6. Modified Procedures for the Colorimetric Estimation of Arginine and Histidine

By **Herbert Taylor Macpherson**, *Animal Diseases Research Association,
Moredun Institute, Gilmerton, Midlothian*

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In the estimation of very small quantities of material there is no question that colorimetric is preferable to gravimetric analysis. The colorimetric method is generally quicker and simpler to carry out—an important factor when numerous analyses are involved—and it is not subject to the manipulative errors incurred in dealing gravimetrically with minute amounts of material. The method, however, must be reliable; it must be specific, sensitive and capable of giving consistent and reproducible results. These conditions are not always fulfilled and the colorimetric method is very often neglected in favour of a relatively more dependable gravimetric method. Such is the case with the estimation of arginine and histidine for, although several colorimetric methods are available, unless certain conditions are rigidly observed and considerable experience has been gained, the methods are prone to give erroneous results and many workers prefer the more laborious gravimetric technique.

With a view to rendering the colorimetric estimation of arginine and histidine thoroughly reliable under less stringent conditions, the modified procedures described in this paper are put forward. It is proposed to employ the methods in the analyses of solutions of the basic amino-acids obtained by electrodialysis or precipitation with phosphotungstic acid or by a combination of both. A subsequent paper will be published in this connection.

THE ESTIMATION OF ARGININE

After experimenting with the α -naphthol and acetylbenzoyl methods and noting the limitations of both, it was decided that the former was more suitable for modification. Arginine in alkaline solution gives a red colour with α -naphthol and an oxidizing agent such as hypochlorite [Sakaguchi, 1925]. The method as generally carried out [Weber, 1930] is, however, unsatisfactory and subject to considerable errors. The reaction is complicated by the rapid destruction of colour by excess hypobromite, which is employed since it gives a more specific reaction than hypochlorite, and the usual procedure is to add urea almost immediately in order to take up the excess hypobromite. This expedient is only partially effective for, although formation of the coloured compound is instantaneous, destruction by hypobromite also begins immediately, and in the several seconds which must elapse during mixing of arginine and reagent and dispersal of urea throughout the solution, considerable loss of colour will have occurred, and full colour development will not be achieved. Unless the interval before addition of urea, the proportions of hypobromite and urea and the efficiency of mixing are always identical, it is impossible to obtain consistent results. Experimentally it is found that there is a certain optimum quantity of hypobromite with respect to the arginine which will give a maximum colour development. Slight deviations from this optimum will yield much lower results. Obviously then, although the time factor may be fairly accurately controlled, it is impossible to assess the optimum amount of hypobromite for a solution in which the arginine content is not known.

The modified procedure given below in which the urea is added before the hypobromite and the colour is built up in two stages, ensures (a) maximum and consistent colour

development, (b) colours stable for a considerable time, (c) results not affected by reasonable deviation from the stated quantities of reagents.

The last point is important in the photometric estimation of the colour, since the standard curve may have been constructed with a different sample of, say, hypobromite from that used in later estimations.

The quantity of arginine which can be accurately estimated, using the Pulfrich photometer, depends on the final volume of solution and the stratum thickness employed. In a volume of 25 ml. and with a 10 mm. cell, 0.02 ± 0.001 mg. can be estimated. With a smaller final volume and a 20 mm. cell, more minute quantities can be measured.

Procedure

Reagents required: 10 % potassium hydroxide;
0.1 % α -naphthol in 50 % alcohol;
40 % urea;
Potassium hypobromite (2 g. bromine in 100 ml. 5 % KOH).

An aliquot of the solution containing 0.04–0.40 mg. arginine is transferred to a standard 25 ml. flask and the volume made up to about 10 ml., if necessary, with distilled water. If the solution is acid add the KOH drop by drop till alkaline to litmus paper and add 1 ml. excess. Add 2 ml. α -naphthol, 1 ml. urea, mix thoroughly and cool under the tap. Now add 1 ml. of the hypobromite solution, swirling the flask to prevent local excess, mix and keep 2–3 min. to develop the colour. This colour is not the maximum obtainable since the excess urea consumes the greater part of the oxidizing agent, but the subsequent repetition of the procedure, adding 1 ml. urea followed by 1 ml. hypobromite, gives the maximum colour development. Make up to volume with distilled water, mix and stand 10–15 min. before reading.

The colour intensity is measured by means of the extinction coefficient, using the Pulfrich photometer, filter S53 and a 10 mm. (or 20 mm.) cell, with distilled water in the compensating cell. The arginine present can then be read off from the standard curve.

The photometer is preferable to the colorimeter. It is more convenient, since standards do not have to be set up for each lot of estimations, and as the colour does not obey Beer's Law, the colorimeter is not so accurate unless standard and unknown are very near one another.

Standard arginine curve

Standard solution prepared from B.D.H. arginine, free from nitrate; nitrogen content checked by micro-Kjeldahl estimation.

Arginine (mg.)	0.000	0.031	0.062	0.155	0.310	0.465
$E_{1\text{ cm.}}(k)$	0.095	0.180	0.265	0.525	0.930	1.270

Critical examination of the method

(1) Urea and hypobromite necessary to give maximum colour.

No. of additions of urea and hypobromite (1 ml. each)	Arginine mg.	$E_{1\text{ cm.}}$
1	0.000	0.070
	0.127	0.300
	0.490	0.700
2	0.000	0.095
	0.127	0.455
	0.490	1.320
3	0.000	0.095
	0.127	0.455
	0.490	1.310

It can thus be seen that whilst one addition of urea and hypobromite is not sufficient to give maximum colour development, there is no increase in colour intensity on a third addition of reagent.

(2) *The effect of temperature.* Two portions of solution each containing the same amount of arginine were set up. In one, colour development was carried out at 10° and in the other at 20°. The resulting extinction coefficient was found to be the same for both solutions, so that variations in the normal room temperature will not cause any discrepancy in the results.

(3) *Effect of traces of organic solvents.* In the solution of bases obtained by decomposing the phosphotungstate precipitate with acidified water and amyl alcohol-ether, there are traces of the organic solvents. It was found that the addition of a little amyl alcohol-ether made no difference to the extinction coefficient.

(4) *Effect of varying quantities of reagents.* No significant difference in colour intensity was occasioned by reasonable deviation from the stated amounts of reagents. The quantity of hypobromite for instance may differ by 10% or more without affecting the result.

(5) *Interference from histidine or lysine.* The estimation of arginine was found to be unaffected by the presence of an equal quantity of histidine and by even larger quantities of lysine.

(6) *Stability of potassium hypobromite.* Potassium hypobromite is only stable when kept in stoppered bottles in a cool dark place. A sample which is used daily in the laboratory gradually loses its properties and fresh hypobromite should either be prepared frequently or fresh portions should be taken from a stock solution kept in the cold.

THE ESTIMATION OF HISTIDINE

The colorimetric methods for the estimation of histidine are open to the same objections as the methods for arginine. In the bromine-ammonium carbonate method [Kapeller-Adler, 1933], for example, the quantity of bromine added must be controlled most precisely; too much or too little, within very narrow limits, will give low results; the colour is not stable. In the estimation by diazotized sulphanilic acid [Hanke & Koessler, 1920; Hanke, 1925; Jorpes, 1932], the colour fades rapidly and in addition to the inconvenience of treating numerous samples in an ice-bath, considerable fluctuation of colour intensity results from slight differences in the alkalinity of the solution.

After extensive experiments on the various methods, that employing sulphanilic acid and sodium nitrite was selected for improvement. Three modifications were introduced.

(1) The reaction between histidine, sulphanilic acid and sodium nitrite is carried out at room temperature (15–20°) instead of at 0°.

(2) Sodium hydroxide is replaced by sodium carbonate; whilst a larger quantity of this is required equal colour development can be obtained with much greater permissible variation in the amount of alkali present.

This is not actually a modification, since carbonate is generally employed, but it has been suggested [Yokoyama, 1936] that hydroxide is preferable. This was not found to be the case and it is to the use of hydroxide that the observation above on the fluctuation of colour with alkalinity applies.

(3) After making alkaline, it is found that the colour can be rendered stable for a considerable time by the addition of alcohol. The alcohol is generally made slightly alkaline as a safeguard against the tendency of alcohol to discharge the colour when the solution is only just alkaline.

Procedure

Reagents required: 1% sulphanilic acid in 10% HCl;
5% sodium nitrite;
20% sodium carbonate;
75% alcohol containing 1 ml. 20% Na₂CO₃/100 ml.

The solution to be analysed should be made just acid to litmus, adding 10% HCl drop by drop. Since an almost neutral solution is also employed in the arginine estimation, it is convenient to adjust the reaction of the whole solution of the bases before making up to volume.

An aliquot of the solution (to 10 ml.) containing 0.01–0.20 mg. histidine is transferred to a standard 25 ml. flask or graduated cylinder. 1 ml. sulphanilic acid followed by 1 ml. sodium nitrite are added and the solution mixed. After 30 min. at room temperature 3 ml. sodium carbonate solution are added. Mix thoroughly and add 10 ml. alcohol, mix and cool under the tap before making exactly to volume with distilled water.

Read in the photometer using filter S53 and a 10 mm. (or 20 mm.) cell with distilled water in the compensating cell. The histidine present is then read off from the standard curve.

Since the colour obeys Beer's Law, it is equally suitable for measurement in a colorimeter.

Note. If the solution under examination is a solution of the bases obtained by decomposing the phosphotungstate precipitate by means of acidified water and amyl alcohol-ether, then there may be sufficient organic solvent present to give a turbidity on the addition of sodium nitrite due to precipitation of the diazonium compound. Whilst this turbidity does not appear to affect the final result, a larger proportion of alcohol is sometimes necessary to clear the final solution, so that, to keep within 25 ml. the aliquot of solution taken may have to be reduced. It is recommended that the bulk of the alcohol and ether be removed by boiling the neutral solution of the bases for a few minutes before making up to volume.

Standard histidine curve

Standard histidine solution prepared from B.D.H. histidine monohydrochloride, nitrogen content checked by micro-Kjeldahl.

Histidine (mg.)	0.0000	0.01845	0.0396	0.0738	0.1107	0.1845
$E_{1\text{ cm.}}$	0.010	0.125	0.230	0.455	0.675	1.110

Critical examination of the method

(1) Reasonable variation in the alkali and alcohol added, more particularly if added in excess, does not affect the results. A very considerable reduction in either gives somewhat lower results.

(2) *Effect of temperature.* Normally, a diazotization is carried out at 0° or only slightly above 0° in order to prevent formation of a phenol. In the estimation of histidine, estimations were carried out at temperatures ranging from 0° to about 80°. Results were only low when a fairly high temperature was reached, and no change was observed between 0° and 20°, so that either there is no phenol formation at room temperatures or it is so incon siderable as not to lower the results. In support of this, it is noted that the colour intensity is slightly increased by keeping at room temperature (15–18°) up to 30 min. before making alkaline.

(3) *Interference from arginine and lysine.* Comparatively large amounts of arginine (up to at least 20 times the amount of histidine) do not affect the results. Similar amounts of lysine have no effect; greater amounts appear to reduce the extinction coefficient slightly.

SUMMARY

Modified procedures are suggested for the colorimetric estimation of arginine and histidine. The procedures are based on the α -naphthol and diazotized sulphanilic acid methods respectively.

The modifications ensure (a) consistent colour development without the necessity for stringent precautions in the addition of reagents, (b) colours stable for a considerable time, (c) minimum interference from other bases.

The methods have been thoroughly tested under varying conditions and yield accurate results.

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