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# The Reaction between Mercury(II) and Organic Compounds. III. Separation and Quantitative Determination of Basic Amino Acids with Mercury(II)-Salt Precipitants

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The particular reactivities between mercury(II) chloride and basic amino acids give such products as [Arg-Hg]Cl·$HgCl_2$, [His-Hg]Cl·$HgCl_2$, and [Lys-Hg]Cl·$HgCl_2$. The possibility of the application of these reactivities to the separation and determination of basic amino acids has now been studied. The products were obtained preferentially from a mixture of various amino acids. The isolation of the mixture of the products could be carried out effectively by the utilization of the different solubilities of the products in aqueous sodium hydroxide. All basic amino acids except His usually do not show any absorption in the ultraviolet region. The above products, however, showed characteristic absorption curves which differed from those of the components. In these spectra, although there were no absorption maxima, calibration curves which were in a good linear relationship could be obtained at any proposed wavelength. By measuring the absorbances of aqueous hydrochloric acid (pH 1.2) or sodium hydroxide (pH 12.0) solutions of the products, quite a small amount of each basic amino acid could be determined spectrophotometrically in the ultraviolet region, within the detectable limits of $4\times10^{-4}$—$6\times10^{-5}$ M. The accuracy obtained by the determination of the basic amino acid content in an ovalbumin makes it possible to recommend the present methods.

The separation, isolation, and quantitative determination of basic amino acids in various proteins have hitherto been studied by many groups of workers. The analyses using phosphotungstic acid,[1] silver salts,[2,3] ion exchange resins[4-11] are well-known established methods, while electrophoresis,[12,13] various chromatographic techniques,[13-15] spectral reflectance[16] and conductivity measurements[17] have also been used in recent years. However, all of these procedures are time-consuming or complicated. Warburg *et al.*[18-20] have investigated the separation of basic amino acids by using mercury(II) chloride, acetate, or sulfate, and reported that His was almost exclusively dealt with using mercury(II) salt, while Arg and Lys were isolated by using silver salts.

The present author has shown in an earlier publication[21] that there is possibility of separating and determining all the basic amino acids by utilizing their particular reactivities with mercury chloride.

The present paper will study this point in more detail and will propose an analytical method for basic amino acids. Finally, it will describe the determination of basic amino acids in an ovalbumin using the proposed method. As a result it will be shown clearly that the present method can make possible a brief and rapid preferential separation and an accurate quantitative determination, and that it is more suitable than the many reported methods mentioned above.

## Experimental

**Materials.** Arg, His, and Lys were obtained from the Nippon Rikagaku Yakuhin Co., Ltd., while reagent-grade mercury(II) salts were obtained commercially.

1) Van Slyke, *J. Biol. Chem.*, **22**, 281 (1915).
2) H. B. Vickery and R. J. Block, *ibid.*, **93**, 105 (1931).
3) G. R. Tristram, *Biochem. J.*, **33**, 1271 (1939).
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10) J. E. Eastoe, *Biochem. J.*, **74**, 8 (1960).
11) S. Jacobs, *ibid.*, **87**, 30 (1963).
12) G. N. Atfield and C. J. O. R. Morris, *ibid.*, **74**, 37 (1960).
13) T. Shinoda and K. Satake, *J. Biol. Chem.*, **50**, 293 (1961).
14) A. Darbre and K. Blau, *Biochem. J.*, **88**, 8 (1963).
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17) A. Nieman, *Z. Anal. Chem.*, **205**, 403 (1964).
18) O. Warburg and W. Christian, *Biochem. Z.*, **310**, 304 (1942).
19) A. Kossel, *Z. Physiol. Chem.*, **22**, 176 (1896).
20) G. L. Foster and D. Shemin, *Org. Syn.*, **18**, 42 (1938).
21) F. Kai, *Nippon Kagaku Zasshi (J. Chem. Soc. Japan, Pure Chem. Sect.)*, **86**, 1040 (1965).

Mercury(II) perchlorate was prepared by a procedure similar to those reported previously.[22] All the other chemicals purchased were of an analytical or of a special grade; they were used without further purification. The buffer solutions for pH 1.2 and 12.0 were prepared by mixing, respectively, 0.2 N potassium chloride and 0.2 N hydrochloric acid, and 0.1 N sodium hydroxide and di-sodium hydrogen phosphate (17.81 g/*l*) in the ratios required. They were used when the spectrophotometric determination was carried out, unless otherwise stated.

**Apparatus.** The pH was measured with a glass electrode (Hitachi-Horiba type M-5). The absorption spectra and the absorbances were measured with a Hitachi recording spectrophotometer, EPS-2 type, and a Hitachi photoelectric spectrophotometer, EPU-2 type, respectively.

The mercury content in the products was determined by a chelatemetric titration[23] or by a colorimetric determination.[24,25]

## Recommended Procedures

**The Selective Separation of Basic Amino Acids in a Mixture of Various Amino Acids.** Crystalline solid mercury(II) chloride, perchlorate, or an aqueous solution of one of these salts was added at pH 3—4 to an acid sample solution containing a mixture of acidic, neutral, and basic amino acids. After the mixture had been stirred to dissolve the mercury salts substantially, aqueous sodium hydroxide (0.1 to 0.01 N) was added drop by drop, into the solution at room temperature until the pH of the supernatant reached 9.0. In this procedure, particular attention was paid not to add an excess amount of the alkaline solution, since the [His-Hg]X·$HgX_2$ (X=Cl or $ClO_4$) thus precipitated was dissolved again and the [Lys-Hg]X·$HgX_2$ precipitates were apt to be decomposed above pH 13.4 at room temperature. In order to verify the complete precipitation, a small volume of the supernatant was taken out and the same procedure was carried out again after the addition of a slight excess of mercury(II) chloride or perchlorate; when no more precipitate was produced by this procedure, the yield was concluded to be satisfactory. The precipitates were filtered under reduced pressure, washed, and suspended in water. The precipitates were always kept wet during these procedures. The suspension was acidified with hydrochloric acid until the precipitates were dissolved thoroughly. The solution thus obtained was then bubbled through with hydrogen sulfide; the black precipitates of mercury sulfide thus produced were filtered off and repeatedly washed with water. The filtrate was combined with washings, acidified to pH 3—4 with hydrochloric acid, and then heated to remove the excess hydrogen sulfide. Thereby, a solution of only the basic amino acids could be obtained.

The isolation procedures which will be described below are restricted to those utilizing mercury-(II) chloride.

**The Isolation of Each Basic Amino Acid from the Mixture.** It was found that the solubility of each basic amino acid as a mercury salt was different at each pH of the solution.

**Arg.** A wet mixture of Arg, His, and Lys as mercury salts was suspended in water, aqueous sodium hydroxide (0.1 to 0.01 N) was added by stirring and the pH was adjusted to 12.2—12.3 at room temperature. By heating this suspension until it boiled, only [Arg-Hg]Cl·$HgCl_2$ was dissolved at this pH; filtration was then carried out keeping the suspension hot, and the residue was washed with hot water at pH 12.2. When the filtrate was then cooled, [Arg-Hg]Cl·$HgCl_2$ was reprecipitated.

**Lys and His.** The residue was suspended again in aqueous sodium hydroxide (pH 12.7—12.8). When the suspension was boiled, only the [Lys-Hg]Cl·$HgCl_2$ was dissolved, while the [His-Hg]-Cl·$HgCl_2$ remained.

**The Quantitative Determination by the Spectrophotometric Method.** [His-Hg]Cl·$HgCl_2$ was dissolved in a dilute hydrochloric acid or sodium hydroxide, and the solutions were adjusted to pH*1 1.2 or 12.0 using a Clark-Lubs buffer (KCl+HCl) or a Kolthoff buffer ($Na_2HPO_4$ +NaOH) respectively; the His content in its mercury salt was determined by measuring the absorbances of the above solutions. [Arg-Hg]Cl·$HgCl_2$ and [Lys-Hg]Cl·$HgCl_2$ were dissolved in a dilute hydrochloric acid and brought to pH 1.2*1 with the above buffer system of Clark-Lubs; then the Arg and Lys contents were similarly determined.

## Results and Discussion

When an aqueous sodium hydroxide was dropped into an aqueous solution of a mixture of mercury-(II) chloride or perchlorate and acidic amino acids (Asp and Glu), mercury oxide was formed immediately. Similar phenomena were also observed when the mixture was prepared using three kinds of neutral amino acids (Ala, Hypro, and Leu). Other neutral amino acids (Met, Phe, Ser, and Try) did not gave any precipitate

22) F. Kai, This Bulletin, **40**, 1136 (1967).
23) K. Ueno, "Chelate Titration" (in Japanese), Nankodo, Tokyo (1964), p. 268.
24) E. B. Sandell, "Colorimetric Determination of Traces of Metals," Interscience Publ., New York (1959), p. 621.
25) F. D. Snell and C. T. Snell, "Colorimetric Method of Analysis," C. Van Nostrand Co., Philadelphia (1959), p. 63.

*1 At about these pH values, the salts could be dissolved as mercury salts in an approximate concentration of $10^{-2}$ M.

at all when thus treated. When Gly, one of the neutral amino acids, was tested similarly, however, amorphous black precipitates were obtained. On the other hand, if basic amino acids (Arg, His, and Lys) were used, only amorphous white precipitates were produced.

Since the phenomena described above had been observed when each testing mixture was composed of mercury(II) chloride or perchlorate and an individual basic amino acid, similar experiments on the aqueous solution of a mixture consisting of mercury(II) chloride or perchlorate, and all the acidic, neutral, and basic amino acids were carried out. Contrary to the above individual case, the mixture gave only amorphous white precipitates formed selectively between mercury(II) salts and basic amino acids, as in the case of an individual test. In addition, unexpectedly, no interference by Gly, which gave a black precipitates, was observed.

On the basis of these results, it seems that this reactivity may be adopted for the preferential separation of basic amino acids in a mixture of various amino acids.

In order to clarify the role of anions, the effect on this peculiar precipitate formation by several mercury(II) slats was investigated. Nitrate, sulfate, and acetate could not utilized, however, because of their decomposition into mercury oxide under these experimental conditions. Mercury(I) salts were not employed either because of their lower solubilities in water or decomposition into mercury oxide.

**The Structures and Properties of the Products between Basic Amino Acids and Mercury(II) Chloride.** The discussions described below are restricted to the results obtained using merucry(II) chloride. The products were analyzed as follows:

**Arg.** Found: C, 9.98; H, 1.59; N, 8.05; Cl, 15.51; Hg, 60.26%. Calcd for $C_6H_{13}N_4O_2$-$Hg_2Cl_3$: C, 10.59; H, 1.92; N, 8.23; Cl, 15.62; Hg, 58.93%. Mp 188°C (decomp.).

**His.** Found: C, 11.05; H, 1.19; N, 6.14; Cl, 16.70; Hg, 59.25%. Calcd for $C_6H_8N_3O_2Hg_2$-$Cl_3$: C, 10.89; H, 1.21; N, 6.35; Cl, 16.73; Hg, 60.64%. Mp 212—213°C (decomp.).

**Lys.** Found: C, 11.15; H, 1.82; N, 4.38; Cl, 16.30; Hg, 60.95%. Calcd for $C_4H_9N_2O_2$-$Hg_2Cl_3$: C, 11.04; H, 2.00; N, 4.29; Cl, 16.29; Hg, 61.46%. Mp 171—173°C (decomp.). Accordingly, the structures of these compounds may be considered to be as follows:

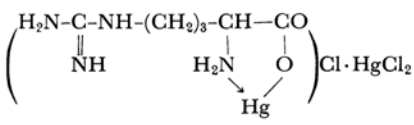
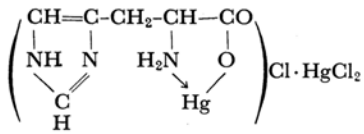
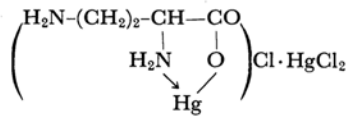


Abb. as [Arg-Hg]Cl·$HgCl_2$



[His-Hg]Cl·$HgCl_2$



[Lys-Hg]Cl·$HgCl_2$

All the products are not soluble in water and organic solvents, but they are soluble in an acid. In an aqueous solution of a strong alkali, [His-Hg]Cl·$HgCl_2$ is soluble at room temperature, while [Arg-Hg]Cl·$HgCl_2$ and [Lys-Hg]Cl·$HgCl_2$ are not soluble at that temperature, but they are soluble in hot alkali.

## Application to the Quantitative Determination of Basic Amino Acids

**The Stoichiometric Study of the Components.** The yields of the products varied with the ratios of the components. The results are illustrated in Fig. 1. The concentrations of both mercury(II) chloride and a basic amino acid in the original standard aqueous solutions were $5\times10^{-3}$ M, and the amount of each amino acid solution used was 10 *ml*, corresponding to $5\times10^{-5}$ mol. Into this solution of mixtures, 0.1 N aqueous sodium hydroxide was added drop by drop while stirring. The yields of the products at a given ratio of two components were determined by the spectrophotometric method, by analyzing the nitrogen content, and by observing ninhydrine reaction in an aliquot of the filtrate. As can be

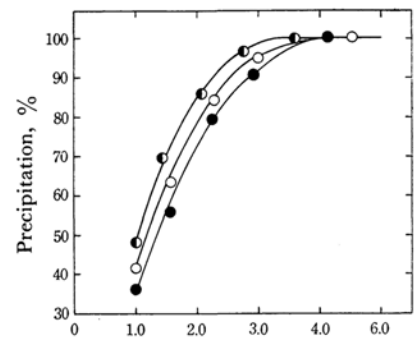



Fig. 1. Effect of varying ratio of component on precipitate formation.
-●- [Arg-Hg]Cl·$HgCl_2$
-○- [His-Hg]Cl·$HgCl_2$
-◐- [Lys-Hg]Cl·$HgCl_2$
Original concn. of each component std. soln. was $5\times10^{-3}$ M

seen from this figure, at the ratio of 1.0 the yields of Arg, His, and Lys were 35, 41, and 48% respectively. Furthermore, at the ratio of 2.0, which was considered to be the theoretical value for a complete precipitate formation, the yields were no more than 73—85%. On increasing the ratio, sufficient yields were obtained at the ratios of 4.1 for Arg, 4.6 for His, and 3.6 for Lys. Therefore, more than five-times as much mercury(II) chloride against each basic amino acid is required at least. The influence of excess mercury(II) chloride (about ten-times as much mercury as each basic amino acid) on the precipitate formation was also studied. The formation, however, was not affected by that, and nor was any production of mercury oxide observed under these conditions.

**The Effect of pH on the Precipitate Formation.** The Yields of the products were affected by the pH of the sample solution. Dilute aqueous sodium hydroxide (0.1—0.01 N) was added, drop by drop, into mixtures consisting of $2\times10^{-4}$ M of each amino acid and $1.4\times10^{-3}$ M of mercury(II) chloride. The results, shown in Fig. 2, suggest that the pH values of the solutions of mixtures which gave substantial yields were 8.9—9.0 for Arg, 7.4—7.6 for His, and 8.6—8.8 for Lys.

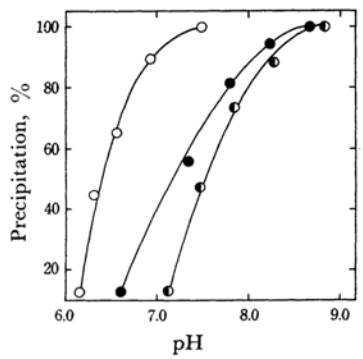



Fig. 2. Effect of pH on precipitate formation.
-◐- [Arg-Hg]Cl·$HgCl_2$
-○- [His-Hg]Cl·$HgC_2$
-●- [Lys-Hg]Cl·$HgCl_2$

**Spectrophotometric Analysis.** The ultraviolet absorption curves for the products and for their components are illustrated in Fig. 3. The spectra of the acidic solutions of [Lys-Hg]Cl·$HgCl_2$ (curve A), [Arg-Hg]Cl·$HgCl_2$ (curve B) and [His-Hg]Cl·$HgCl_2$ (curve C) show characteristic curves markedly different from those of the components, Lys (curve A'), Arg (curve B'), His (curve C'), and mercury(II) chloride (curve D). As can also be seen from Fig. 3, since the corresponding free amino acid content in these samples is small ($8.1\times10^{-5}$ M for Lys, $7.2\times10^{-5}$ M for Arg, and $4.0\times10^{-5}$ M for His), the quantitative determination by the spectrophotometric method can be employed for a micro amount of basic amino acids.

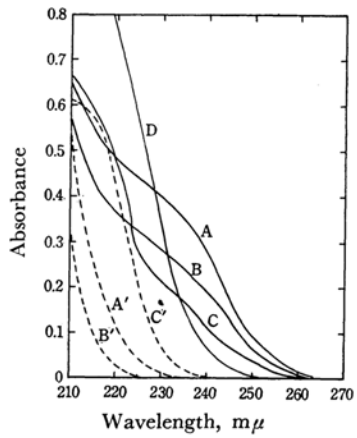



Fig. 3. The absorption spectra in acidic solutions (pH 1.2):

| | | |
|---|---|---|
| A | [Lys-Hg]Cl·$HgCl_2$ | ($8.1\times10^{-5}$ M) |
| B | [Arg-Hg]Cl·$HgCl_2$ | ($7.2\times10^{-5}$ M) |
| C | [His-Hg]Cl·$HgCl_2$ | ($4.0\times10^{-5}$ M) |
| A' | Lys | ($10^{-2}$ M) |
| B' | Arg | ($10^{-2}$ M) |
| C' | His | ($10^{-4}$ M) |
| D | $HgCl_2$ | ($2\times10^{-4}$ M) |

**Calibration Curves.** The optimum wavelength for the quantitative determination must be ascertained because of no maxima in all the absorption curves of the products. After many trials, the calibration curves which were in a good linear relationship at the proposed wavelength were obtained, as will be seen below. The amino acid concentrations scaled in Figs. 4—7 are shown as calculated amounts as free amino acids in the corresponding mercury salts of basic amino acids respectively.

**His.** The calibration curves on His in an acidic aqueous solution (HCl, pH 1.2), shown in

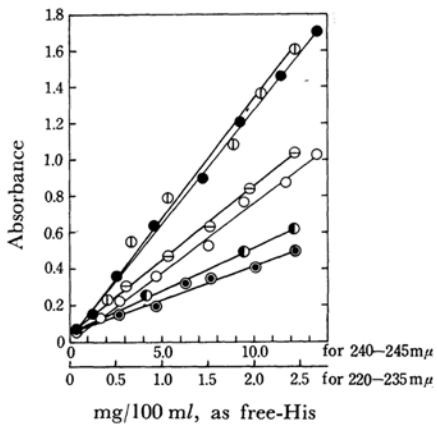



Fig. 4. The calibration curves on His in acidic solutions (pH 1.2):
-◐- 220 mμ; -⊖- 225 mμ; -◐- 230 mμ;
-⊙- 235 mμ; -●- 240 mμ; -○- 245 mμ

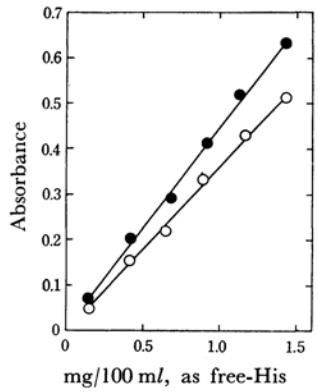



Fig. 5. The calibration curves on His in a basic solutions (pH 12.0).
-●-, 228 mμ -○-, 234 mμ

Fig. 4, are obtained at a wavelength fixed arbitrarily in the region between 220—245 mμ. About $1.6\times10^{-4}$ M of free His can be determined in the 220—235 mμ region, while about $9\times10^{-4}$ M of free His can be determined in the 240—245 mμ range. Experiments on His in sodium hydroxide solutions were carried out similarly; the results obtained are summarized in Fig. 5. About $10^{-4}$ M of His can also be determined in aqueous sodium hydroxide solutions (pH 12.0) in the 228—234 mμ region.

**Arg.** Figure 6 shows the curves of Arg in the range of 230—250 mμ. Free Arg up to about $4\times10^{-4}$ M in the 230—235 mμ region and up to about $8\times10^{-4}$ M in the 240—250 mμ region can be determined.

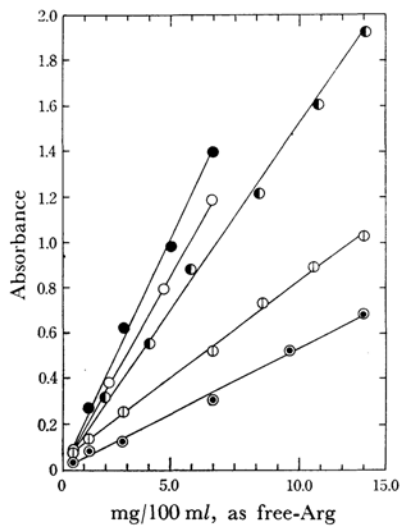



Fig. 6. The calibration curves on Arg in acidic solutions (pH 1.2).
-●- 230 mμ; -○- 235 mμ; -◐- 240 mμ;
-◑- 245 mμ; -⊙- 250 mμ

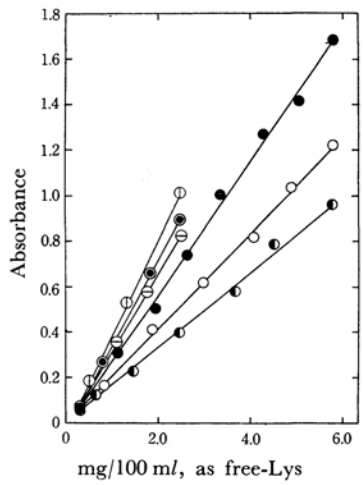



Fig. 7. The calibration curves on Lys in acidic solutions (pH 1.2).
-◑- 220 mμ; -⊙- 225 mμ; -⊖- 230 mμ;
-●- 240 mμ; -○- 243 mμ; -◐- 245 mμ

**Lys.** The curves for free Lys up to $1.7\times10^{-4}$ M in the 220—230 mμ region and to $4\times10^{-4}$ M in the 240—245 mμ region show a fair linearity (Fig. 7).

**Recoveries by the Spectrophotometric Determination.** The recoveries of basic amino acids by the present method were examined; and the data obtained are listed in Tables 1 and 2. Table 1 shows the results obtained when each aqueous testing solution was composed of a mixture of mercury(II) chloride and an individual basic amino acid, while Table 2 shows those obtained from an aqueous solution of a mixture of mercury-(II) chloride and all the basic amino acids. Sufficient recoveries, almost 100%, were shown in all cases.

**Determination of Basic Amino Acids in an Ovalbumin.** Further confirmation of the usefulness of the proposed method was carried out by the determination of basic amino acids in an ovalbumin. The procedure was as follows: 100 ml of water and 20 ml of concentrated sulfuric acid were added to about 50 g of the ovalbumin in a 300 ml glass-stoppered flask equipped with a condenser; then the mixture was boiled for about 24 hr. A deep brown digestion liquor was obtained in proportion to the hydrolization. After cooling, the excess sulfate ions in the liquor were removed by the addition of aqueous saturated barium hydroxide and the solution was brought to pH 7—8. The barium sulfate which precipitated out and a humine were separated by centrifuging; then about 20 g of mercury(II) chloride was added to the supernatant layer (about 500 ml), and the mixture was stirred until the mercury(II) chloride had dissolved completely. By the drop by drop addition of aqueous sodium hydroxide

TABLE 1. RECOVERIES OF A BASIC AMINO ACID FROM AN EACH AQUEOUS SOLUTION BY THE SPECTROPHOTOMETRIC DETERMINATION

| Arg | | | His | | | Lys | | |
|---|---|---|---|---|---|---|---|---|
| Added mg | Found mg | Recoveries % | Added mg | Found mg | Recoveries % | Added mg | Found mg | Recoveries % |
| 2.2 | 2.3 | 104.5 | 5.7 | 5.3 | 93.0 | 1.0 | 0.9 | 90.0 |
| 4.6 | 4.1 | 89.7 | 3.3 | 3.0 | 90.8 | 3.2 | 3.3 | 103.1 |
| 5.8 | 5.7 | 98.2 | 4.0 | 4.1 | 102.4 | 4.8 | 4.8 | 100.0 |
| 11.3 | 11.0 | 97.3 | 15.3 | 14.7 | 96.0 | 9.8 | 9.9 | 101.0 |
| 15.0 | 15.3 | 102.0 | 2.8 | 2.7 | 98.9 | 17.1 | 16.5 | 96.5 |

TABLE 2. RECOVERIES OF A BASIC AMINO ACID FROM AN AQUEOUS SOLUTION CONTAINING MIXTURE OF THE BASIC AMINO ACIDS BY THE SPECTROPHOTOMETRIC DETERMINATION

| Added, mg | | | Found, mg | | | Recoveries, % | | |
|---|---|---|---|---|---|---|---|---|
| Arg | His | Lys | Arg | His | Lys | Arg | His | Lys |
| 2.7 | 15.4 | 5.8 | 2.3 | 15.8 | 5.3 | 85.1 | 102.6 | 91.4 |
| 3.9 | 4.6 | 12.6 | 4.0 | 4.5 | 12.2 | 102.5 | 97.8 | 96.9 |
| 9.2 | 3.3 | 7.6 | 9.0 | 3.1 | 7.3 | 97.8 | 93.5 | 96.0 |

(0.1 N) to this solution, and by then allowing it to stand overnight in a refrigerator, white products were formed between mercury(II) chloride and the basic amino acids. The succeeding isolation and quantitative determination were carried out in a manner similar to those described hitherto. The results, together with the data reported by Folin *et al.* and Wieland, are listed in Table 3.

TABLE 3. THE BASIC AMINO ACIDS IN OVALBUMIN

| | Arg, % | His, % | Lys, % |
|---|---|---|---|
| a) | 7.80 | 2.29 | 7.71 |
| b) | 7.6 | 2.57 | 10.0 |
| c) | 5.72 | 2.35 | 6.30 |

a) The spectrophotometric determination by the present method

b) O. Folin, *J. Biol. Chem.*, **56**, 377 (1922); O. Folin, *ibid.*, **51**, 393 (1922); E. G. Frame, J. A. Russell and A. E. Wilhelmi, *ibid.*, **149**, 255 (1943); N. H. Furman, G. H. Morrison and A. F. Wagner, *Anal. Chem.*, **22**, 1561 (1950).

c) T. Wieland and L. Wirth, *Ber.*, **76**, 823 (1943).

From these results it can be proposed that the present methods for the separation, isolation, and determination of basic amino acids make a very good comparison with the other procedures, which are complicated and time-consuming. In addition, there is no procedure except that using phosphotungstic acid for the simultaneous separation of three kinds of basic amino acids in various amino acids.

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