CLXXVIII. THE COLORIMETRIC DETERMINATION OF HISTIDINE.

By ERIK JORPES.

*From the Physiological Chemistry Department of the Caroline Institute,
Stockholm, Sweden.*

(Received August 6th, 1932.)

THE reaction of iminazoles with diazobenzene sulphonic acid, which was introduced into protein chemistry by Pauly [1904], was used for an approximate quantitative estimation of histidine for the first time by Weiss and Ssobolew [1914]. Later Koessler and Hanke [1919] elaborated their colorimetric method for the determination of histidine in protein hydrolysates. The diazo-compound formed was also determined by volumetric titration by Lautenschläger [1918]. The compound was reduced by titanous chloride, and the excess of titanous salt was oxidised by ferric alum, thiocyanate being used as indicator.

Weiss and Ssobolew were well aware of the difficulties involved in the use of the reaction for quantitative purposes owing to the instability of the colour produced. They gave the following directions for the performance of the test. To 10 cc. of a histidine solution, containing 0.1 mg. histidine per cc., 1.5 cc. of the solution of the diazonium salt and then 3 cc. of a 10 % sodium carbonate solution should be added, thus assuring an optimum of purity and stability of colour.

Nevertheless Koessler and Hanke on theoretical grounds recommended a different procedure, which was also adopted by Hunter [1925] for the determination of iminazoles in the urine. They considered the reaction between the amino-group in histidine and the nitrous acid in the acid diazonium solution as undesirable. They therefore first mixed the diazonium salt with the carbonate solution and then after one minute added the histidine solution. Another reason in favour of this procedure was that the concentration of diazotate became maximum before the addition of the histidine solution. It is very probable according to them that the compound entering into reaction with histidine is a diazohydrate, $\text{NaOSO}_2\text{C}_6\text{H}_4\text{N}=\text{NOH}$ or an isomeric nitrosamine $\text{NaOSO}_2\text{C}_6\text{H}_4\text{NH}$, which is formed through hydrolysis of the

diazotate. By carefully noting the time for the addition of the histidine solution and the colorimetric reading it should be possible to obtain close agreement between the histidine content and the colour produced. Hanke

and Koessler [1920] used their method for the determination of the histidine content of protein hydrolysates. The histidine is of course to be separated by phosphotungstic acid or by the Kossel-Patten procedure from tyrosine, which also gives the reaction.

In analysing some blood-proteins for their content of histidine by the Koessler and Hanke method, using the Zeiss step-photometer, I have found it difficult to obtain reproducible results with their technique. With the photometer the development and fading of the colour could be followed much more easily than with a colorimeter, and furthermore the optimum field of the spectrum could be selected. Immediately after mixing the reagents a yellow colour appears which is less suitable for reading than the reddish colour which develops in the course of 2–3 minutes.

On attempting to determine the degree of light absorption, using solutions containing 0.08–0.01 mg. *L*-histidine dihydrochloride per cc., no definite coefficient of extinction was obtained. The readings were made with filter S. 50 where the reddish colour has its maximum of absorption. According to the descriptions given by Koessler and Hanke the histidine solution was added exactly one minute after mixing the other two solutions. The intensity of the colour should be maximum after 6 minutes and remain at this level for 2–3 minutes. However, the colour was found to be maximum in 3–3½ minutes after addition of the histidine solution and fading could be observed 5–6 minutes after mixing. Furthermore it was indifferent whether the histidine solution was added immediately after the diazo-solution or 2 minutes later. The coefficient of extinction, ϵ or $-\log_{10} \frac{I_p}{I_a}$, d being 1 cm., varied between 2.09 and 2.31 as calculated for an amount of 0.1 mg. histidine in 8 cc. of the final reaction mixture. The mean figure for 32 determinations was 2.19 ± 0.0140 . The error of the reading is very small and does not influence the results. In spite of its very common use during the last few years the reaction is apparently too labile to be considered reliable.

Without making any statements about the possible cause of this lability in the reaction it is possible to trace the influence of the diazotate solution. If the transformation of diazonium salt to diazotate, *i.e.* the alkalisation of the diazonium solution, proceeds in the presence of histidine a much better agreement between histidine content and colour intensity is obtained. When the diazonium solution was added last, the corresponding figure for ϵ was 2.16 ± 0.0015 (mean of 32 determinations). A still closer agreement was obtained when the original procedure of Weiss and Ssobolew was followed and the carbonate solution added last even when the reagents of Koessler and Hanke were used. With this technique it was observed that the absorption of light increased with prolongation of the interval before the carbonate solution was added. If 20 seconds elapsed between the admixture of the diazonium and the carbonate solutions, the figure for ϵ (calculated as stated above) was found to be 2.34; if 10 minutes 2.46, and if 20 minutes 2.51. After

an hour no further increase was obtained. The same values were obtained even when the specimens had been standing with the diazonium solution for 4 hours before its alkalisation. The final figure for ϵ found was 2.66 ± 0.0054 (mean of 23 determinations).

The colour reached its maximum of intensity in $3\frac{1}{2}$ –4 minutes and fading began not less than 8 minutes after adding the carbonate solution.

With the technique given below, this reaction seems to be the most reliable of all the colorimetric reactions for amino-acids used for quantitative purposes, due regard being given of course to its non-specificity in the presence of tyrosine, guanine, adenine, phenols, iminazoles, sulphides and ammonium salts.

Whatever the explanation of this difference in the colour development may be, it seems probable that a reaction proceeds between histidine and diazobenzenesulphonic acid even in a slightly acid solution, as is evident from the increasing colour intensity with an increasing interval before alkalisation. It seems probable that this reaction does not consist only in a deamination of histidine in the presence of nitrous acid but that the diazo-compound is coupled with the iminazole nucleus.

At least the conditions for a stoichiometric course of the colour reaction are more favourable here than in the alkaline diazotate solution in which apparently several undesirable reactions take place. The diazotate solution itself acquires a yellow tint in half an hour which indicates the occurrence of such changes.

As pointed out by Weiss and Ssobolew the intensity of the colour is largely influenced by changes in the reacting solutions. The maximum of colour intensity is not obtained with the reagents recommended by Koessler and Hanke, but these nevertheless give the most easily reproducible figures. If a 10 % carbonate solution is used instead of 1.1 %, no colour at all is obtained, but if the stronger carbonate solution is used, together with a fivefold stronger diazonium solution, the colour obtained is 40 % stronger than with the ordinary reagents.

Technique of the reaction.

Solutions.

(1) A neutral or faintly acid histidine solution containing 0.05–0.005 mg. histidine per cc.

(2) A diazonium solution prepared according to the descriptions of Koessler and Hanke. To 1.5 cc. of a solution containing 0.9 g. sulphanilic acid and 9 cc. conc. HCl in 100 cc. are added 1.5 cc. of a 5 % sodium nitrite solution. The mixture is cooled on ice for 5 minutes. Then 6 cc. of the nitrite solution are added with shaking, and after cooling for 6 minutes water is added to 50 cc. The diazonium solution should be kept on ice. It keeps for 24 hours. The solution of sulphanilic acid keeps for years. The best results are obtained if the 5 % solution of sodium nitrite is freshly prepared.

(3) 1.1 % sodium carbonate solution (Kahlbaum's anhydrous sodium carbonate).

To 1 cc. of solution 1 (histidine) 2 cc. of solution 2 (diazonium) are added. After 1–3 hours 5 cc. of solution 3 (carbonate) are added. The reading is made with filter S. 50 of the step-photometer 4–8 minutes after the addition of solution 3.

The amount of histidine present in the specimen is calculated from the figure found for ϵ or $-\log_{10} \frac{I_p}{I_a}$. If 1 cc. solution or 8 cc. reaction mixture contains 0.1 mg. histidine the figure for $-\log_{10} \frac{I_p}{I_a}$ is 2.66, d being 1 cm. If the tests are performed as described above the colour intensity is directly proportional to the histidine content when this is from 0.05 to 0.005 mg. per cc. Almost the same figure for ϵ was obtained on three different occasions with a 3 to 6 months' interval, *viz.* 2.66, 2.69 and 2.66. These figures were obtained with a specimen of *l*-histidine dihydrochloride, which contained 18.57, 18.41 % N (Kjeldahl), calc. 18.43 %.

Influence of foreign substances.

It is known that the development of the colour is prevented by alkalis or acids as well as in the presence of heavy metal salts. If the histidine is dissolved in 1 cc. of 0.1 *N* NaOH no colour is obtained. The solution must not have an alkalinity stronger than 0.01–0.001 *N*. The acidity must not exceed 0.01 *N*. The presence of 0.01 mg. HgCl₂ impaired the colour. The same applies to the copper and silver salts. Small amounts of ferric salts on the other hand do not inhibit the reaction, thus 1 mg. FeCl₃ does not influence the test. A moderate concentration of sodium chloride has no influence.

Owing to the necessity of separating the basic amino-acids the influence of phosphotungstic acid is important. Fortunately the colour intensity is not affected, if the histidine is dissolved in a 1–0.5 % solution of phospho-24-tungstic acid (Wu). When the concentration is increased to 3 % a slight impairment is apparent.

There are apparently no disadvantages in using this method for the determination of histidine after dissolving the basic fraction of the amino-acids with alkali in the ordinary Van Slyke procedure. In previous work [1932] the histidine content of oxyhaemoglobin was determined in this way in the basic fraction of the amino-acids. The figures found corresponded closely to those given by Vickery and Leavenworth [1928].

SUMMARY.

The colorimetric determination of histidine by the Pauly reaction has been studied. By means of the Zeiss photometer the development and the stability of the colour were followed. It was found that the procedure, recommended by Hanke and Koessler, which has been largely applied during recent years, gives very unsatisfactory results. Using their reagents, however, it is possible by changing the technique to get easily reproducible figures.

A true proportionality is obtained between the intensity of the colour and the amount of histidine present in the range 0.05–0.005 mg. histidine per cc. The colorimetric method, thus modified, is recommended for the determination of histidine in the basic fraction of the amino-acids in the protein analysis according to Van Slyke.

REFERENCES.

- Hanke and Koessler (1920). *J. Biol. Chem.* **43**, 527.
Hunter (1925). *Biochem. J.* **19**, 25.
Jorpes (1932). *Biochem. J.* **26**, 1488.
Koessler and Hanke (1919). *J. Biol. Chem.* **39**, 497.
Lautenschläger (1918). *Z. physiol. Chem.* **102**, 226.
Pauly (1904). *Z. physiol. Chem.* **42**, 508.
Vickery and Leavenworth (1928). *J. Biol. Chem.* **79**, 377.
Weiss and Sobolew (1914). *Biochem. Z.* **58**, 119.