem.*, **13**, 485 (1913); *THIS JOURNAL*, **35**, 290 (1913).

method will indicate the amount of hydrolysis at any time. (3) On treating the copper complexes with barium hydroxide, as specified, they will give the amount of the final products of hydrolysis, the free amino acids. All this can be easily accomplished on 5 mg. of protein.

For the study of proteases with substances of known structure like the polypeptides, this method is also well adapted. Not only can the rate of hydrolysis be estimated accurately with small amounts of material but the molecular weights of the products can be determined.

In the following two tables, mixtures of amino acids and peptides were used. The molecular weights of the substances cannot be determined from these data as the amounts are not equivalent, as they would be in actual proteolysis. However, they show clearly enough that very small amounts of amino acids can be detected in the presence of their derivatives, the

AMINO ACID AND PEPTIDE MIXTURES.

Mixtures.	Weight taken, $\frac{1}{2}$ of mg.	CuO found in filtrate. Mg.	CuO found in residue. Mg.	Total CuO found. Mg.	Theoretical amt. CuO. Mg.	Ratio of CuO found to theoretical. Per cent.	Increase in CuO of residue. Mg.	Expected increase in residue. Mg.	Increase in CuO of filtrate. Mg.	Expected increase in filtrate. Mg.
Glycyl-glycine.....	10.22	2.47	0.57	3.04	3.08	98.9	0.00	0.00
Precipitability of 23.1%.										
Glycyl-glycine.....	10.22	2.48	0.58	3.06	3.14	97.5	0.01	0.06
Glycine.....	0.25
Glycyl-glycine.....	10.22	2.37	0.76	3.13	3.21	98.0	0.19	0.13
Glycine.....	0.51
Glycyl-glycine.....	10.22	2.40	0.88	3.28	3.34	98.1	0.21	0.26
Glycine.....	1.02
Glycyl-glycine.....	10.22	2.39	1.28	3.67	3.75	98.0	0.71	0.64
Glycine.....	2.54
Glycyl-glycine.....	10.22	2.34	1.95	4.29	4.42	97.2	1.38	1.29
Glycine.....	5.08
Glycyl-glycine.....	10.22	2.36	3.25	5.61	5.77	97.3	2.68	2.58	2.27	2.31
Glycine.....	10.16
Glycyl-glycine.....	5.11	1.09	3.05	4.14	4.23	98.0	1.00	1.15
Glycine.....	10.16
Glycyl-glycine.....	2.55	0.59	2.84	3.43	3.46	99.1	0.50	0.59
Glycine.....	10.16
Glycyl-glycine.....	1.02	0.26	2.70	2.96	3.00	98.6	0.17	0.18
Glycine.....	10.16
Glycyl-glycine.....	0.50	0.16	2.66	2.82	2.84	99.2	0.07	0.09
Glycine.....	10.16
Glycyl-glycine.....	0.25	0.09	2.58	2.67	2.77	96.5	0.00	0.00
Glycine.....	10.16
Glycine.....	10.16	0.09	2.58	2.67	2.69	99.1	0.00	0.00
Precipitability = 96.5%.										

AMINO ACID AND PEPTIDE MIXTURES.

Mixtures.	Weight taken $\frac{1}{3}$ of mg.	CuO in filtrate. Mg.	CuO in residue. Mg.	Total CuO found. Mg.	Theoretical amt. Mg.	Ratio of CuO found to theoretical. Per cent.	Increase in CuO of residue. Mg.	Increase expected in residue. Mg.	Increase in CuO of filtrate. Mg.	Increase expected in filtrate. Mg.
1 Alanine.....	5.12	0.032	1.096	1.128	1.144	98.6
2 Alanylglycine.....	5.04	1.018	0.323	1.341	1.373	97.7
3 Glyc-al-glycine	5.09	0.918	0.080	0.998	0.999	99.9
1	0.00
2	5.04	1.94	0.40	2.34	2.37	98.8	0.00	0.00
3	5.09
1	0.25
2	5.04	1.93	0.45	2.38	2.43	98.2	0.05	0.05
3	5.09
1	0.51
2	5.04	1.99	0.45	2.44	2.49	98.1	0.05	0.11
3	5.09
1	1.02
2	5.04	2.08	0.47	2.55	2.60	98.3	0.07	0.21
3	5.09
1	2.56
2	5.04	2.11	0.80	2.91	2.94	98.9	0.40	0.54
3	5.09
1	5.12
2	5.04	1.96	1.52	3.48	3.52	98.9	1.12	1.10	1.93	1.94
3	5.09
1	5.12
2	2.52	1.03	1.29	2.32	2.33	99.5	1.00	0.97
3	2.55
1	5.12
2	1.01	0.45	1.14	1.59	1.62	98.1	0.42	0.39
3	1.02
1	5.12
2	0.50	0.27	1.12	1.39	1.38	100.7	0.24	0.19
3	0.51
1	5.12
2	0.25	0.16	1.09	1.25	1.26	99.2	0.13	0.10
3	0.25
1	5.12	0.03	1.10	1.13	1.14	98.6	0.00	0.00
2	0.00
3	0.00

polypeptides. Attention should be called to the fact indicated with the other tables, that the results obtained are for only one-half of the amount given in column 2.

(b) *In Blood*.—The concentration of interfering substances in blood is

so small that the reagent could be applied directly without preliminary treatment, were it not that the proteins delay the filtering and the red hemoglobin conceals the starch-iodine color. To circumvent this difficulty the blood proteins may be precipitated and the serum clarified according to the method of Folin¹ or Bang.² The former has the advantage that urea and the total non-protein nitrogen can be determined according to Folin's technic, conjunctively with the determination of amino acids on the same sample of blood (10 cc.).

The following figures were obtained with 5 or 10 cc. of blood treated according to the method of Folin, and according to the method of Bang, with the exception of some slight modifications. In the Folin method of precipitating protein with methyl alcohol, the volume was changed from 50 to 100 cc. In the Bang method of precipitating protein the following details were employed: 50 cc. of water were placed into an Erlenmeyer flask with 2 cc. of saturated magnesium sulfate (the chloride is preferable) and 1 cc. of the glacial acetic acid. After bringing this solution to a boil, 10 cc. of the blood were added and allowed to heat for about a minute. After cooling the solution was made up to 100 cc. and filtered.

In the table below three specimens of human blood were used and in each case the blood was precipitated with alcohol at once. Two specimens of slaughter house blood were also determined, but considerable time one to two hours, was consumed in transit, before the analysis was begun.

Kind of blood.	Amount.	Method.	Amount of filtrate used.	Total amount of 0.001 <i>N</i> thio-sulfate solution used. Cc.	Per cent. of amino acid nitrogen found. Vol. per cent.
Human pathological.....	10 cc.	Folin	1/2	2.4	0.0013
Human pathological.....	10 cc.	Folin	1/2	4.0	0.0022
Human normal.....	5 cc.	Folin	1/2	1.6	0.0009
Calf's.....	10 cc.	Folin	1/2	6.3	0.0035
Calf's.....	10 cc.	Bang	1/2	12.8	0.0072
Lamb's.....	10 cc.	Bang	1/2	11.2	0.0063
Lamb's.....	10 cc.	Folin	1/2	4.6	0.0026

(c) *In Urin.*—In non-diabetic urin, uric acid seems to be the only interfering substance.

To circumvent the action of uric acid and to estimate the amino acids in the urin, the following procedure is recommended: To 20 cc. or less of urin, measured into a 50 cc. graduated flask, are added 0.5 cc. saturated magnesium chloride solution and two cc. of 5% cupric chloride solution. After standing 1 or 2 minutes, 4 drops of 0.1% phenolphthalein indicator

¹ *J. Biol. Chem.*, 11, 528 (1912).

² *Biochem. Z.*, 49, 22 (1913).

and 1 *N* CO₂-free sodium hydroxide are added until the color changes to a purple (*i. e.*, until the solution is slightly alkaline). The "amino acid reagent" is now added to the mark and the solution is shaken at intervals for 5 minutes. After filtering through a dry paper and funnel, an aliquot portion of the filtrate (25 cc.) is withdrawn and titrated directly, iodometrically, to obtain the total amino acid nitrogen or, it is precipitated with 5 cc. 0.360 *N* Ba(OH)₂ as described and the residue and filtrate titrated separately, for the free amino acid nitrogen and the peptide nitrogen.

The following results on 24-hour specimens of urin were obtained with a mixed diet:

Specimen No.	0.004 <i>N</i> thiosulfate solution for filtrate. Cc.	0.004 <i>N</i> thiosulfate solution for precipitate. Cc.	Total cc. 0.004 <i>N</i> Na ₂ S ₂ O ₃ .	Total nitrogen in 10 cc.	Amino acid nitrogen in 10 cc.	Ratio of amino N to T-N.
A.	2.14	10.69	12.8	0.0508	0.00138	2.7
B.	(2.75)	11.05	13.8	0.0404	0.00149	(3.7)
C.	1.88	10.17	12.0	0.0444	0.00129	2.9
D.	1.06	6.12	7.2	0.0258	0.00077	3.0
E.	1.23	6.81	8.0	0.0320	0.00086	2.7
F.	2.16	12.09	14.2	0.0502	0.00153	3.0

The results show that in this normal urin the ratio of amino acid nitrogen to total nitrogen is, similarly to the urea ratio, quite constant. The ratio between the amount of copper in the residue and the amount in the filtrate (80–85%) shows that there is very little, if any, polypeptides in the urin.

In diabetic urin the sugar prevents the separation of free amino acid nitrogen from the peptide nitrogen, but does not hinder the estimation of the total amino acid nitrogen, including the peptide nitrogen.

The following procedure is applicable to stomach contents and other material containing sugars and polyhydroxy substances, for the purpose of estimating the peptide nitrogen. When the total amino acid is to be determined in the presence oxalic, citric and tartaric acids, this procedure must be slightly modified. This is due to the fact that the reagent used, ammoniacal lead acetate, throws down in moderate dilutions (1 part in 500) a few amino acids; histidine, tryptophane, and tyrosine, although it removes all of the interfering acids. In very dilute solutions this reagent does not precipitate appreciable amounts of amino acids (1 part in 10,000).

To 20 cc. of diabetic urin, are added 5 cc. of strong ammonia (sp. gr. 0.90, 28–29%) and 25 cc. of 10% lead acetate, which precipitates the sugars and most other polyhydroxy substances quantitatively, and leaves in the filtrate most of the amino acids and all of the peptides, the excess of lead acetate and ammonia. As ammonia dissolves copper and lead forms a yellow iodide which interferes in the iodometric titration, it is necessary to remove these, before going ahead with the "copper" technic.

The filtrate is then treated with 10 cc. of 10% potassium sulfide solution, and filtered, from the lead sulfide. As the sulfide is strongly alkaline one can get rid of the ammonia, by placing the filtrate in a Claisson side neck distillation flask, and distilling *in vacuo* until all of the ammonia has been removed (20-25 minutes).

The solution is now transferred to a 50 cc. graduated flask, and titrated with an aqueous solution of iodine, and starch, until a permanent starch-iodine color is produced. This is done to remove any reducing thio-substances in the filtrate. After this color has been discharged with 0.004 *N* thiosulfate, added drop by drop, the solution is, neutralized, and the "amino acid reagent" is added.

Instead of this potassium sulfide treatment to remove the lead and ammonia, one can use a small amount of sulfuric acid or K_2SO_4 , subsequently distilling the ammonia with a slight excess of barium hydroxide, *in vacuo*.

10. Comparisons with Other Methods.

In the following pages a brief comparison will be given of the Sørensen,¹ the Van Slyke² and the Abderhalden methods³ in regard to their applicability and sensitiveness for qualitative and quantitative estimations of amino acids.

(a) *Qualitative Methods*.—Of these, the one most recently used for qualitative estimations of amino acids, is Abderhalden's "ninhydrin" method. Abderhalden claims that this reagent will detect amino acids in one to ten thousand dilution. By increasing the amount of reagent about 25 times and by boiling three minutes instead of one, as Abderhalden recommends, we have been able to detect one part alanine in fifty thousand with certainty, but thus far one part in a hundred thousand is beyond recognition with this reagent. Nevertheless, we believe that with a stronger solution of this reagent a very delicate reaction may be developed and one which may even be made the basis of a colorimetric method. The chief obstacle in the use of this reagent for amino acids is the interference of proteins and peptones, which give a decided color with it on boiling. Since the method is only qualitative, it is hard to estimate the amount of color due to proteins, and thus it is impossible to detect amino acid in the presence of protein. To circumvent this difficulty Abderhalden recommends dialysis. On cooling a solution of this color it fades appreciably. At present the cost of material may prevent its general adoption.

As a qualitative test our reagent will easily detect one part of amino acid in 500,000, and without much difficulty detect one part in 1,000,000. The qualitative, as well as the quantitative test seems limited only by

¹ *Z. physiol. Chem.*, 64, 120 (1910).

² *J. Biol. Chem.*, 12, 275 (1912).

³ *Z. physiol. Chem.*, 72, 37 (1911); 85, 143 (1913).

the delicacy and accuracy of the test for copper. It has the advantages that it gives no reaction with native protein whatsoever; that it requires no boiling and can be done in an ice mixture, and that it is easily obtainable and inexpensive.

(b) *Quantitative Methods.*—In the formol titration of Sørensen, free ammonia must be removed, but urea and other substances do not interfere to any extent. Theoretically, the method seems simple and accurate but practically it is difficult, especially in estimating amino acid in material such as urin and blood. Löb¹ found it possible by using 0.01 *N* NaOH to estimate 0.005 gram of glycine in 8 cc., or one part in 1600, but it seems doubtful that less than a milligram can be estimated with any degree of accuracy, even in a small volume.

In the Van Slyke method for the estimation of amino nitrogen in aliphatic substances, using nitrous acid, ammonia, urea, glucosamine, amino purines and pyrimidines react, while glycine and glycyglycine give more than the theoretical results, owing to further decomposition. As the method is specific for the amino group and not for amino acids and only in the absence of interfering substances gives suitable results, the method is applicable, only with difficulty to the estimation of amino acids in physiological material. According to Van Slyke, the smallest amount of amino acid nitrogen that can be detected by his method, is 0.05 mg., which under the best conditions, that is 10 cc., is one part in 200,000. For quantitative work within 5%, at least one milligram of amino acid nitrogen is necessary, which is one part in 10,000.

For microchemical estimations, our method seems to be well suited. It is in common with the formol titration specific for amino acids, but has the advantage of being more sensitive and in practical work of easier operation. As a matter of fact, the reaction, operation and calculation are simple enough to be called a "clinical method." Ammonia in such quantities, as occurs in physiological material, urea, glucosamin, and other amino substances that are not of amino acids do not interfere with this method. By using a 0.001 *N* thiosulfate solution we had no difficulty in estimating 0.5 mg. of amino acids or about 0.05 mg. of amino acid nitrogen in 25 cc. within 3 to 5%, which means one part in 500,000. The copper method has other advantages; the reagent is a very insoluble substance, an excess of which is easily removed by filtration, and the reaction between the substances produces no permanent change in the molecule of the substances. Therefore, without much more work, the amino acids and their derivatives can be used for the same purpose again. This demonstrates the non-vigorous character of the reagent and the advantages of a reaction based chiefly on the so-called "secondary" valences.

¹ *Ber.*, 46, 696 (1913).

11. General Discussion.

The results in this paper with small amounts of substances (from 1 to 25 mg.) confirm our figures of previous papers made with larger amounts (0.100 gram). The values, as a rule, are slightly lower and more nearly theoretical, due no doubt to the elimination of the carboamino reaction. Three exceptions in the stoichiometrical relationships of the amino acids were found. As has been pointed out and explained glutamic and aspartic acids function as monobasic acids with our reagent. Histidine forms a complex which corresponds to the formula $(\text{Hist.})_3\text{Cu}_2$ but which may be only a partial formation of $(\text{Hist.})_4\text{Cu}_3$. This we expect to study in the near future. So far as we are aware these are the first attempts to make copper complexes with histidine. In this connection it is of interest to note that these insoluble complexes of normal amino-caproic acid and of phenyl glycine have also not been described heretofore. A salt of the latter is mentioned by Schwebel¹ in 1877, but probably is not the same substance. Schwebel's salt is soluble giving a greenish solution. Our complex is so insoluble that the filtrate is perfectly colorless and gives no test for copper with all the known reagents.

Since the appearance of our last paper, which showed that all the peptides which we had, gave the stoichiometrical relationship of one peptide to one copper, Abderhalden² and Fodor have published formulas on tri-peptide complexes that are for the most part not in agreement with those of Fischer's and ours. Abderhalden and Fodor used a technic which we found to be insufficient.³ Fischer's results on five isolated crystallin complexes of peptides showed a relationship of one molecule of copper to one molecule of peptide. *The technic developed in this laboratory for the quantitative formation of complexes in solution gives results that are identical with those of known isolated complexes.* It is to be noted that all abnormal results of Abderhalden were obtained on non-crystallin residues, and that in each case where he succeeded in crystallizing the complex, the formula turned out to be one to one. As a matter of fact with the racemic form⁴ of one of the tri-peptides (1-leucyl-d-alanyl-glycine) with which Abderhalden and Fodor obtained these exceptional results, we have obtained the usual ratio of one molecule of copper to one of peptide.

As to the color of these complexes we will mention only one important observation: The "biuret" or "semi-biuret" color produced by making the solution of a peptide copper complex (containing three or four nitro-

¹ *Ber.*, 10, 2046.

² *Z. physiol. Chem.*, 81, 1 (1912).

³ *J. Biol. Chem.*, 13, 12 (1912).

⁴ Experience in general (see Walden, *Ber.*, 29, 1692 (1896)) and ours in this work (see table of precipitabilities) have shown no difference in the chemical action between the optically active and inactive substances (see Stewart, "Stereochemistry," p. 42 (1907)).

gen atoms in ring formation) alkaline, *is dependent, not only on the substance and the temperature, but also on the concentration of OH⁻ ions.* In other words, each peptide requires a certain concentration of OH⁻ ions for the maximum development of the "semi-biuret or biuret" reaction. We have found the "buffer" solution used in this method to have a very favorable concentration of OH⁻ ions ($C_H = 10^{-8.8}$) for the "semi-biuret" reaction with tri-peptides, while with tetra-peptides the concentration is not at all favorable. On adding stronger alkali (5 cc. 0.360 N Ba(OH)₂) to the tri-peptide complexes having a very marked "semi-biuret" color, the tint turns towards the blue, *i. e.*, the semi-biuret color becomes fainter. With tetra-peptides the reverse is true. The reddish "biuret" color only appears with stronger alkali. *Therefore, in the future, it will be necessary to designate the OH⁻ ion concentration when comparing the colors of alkaline complexes of peptides and peptones.* This observation helps to explain many discrepancies in the literature on biuret reaction where the concentration of alkali is never the same. Thus Fischer¹ using strong alkali, states that di-glycyl-glycine is the only tri-peptide which does not give a biuret tint, and this substance, according to our work, gives the same characteristic "semi-biuret" color as the other tri-peptides, provided the concentration of OH⁻ ions is not too strong. *The recommendation of Fischer,¹ to use rather strongly alkaline solution for making biuret reactions with peptides does not give good results with tri-peptides.* On the spectrographic variations of biuret color with temperature and alkalinity, we hope to report in the near future.

The slight variations in our results may be due to one or more of the five possible causes: (1) variations in temperature; (2) impurities in some of the substances; (3) faulty filtering; (4) instability or hydrolysis of the complexes; (5) error of iodometric titrations.

The influence of temperature on the precipitabilities according to the table is very slight but the influence of temperature on the formation of the complexes may be the cause of some slight variations. The purity of some of these substances cannot be questioned as all known tests on the substances gave good agreements with the theoretical. On the other hand, some of the peptides contain appreciable impurities. They are, as a rule, very difficult to purify and in many cases the cost of the substances prevents further purification. As the results indicate, most of the substances made by the late Dr. A. H. Koelker and ourselves, are quite pure. In several cases where the copper results showed low figures the percentage of total nitrogen was found low in proportion. It should be noted that the values given in this paper were obtained practically as recorded, and unless specified represent single values and not average values. The results recorded in this paper were obtained on single filtrations, that is, the fil-

¹ Untersuchung über Aminosäuren, Polypeptide und Proteine, 50 (1906).

trates were never refiltered. How much of an error this may make, we do not exactly know. Owing to barium carbonate being formed from carbon dioxide of the air and barium hydroxide and producing a cloud, any suspended cupric hydroxide could not be estimated nephelometrically. The results show that the error can only be appreciable with the amounts under 5 mg. A tendency to hydrolyze was only noticed with one mg. of leucine in 25 cc. but this may be only due to slower reaction, which in the time given, was not sufficient to be complete.

The table given elsewhere shows that the method of titrating is quite accurate but that with smaller amounts there is a tendency to overtitrate. These and other irregularities we hope to eliminate in the near future.

The average results on the precipitabilities are as follows:

No. of substances.	Substances.	CONCENTRATIONS.					Average. Per cent.
		0.001 g. in 50 cc. Ca 0.000008 gram-equiv. Per cent.	0.005 g. in 50 cc. Ca 0.00004 gram- equiv. Per cent.	0.010 g. in 50 cc. Ca 0.00008 gram- equiv. Per cent.	0.025 g. in 50 cc. Ca 0.00021 gram- equiv. Per cent.	Ba(OH) ₂ 5 cc. 360 N. Ca. 0.0012 gram-equiv.	
19	Amino acids	87.8	87.3	86.6	83.4	..	86.5
27	Di-peptides	24.2	17.9	..	9.9	..	17.3
18	Tri-peptides	15.6	11.1	..	5.8	..	10.8
4	Tetra-peptides	11.7	9.8	..	6.0	..	9.2
Total 68	Average	17.1	12.9	..	7.2	..	12.4

The precipitabilities of the amino acids vary only in proportion to the amount of free barium hydroxide which is appreciably reduced in the larger concentrations of substances. No particular explanation can be given now for the decrease in precipitabilities of the peptides.

Experimental.

(1) Experiments with Phosphate Solutions; (2) Experiments with Borate Solution; (3) Equilibrium Studies with Barium Hydroxide; (4) Experiments with Temperature; (5) Experiments with Time; (6) Experiments in Making Cupric Hydroxide; (7) Experiments with Histidine; (8) Experiments with Proteins, Peptones and Bromo-acid Derivatives of Polypeptides; (9) Experiments with Small Amounts of Copper; (10) Experiments with Lead Acetate and Ammonia; (11) Total Nitrogen Estimations.

(1) *Experiments with Phosphate Solutions.*—To show that low results with the phosphate mixture are due to the formation of an insoluble phosphate 0.15 gram of glycine copper complex were allowed to stand for 24 hours with 50 cc. of "7-9.65" phosphate mixture. At the end of that time most of the copper was in the form of a bluish green precipitate. This was examined after filtering and washing, for glycine, phosphates and copper. No traces of glycine, but large amounts of phosphates and copper were found. The precipitate is without doubt a phosphate of

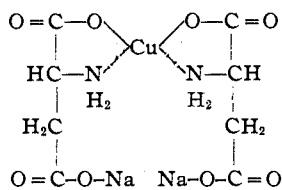
copper. For these reasons we abandoned the attempt to use phosphates as a "buffer" solution and tried the following mixtures of borates as a preliminary experiment:

BORATE EXPERIMENTS.

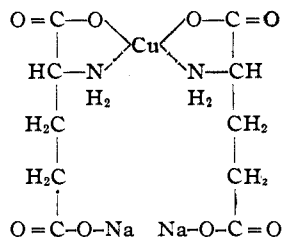
Mixture No. 1:	Substance.	Gram.	CuO found.	Theory.	Difference.	Per cent.
25 cc. <i>m/15</i> ; "sodium borate;" conc. of H = $10^{-9.24}$.	Glycine	0.00506	0.00271	0.00268	+0.00003	101.1
	Alanine	0.00505	0.00225	0.00226	-0.00001	99.6
	Aspartic acid	0.00501	0.00151	0.00150	+0.00001	100.7
	Glyc-glyc	0.00516	0.00301	0.00311	-0.00010	96.8
	Blank	0.00005
Mixture No. 2: 7.5 pts. <i>m/15</i> "Na borate;" 2.5 pts. 0.1 <i>N</i> HCl; conc. of H = $10^{-8.80}$.	Glycine	0.00506	0.00271	0.00268	+0.00003	101.1
	Alanine	0.00505	0.00222	0.00226	-0.00004	98.2
	Aspartic acid	0.00501	0.00153	0.00150	+0.00003	102.0
	Glutamic	0.00504	0.00135	0.00137	-0.00002	98.5
	Glyc-glyc	0.00516	0.00304	0.00311	-0.00007	97.8
Blank	0.00004	
Mixture No. 3: 5.0 pts. <i>m/15</i> ; "sodium borate;" 5.0 pts. 0.1 <i>N</i> HCl; conc. of H = $10^{-6.65}$.	Glycine	0.00506	0.00318	0.00268	+0.00050	118.7
	Alanine	0.00505	0.00285	0.00226	+0.00059	126.1
	Aspartic acid	0.00501	0.00225	0.00150	+0.00075	150.0
	Glyc-glyc	0.00516	0.00307	0.00311	-0.00004	98.7

Here, too, the volume of the "buffer" solution was 25 cc. and the total 50 cc.

The results with borate mixtures 1 and 2 show that the dibasic acids, aspartic and glutamic, under these conditions function as monobasic acids. This is consistent with the ideas and configurations given in a previous paper, namely, that the complexes are 5- and 6-membered rings of copper with α - and β -amino acids, and that 6- and 7-membered ring formations were less stable than 5-membered rings. In these "buffer" solutions we have a very slight alkalinity which probably forms a sodium salt of the second carboxyl group, thereby increasing the basicity of the amino group to make the stable complexes as represented below:



Aspartic acid complex.



Glutamic acid complex.

The stoichiometrical results certainly agree well with the theoretical. This change would not apply to the only other dibasic acid, cystine, as it is also a di-amino acid; the two amino groups being in α -positions.

(3) *Equilibrium Studies with Ba(OH)₂*.—The following results were obtained when the "buffer" solution was omitted in order not to further complicate the calculation of the amount of free barium hydroxide at equilibrium. The free barium hydroxide represents the amount obtained by subtracting that used by the copper, in precipitating as hydroxide, from the total added. 0.010 gram of glycine and glycyglycine were used, in each equilibrium.

TABLE XX.

Volume, ¹ Cc.	Percent. of glycine as Cu(glyc) ₂ in filtrate. (a).	0.360 N Ba(OH) ₂ in solution. (b).	Cu(OH) ₂ in solu- tion. (c).	Percent. of glycine as Ba(glyc) ₂ in filtrate. (d).	If $\frac{a \cdot b}{d} = K_1$.	If $\frac{A \cdot b}{d} = K_{11}$.
					(K ₁).	(K ₁₁).
51.00	37.4	0.71	Very	62.6	0.42	
52.50	10.9	2.11	small	89.1	0.26	
55.00	6.4	4.59	and	93.6	0.31	
60.00	3.7	9.59	assumed	96.3	0.37	
65.00	3.3	14.59	constant	96.8	0.48	
				Average	0.37	
	Per cent. glyc- glyc as Cu(g-g) ₂ in filtrate. (a).			Per cent. of g-g as Ba(g-g) ₂ in filtrate. (d).		
51.00	94.5	0.97	Very	5.5	17.0	16
52.50	91.2	2.40	small	8.5	26.0	24
55.00	83.2	4.87	and	16.8	23.0	20
60.00	75.0	9.84	assumed	25.0	29.0	22
65.00	77.0	10.84	constant	23.0	50.0	38
				Average		24

To attempt an accurate estimation of equilibrium concentrations with these substances, is beyond the scope of this paper, as our only purpose is to show that for the different amounts of alkali and of substance, a definite relationship exists as to the amount of cupric hydroxide precipitated.

As these are probably either bimolecular or trimolecular heterogeneous equilibria, there are many points which ought to be taken into consideration in a complete study, but we can only briefly mention a few of them here.

¹ About 20 cc. must be added to these volumes, due to wash water.

(1) The assumption of one substance having a constant active mass, which is this case would be $\text{Cu}(\text{OH})_2$ and the influence of such an assumption, in a reaction involving two molecules of $\text{Cu}(\text{OH})_2$.

(2) To what extent is the reaction ionic?

(3) Is the reaction one of consecutive smaller reaction, or not?

The values given for K_{I} and K_{II} indicate that equilibrium practically exists. Whether the exact reaction as given is correct or whether it only represents the reaction, cannot be decided by these experiments.

It is of interest to note that when 2.5, 5.0, and 10 cc. of $\text{Ba}(\text{OH})_2$ were used, the results are satisfactory; being for glycine 0.26, 0.36, 0.37, average = 0.31; and for glycyl-glycine, 26, 23, 29, average = 26; and that the precipitability of glycine in this experiment is 84 times that of glycyl-glycine.

In most of these preliminary experiments, the total filtrate with wash waters was concentrated to about 15 cc. and titrated iodometrically. Most of the experiments when not otherwise indicated were with an initial volume of 50 cc.

Having shown that the reaction comes to an equilibrium when barium hydroxide is added to the copper complex, already completely formed, it was of interest and an absolute proof of the reversibility of the reaction to add the barium hydroxide first and show the reverse action.

The figures given below from our note book are not immediately comparable to the ones given in the table of precipitability, as a different concentration of the alkali makes an appreciable difference. The concentration of alkali in these experiments instead of being 0.06 N is 0.072 N .

Substance.	Weight of substance. Gram.	Volume of solution. Cc.	Conc. of $\text{Ba}(\text{OH})_2$.	CuO found in filtrate. Gram.	Theoretical amt. Gram.	"Precipitability."	
						Reverse action 0.072 N $\text{Ba}(\text{OH})_2$.	Direct action 0.06 N $\text{Ba}(\text{OH})_2$.
Glycine.....	0.00509	25	0.072 N	0.00009	0.00270	97.0	95.0
Alanine.....	0.00504	25	0.072 N	0.00005	0.00225	98.0	95.0
Glycyl-glycine....	0.00506	25	0.072 N	0.00207	0.00305	32.0	25.0
Alanyl-glycine....	0.00512	25	0.072 N	0.00190	0.00278	32.0	25.0

(4) *Experiments with Temperature.*—To show the influence of temperature on the precipitability of these complexes, the following experiments were made: 0.0101 gram of glycine as copper complex were treated in a volume of about 70 cc. with 10 cc. 0.360 N $\text{Ba}(\text{OH})_2$ at different temperatures for about 5 minutes:

Temperature.	Amount of glycine. Gram.	CuO found in residue. Gram.	CuO found in filtrate. Gram.	Per cent. of residue to total.
0°	0.0101	0.00279	0.00254	52.1
20°	0.0161	0.00433	0.00100	80.8
40°	0.0101	0.00406	0.00130	(70.6)
60°	0.0101	0.00437	0.00096	81.4
80°	0.0101	0.00474	0.00057	88.4
100°	0.0101	0.00510	0.00029	92.3

with glycyl-glycine, similar experiments gave the following results:

Temperature.	Amount of glycyl-glycine. Gram.	CuO found in residue. Gram.	CuO found in filtrate. Gram.	Per cent. of residue to total.
0°	0.0100	0.00070	0.00531	11.6
20°	0.0100	0.00061	0.00536	10.1
40°	0.0100	0.00050	0.00552	(8.3)
60°	0.0100	0.00073	0.00534	12.1
80°	0.0100	0.00079	0.00518	13.1
100°	0.0100	0.00096	0.00504	15.9

The irregularity of results was probably due to variations in volume of solutions and temperature changes during filtration.

(5) *Experiments with Time.*—To show the influence of time on the precipitability, the following experiments were made at room temperature, changing the time in which the $\text{Ba}(\text{OH})_2$ was allowed to act on the copper complexes.

Although slight variations exist, the results show clearly that there is no appreciable increase in the action of $\text{Ba}(\text{OH})_2$ for 45 minutes, over that obtained in 15 minutes, and, therefore, equilibrium is practically reached in 15 minutes.

The total amount of substance in each case is given in columns 2 and 5, but the figures given in the other columns represent one-half of the total filtrate and, therefore, one-half of the substance given in columns 2 and 5. The amount of barium hydroxide was 5 cc. of 0.360 *N* $\text{Ba}(\text{OH})_2$ added to 25 cc. of filtrate, so that the complexes were in equilibrium with 0.06 *N* $\text{Ba}(\text{OH})_2$ as given in the tables of "precipitabilities."

(6) *Experiments in Making Cupric Hydroxide.*—The object of these experiments was to ascertain whether in making cupric hydroxide, from cupric chloride, by neutralization, at a low temperature, over-titration would make any difference in the results with amino substances and in the results of the blanks. In the first experiment 10 cc. of 5% cupric chloride were diluted with 100 cc. of water and sufficient ice was added to bring the temperature below 5° C. After adding three or four drops of 0.1% phenolphthalein indicator the solution was neutralized with (carbon dioxide-free) normal sodium hydroxide. Then 1 cc. of the same alkali was added in excess and washed three times with ordinary distilled water. The cupric hydroxide was then taken up in 50 cc. of water and used as

Substance.	Weight of substance taken. Gram.	Ba(OH) ₂ for 15 min.		Wt. of sub. Gram.	Ba(OH) ₂ for 45 min.		Per cent. precipitated.	
		Precipitated CuO. Gram.	Filtrate CuO. Gram.		Precipitated CuO. Gram.	Filtrate CuO. Gram.	In 15 min.	In 45 min.
Glycine.....	0.00103	0.00024	0.000046	0.00103	0.00023	0.000015	87.9	84.2
Glycine.....	0.00516	0.00130	0.000062	0.00516	0.00130	0.000040	94.8	94.8
Glycine.....	0.01035	0.00260	0.000139	0.01035	0.00260	0.000105	94.8	94.8
Alanine.....	0.00100	0.00020	0.000056	0.00102	0.000160	0.000046	89.3	(70.2)
Alanine.....	0.00502	0.00105	0.000058	0.00511	0.00100	0.000087	93.7	87.7
Alanine.....	0.01005	0.00211	0.000105	0.01023	0.00217	0.000087	93.8	94.7
						Average	92.2	91.2
Glycl-glycine.....	0.001055	0.000133	0.000220	0.00105	0.000118	0.000210	41.8	37.4
Glycl-glycine.....	0.00527	0.000372	0.00121	0.00527	0.00034	0.00121	23.4	21.4
Glycl-glycine.....	0.01055	0.000526	0.00261	0.01055	0.00061	0.00255	16.5	19.2
						Average	27.2	26.3

a suspension at room temperature. This cupric hydroxide is designated as "a" in the table below.

Another portion of cupric hydroxide was prepared in exactly the same way, except that 10 cc. of normal alkali were used as excess and that the precipitate was washed six times; until the wash water was not alkaline to phenolphthalein. This preparation is designated by "b" in the table below.

In the experiments given below 5 cc. of these suspensions of cupric hydroxide were used in each case. In the first two experiments Nos. 1 and 2, 45 cc. of water were added to the hydroxide, the mixture thoroughly shaken and filtered. In the third, fourth and fifth experiments, 25 cc. of this water were replaced with 25 cc. of "buffer" solution. In the sixth, seventh and eighth experiments the water was replaced by "buffer" solution (25 cc.) and by solutions of alanine and glycyl-glycine. In all experiments, therefore, the total volume was 50 cc. In the second column, the time is given during which the solutions were exposed to the air, before filtering. In the third column the results are given in terms of cupric oxide.

As the results show, water without any "buffer" solution dissolved an appreciable amount of copper from the cupric hydroxide marked "a" whereas with the "buffer" solution the amount was not appreciable. With preparation "b" the results on water, with and without "buffer" solution, indicate that practically no copper is dissolved. The residue on alanin and glycyl-glycine show that the preparations are equally suitable.

Substance and solution. Grams.	Time of exposure to air. Minutes.	Amount of CuO found in filtrate. Grams.		Theoretical amount.
		"a."	"b."	
I. 45 cc. H ₂ O, 5 cc. Cu(OH) ₂ . . .	5	0.000031	0.00002	0.00000
II. 45 cc. H ₂ O, 5 cc. Cu(OH) ₂ . . .	15	0.000062	0.00000	0.00000
III. 20 cc. H ₂ O, 25 cc. "Buffer" solution, 5 cc. Cu(OH) ₂ . . .	5	0.000010	0.00000	0.00000
IV. 20 cc. H ₂ O, 25 cc. "Buffer" solution, 5 cc. Cu(OH) ₂ . . .	10	0.000020	0.00000	0.00000
V. 20 cc. H ₂ O, 25 cc. "Buffer" solution, 5 cc. Cu(OH) ₂ . . .	20	0.000010	0.00000	0.00000
VI. 0.01005 gram alanine, 20 cc. H ₂ O, 25 cc. "Buffer" solu- tion, 5 cc. Cu(OH) ₂	5	0.00448	0.00448	0.00449
VII. 0.00502 gram alanine other- wise as "VI"	5	0.00224	0.00223	0.00224
VIII. 0.00517 gram glycyl-glycine, 20 cc. H ₂ O, 25 cc. "Buffer" solution, 5 cc. Cu(OH) ₂ . . .	5	0.00314	0.00312	0.00318

(7) *Experiments with Histidine.*—To remove the copper quantitatively from all of the complexes, the solutions may be acidified with a little 0.1 N H_2SO_4 . After warming, hydrogen sulfide is passed through the solution until all of the copper has been converted into the sulfide. The cupric sulfide is then filtered and treated as described in a previous paper¹ and the amino substances may be recovered by removing the sulfate.

In order to precipitate all the copper except that of the histidine, we have found an excellent precipitant in normal amino caproic acid and also in phenyl glycine. These amino acids even in the slightest excess, will precipitate the copper of all the amino acids, polypeptides and peptones, quantitatively, with the exception of histidine copper. Even the copper from alkaline tartrates and citrates (Fehling's and Benedict's reagent for sugar) can be thrown down as an insoluble complex with these amino acids. This insoluble copper complex may then be dissolved in a little warm acetic acid and titrated iodometrically. In the near future we expect to report on the advantages of these two amino acids in analytical work with copper.

Therefore, to determine histidine the procedure is as follows: The solution containing histidine is treated, as has been described, with the "amino acid reagent" and filtered. To an aliquot portion of the filtrate is added a slight excess of n -amino caproic acid solution (in most cases 2 to 5 cc. of a 1% solution is ample) and the solution is filtered. After washing with distilled water and concentrating filtrate and wash waters *in vacuo* to about 15 cc. copper histidine may then be titrated iodometrically. The following table shows that the copper of histidine is not precipitated by these amino acids but is partially precipitated (about one-third) by boiling and by barium hydroxide.

HISTIDINE EXPERIMENTS.

Amount taken. Gram.	CuO found in filtrate. Gram.	CuO found in residue with 5 cc. 0.360 N $Ba(OH)_2$.	Total CuO found. Gram.	Theoretical CuO. Gram.	Ratio CuO found to theor. Per cent.	"Precipitability." Per cent.
0.01010	0.00118	0.00118	100.0	..
0.01010	0.00067	0.00050	0.00117	0.00118	99.2	34.2
0.01010	0.00118	0.00118	100.0	..
0.01010	0.00115	0.00118	97.5	..
After adding 2 cc. of 1% solution of n -amino caproic acid to the complex, instead of the barium hydroxide.						
0.01010	0.00075	0.00000	0.00118	0.00118	100.0	0.0
0.01010	0.00116	0.00000	0.00116	0.00118	98.3	0.0
After boiling the "neutral" complex 5 minutes.						
0.01010	0.00075	0.00041	0.00116	0.00118	98.3	35.3
After boiling the complex 5 minutes in Buffer solution.						
0.01010	0.00107	0.00009	0.00116	0.00118	98.3	7.8

In making the estimations of precipitabilities of all the other substances

¹ K. Sugiura and Kober, THIS JOURNAL, 34, 818 (1912).

given in this paper, the precipitability with an excess of these amino acids used as reagents was in each case also carried out. As stated before, only with histidine was any copper found in the filtrate after adding these substances. With all the other substances the copper was found as an insoluble, washable, filterable complex.

(8) *Experiments with Proteins, Peptones and Bromo Acid Derivatives of Polypeptides.*—In the following experiments the substances were dissolved in water, adding a little 0.1 *N* sodium hydroxide, if necessary, and treated with our amino acid reagent as described. The total volume (50 cc.) was then divided into two parts. The first part was filtered directly and the results of these are given in column 3. The second part was allowed to stand in a stoppered flask for 15 minutes with 5 cc. of 0.38 *N* Ba(OH)₂, and these are given in column 4.

PROTEINS AND PEPTONES.

Substance.	Amount taken. Gram.	CuO in filtrate without alkali. Gram.	CuO in filtrate af- ter adding 5 cc. 0.38 <i>N</i> Ba(OH) ₂ . Gram.	CuO in filtrate af- ter adding 5 cc. 0.38 <i>N</i> NaOH. Gram.
Egg albumin (Thomas & Co.).....	0.02538	0.00000	0.00069	0.00095
Egg albumin (E. & A.).....	0.02678	0.00000	0.00088	0.00080
Casein (Merck).....	0.02578	0.00011	0.00095	0.00067
Edestin.....	0.02568	0.00000	0.00093	0.00102
Roche peptone.....	0.02533	0.00455	0.00353
Meat peptone.....	0.02645	0.00180	0.00107
Witte's peptone.....	0.02675	0.00093	0.00158
Merck peptone.....	0.02630	0.00081	0.00131

These results show that the native proteins give no appreciable copper complex with our reagent. This is of great value in proteolysis, as an increase in the amount of copper dissolved will indicate the amount of hydrolysis.

BROMO-ACID DERIVATIVES.

Substance.	Amount taken. Gram.	CuO in filtrate without alkali. Gram.	CuO in filtrate after adding 5 cc. 0.38 <i>N</i> Ba(OH) ₂ . Gram.	Theoretical CuO. Gram.	Ratio of CuO in filtrate to theor. Per cent.	Ratio ¹ when 20 cc. 1 <i>N</i> NaOH was used. Per cent.
Brom-propionylglycine.....	0.02538	0.00000	0.00000	0.00481	0.0	..
Brom-butryl-glycine.....	0.02570	0.00000	0.00000	0.00456	0.0	..
Brom-propionylglycyl-glycine.....	0.02538	0.00017	0.00081	0.00378	21.4	76.4
Brom- <i>n</i> -caproyl-glycyl-glycine.....	0.02568	0.00000	0.00041	0.00330	12.4	67.8
Brom-propionyl-diglycyl-glycine.....	0.02640	0.00016	0.00300	0.00323	92.9	95.0
Brom-butryl-diglycyl-glycine.....	0.02555	0.00015	0.00275	0.00300	91.7	95.0

¹ Taken from a previous paper, *Am. Chem. J.*, 48, 387, 389 (1912).

(9) *Experiments with Small Amounts of Copper.*—To show that small amounts of copper can be determined, by iodometric titrations, the following experiments were made:

Concentration of $\text{Cu}(\text{NO}_3)_2$ solution.	Amount used in each test. Cc.	Titrated after neut. with NaOH acetic acid. Cc. $\text{Na}_2\text{S}_2\text{O}_8$.	Titration of ppt. after adding $\text{Ba}(\text{OH})_2$. Cc. $\text{Na}_2\text{S}_2\text{O}_8$.	Ratio of residue to theoretical Per cent.
M/25	50.00	49.04 M/25	49.01 M/25	100.0
M/250	25.00	24.54 M/250	24.52 M/250	100.0
M/1000	25.00	24.64 M/1000	24.53 M/1000	100.0
M/1000	10.00	9.92 M/1000	9.87 M/1000	100.6

These figures demonstrate that one is apt to over-titrate very small amounts of copper and, therefore, they will help to explain slight variations in our work with amino substances.

(10) *Experiments with Lead Acetate and Ammonia.*—Five cc. of strong ammonia (sp. gr. 0.90) and 25 cc. of 0.10% lead acetate were added to a 2% glucose solution (25 cc.). After filtering off a heavy precipitate the filtrate was tested for sugar with Benedict's reagent; no appreciable reduction was noticed. The same result was obtained with levulose. As a few milligrams in 25 cc. H_2O of oxalic, citric and tartaric acid gave a precipitate it was assumed that these substances could be removed by this reagent.

To test the effect of lead acetate and ammonia, and the reagent necessary for the subsequent removal of the lead acetate and ammonia, on the estimation of amino acids and peptides, the following experiments were made:

LEAD ACETATE AND AMMONIA EXPERIMENTS.

I. Substance.	Amount taken. Gram.	CuO found in filtrate. Gram.	CuO found in residue. Gram.	Total CuO found. Gram.	Theoretical CuO . Gram.	Per cent.
(Using K_2S .)						
Glycine.....	0.00521	0.001366	0.001382	98.8
Glycine.....	0.01043	0.00275	0.00276	99.6
Alanine.....	0.00508	0.001174	0.001136	103.3
Alanine.....	0.01017	0.00008	0.00213	0.00221	0.00227	97.4
Alanine.....	0.01017	0.00006	0.00214	0.00220	0.00227	96.9
Alanine.....	0.00508	0.000021	0.001135	0.001156	0.001136	101.8
Glycyl-glycine.....	0.00504	0.001488	0.001519	98.5
Alanyl-glycine.....	0.00507	0.001304	0.001380	94.5
Alanyl-glycine.....	0.01015	0.00232	0.00035	0.00267	0.00276	96.7
Alanyl-glycine.....	0.01015	0.00245	0.00030	0.00275	0.00276	99.6
Glycyl-alanyl-glycine.....	0.01006	0.00191	0.00197	97.0
Glycyl-alanyl-glycine.....	0.01006	0.00182	0.00012	0.00194	0.00197	98.5
(Using K_2SO_4 .)						
Glycine.....	0.00521	0.001318	0.001382	95.4
Glycyl-glycine.....	0.00504	0.001467	0.001519	96.6

Having shown that the aliphatic amino acids and their polypeptides

(Using K_2S .)

Substance mixtures contained each.	Amount taken. Gram.	CuO found in filtrate. Gram.	CuO found in residue. Gram.	Total CuO found. Gram.	Theoretical CuO Gram.	Per cent.
Glycine.....	0.001034	0.000274	...
Alanine.....	0.001004	0.000224	...
Valine.....	0.001014	0.000172	...
Leucine.....	0.001042	0.000158	...
Cystine.....	0.001022	0.000169	...
Tyrosine.....	0.001020	0.000112	...
Lysine picrate.....	0.001068	0.000057	...
Arginine-di- HNO_3	0.001072	0.000071	...
Histidine-di-HCl.....	0.001663	0.000146	...
Copper proline.....	0.001028	0.000132	...
Copper tryptophan.....	0.000472	0.000039	...
Aspartic acid.....	0.001048	0.000157	...
Glutamic acid.....	0.001209	0.000164	...
Mixture A.....	0.01369	0.00171	0.00187	87.3
Mixture B.....	0.01369	0.000000	0.00157	0.00157	0.00187	83.9
Mixture C, 0.5 gram dextrose.....	0.01369	0.000000	(0.00234)	(0.00234)	0.00187	(124.8) ¹
Mixture D, 0.5 gram levulose.....	0.01369	0.000000	0.00188	0.00188	0.00187	100.4
Mixture E.....	0.01369	0.00177	0.00187	94.7
Mixture F.....	0.01369	0.00178	0.00187	94.8
Mixture G.....	0.01369	0.000269	0.00149	0.00176	0.00187	93.9

III.

Substance Mixtures.	Amount taken. Gram.	H_2O . Cc.	Conc. NH_4OH . Cc.	10 per cent. $Pb(C_2H_3O_2)_2$. Cc.	Precipitation. + or --.
Glycine					
Alanine	0.075	25	5	25	—
Valine					
Leucine					
Cystine	0.075	25	5	25	+
Tyrosine					
Lysine picrate					
Arginine-di- HNO_3	0.075	25	5	25	+ Orange
Histidine-di-HCl					
Copper proline	0.050	25	5	25	+
Copper tryptophan					
Aspartic acid	0.050	25	5	25	—
Glutaminic acid					
Glycine					
Alanine	0.030	25	5	25	—
Valine					
Leucine					
Cystine	0.030	25	5	25	—
Tyrosine					

¹ This high result is probably due to insufficient K_2S and, therefore, some NH_3 remained, which dissolved a small amount of copper.

IV. Substance.	Amount taken. Gram.	H ₂ O. Cc.	Conc. NH ₄ OH. Cc.	10% Pb(C ₂ H ₃ O ₂) ₂ . Cc.	Precipitation. + or —.
Lysine picrate	0.030	25	5	25	+
Arginine-di-NO ₂					
Histidine-di-HCl					
Copper proline	0.030	25	5	25	+
Copper tryptophan					
Aspartic acid	0.030	25	5	25	—
Glutaminic acid					
All-above-mixture.....	0.0250	25	5	25	—
Leucine.....	0.050	25	5	25	—
Cystine.....	0.050	25	5	25	—
Tyrosine.....	0.050	25	5	25	+
Lysine picrate.....	0.050	25	5	25	Yellow +
Arginine-di-NO ₂	0.050	25	5	25	trace
Histidine-di-HCl.....	0.050	25	5	25	+
Copper proline.....	0.050	25	6	25	—
Copper tryptophan.....	0.050	25	5	25	+
Glycyl-glycine.....	0.050	25	5	25	—
Alanyl-glycine.....	0.050	25	5	25	—
Gly-alanyl-glycine.....	0.050	25	5	25	—
Glycyl-glycyl-leucine.....	0.050	25	5	25	—
Witte's peptone.....	0.050	25	5	25	+
Roche peptone.....	0.050	25	5	25	—

can be determined after using the "lead acetate and ammonia" technic, it was of importance to try the other amino acids in the same way. Table II shows that very small amounts of all amino acids may be determined after treatment with lead acetate and ammonia. In Table III various mixtures were tried in a qualitative way. In Table IV, smaller amounts of mixtures gave slightly different results. In Table IV, about 50 milligrams of various amino acids and peptides were tested with this technic.

(11) *Total Nitrogen Estimations.*—In a few cases where the copper method gave low results a Kjeldahl estimation was made on the same substances as a check on the purity of the substance. In the following table the results show that low results with the copper method as a rule, were accompanied by low results in the total nitrogen estimation and where good re-

Substance.	Per cent. of nitrogen theoretical.	Per cent. of nitrogen found by Kjeldahl method.	Ratio of CuO found to theoretical. Per cent.	Ratio of total nitrogen found to theoretical. Per cent.
Proline.....	12.7	11.16	91.8	91.6
Glycyl-tryptophan.....	16.10	13.54	87.0	83.7
Phenylalanine 1.....	8.48	7.86	90.0	92.7
Phenylalanine 2.....	8.48	8.60	100.0	101.4
Glutamic acid.....	9.53	9.58	100.4	100.7
Amino butyric acid.....	13.58	13.42	99.0	98.8
Alanyl-glycine.....	19.17	19.01	98.6	99.1
Alanine.....	15.70	15.30	99.2	97.3

sults were obtained with the copper, the total nitrogen estimations also agreed well with the theoretical.

11. Summary.

(1) The method of forming copper complexes of amino acid, peptides and peptones in neutral or slightly alkaline solution ($C_H = 10^{-7.07}$ to $10^{-8.5}$) has been improved so that quantitative results can be obtained in very dilute solution (one part in 500,000).

(2) The "precipitability" of the copper in the complexes of 19 amino acids, 27 di-peptides, 18 tri-peptides, and 4 tetra-peptides is given, when in equilibrium with 0.06 *N* Ba(OH)₂ and it is shown that the amount of copper dissolved by the free amino acid as well as the amount dissolved by the peptides and peptones, can be separately determined; thus, estimating these substances in micro-chemical mixtures.

(3) Experiments are given to show that under the conditions of this method, very few other substances react with the reagent and that these can easily be removed, without interfering with the estimation of amino acids and their homologues.

(4) The method is applied to proteolysis, blood and urin and it is shown that amino acids, peptides and peptones can be estimated accurately and quickly in small amounts of material. It may, therefore, be called a "clinical" method.

(5) Two *very insoluble copper complexes*, *n*-amino caproic acid and phenyl glycine copper are found which may be useful in analytical work with copper.

(6) Ammoniacal lead acetate (5 cc. strong NH₃, sp. gr. 90, to 25 cc. 10% lead acetate) is shown to precipitate sugars, dicarboxylic acids, such as oxalic, citric, tartaric and in moderate dilutions (one part to 5,000) the amino acids, histidine, tyrosine and tryptophan. Under the same conditions none of the other amino acids and of the peptides, are precipitated. In very dilute solution (one to 25,000) this reagent does not seem to precipitate any amino acids.

(7) By means of the amino acids, *n*-amino caproic acid and phenyl-glycine, the copper of all the other complexes including those of the amino acids and all the polypeptides thus far studied, with the exception of histidine, can be thrown down quantitatively. Therefore, by means of these reagents, we can determine histidine in very small quantities, accurately and quickly.

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