A New Colorimetric Method for the Estimation of Histidine and Histamine

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Histidine occurs widely in nature both in the free and combined form. In order to study histidine in its various roles it is necessary therefore to have a sensitive and specific method for its estimation. The colorimetric method described initially by Pauly (1904) and since modified by various workers (Koessler & Hanke, 1919; Jorpes, 1932; Macpherson, 1942; Sanger & Tuppy, 1951), which depends on the reaction of histidine with an aromatic diazonium salt to form a red azo dye, is widely used but suffers from its low specificity. Kapeller-Adler (1933) developed a quantitative colorimetric method based on a reaction discovered by Knoop (1908), which depends on a bromination of histidine in acetic acid. This method, which is specific for histidine and closely related compounds, has not proved entirely satisfactory and many modifications have been suggested (Tsverkalov & Torban, 1851; Hunter, 1952, 1955; Voigt, 1958).

We have discovered a reaction of histidine with iodine and pyridine in which a violet colour is developed. This has led to a convenient and reliable method for the estimation of histidine and histamine which may be used for the analysis of solutions containing as little as 1 μg./ml.

EXPERIMENTAL

Materials. L-Histidine hydrochloride monohydrate was obtained from British Drug Houses Ltd. and was used without further purification. Technical-grade pyridine was fractionally distilled in the presence of phosphorus pentoxide until a fraction of constant boiling point (115°) was obtained. This was stored over solid sodium hydroxide in the dark. A solution of potassium tri-iodide was prepared by dissolving 0·075 g. of AnalaR iodine in 0·4 g. of AnalaR potassium iodide in 10 ml. of water. Sodium dithionite was AnalaR grade and a 0·02 M solution was prepared.

Method. The absorption spectra and extinction of the coloured reaction mixture were determined with a Unicam SP. 500 spectrophotometer (Cambridge Instrument Co.). Introduction of small quantities of reagent to the reaction mixture was accomplished with the aid of an Agla micrometer syringe (Burroughs Wellcome and Co.) fitted with a long delivery capillary.

The method of colour production used in the experiments described in this paper was as follows. A solution of no more than 3 ml. volume containing 1·35 × 10⁻⁴ μmoles (1·55 μg.) of histidine was pipetted into a 5 ml. graduated flask. From a burette, 1·2 ml. of pyridine was added and the solution was adjusted to pH 11·0–12·0 with 0·2N-sodium hydroxide. The bulk of the solution was made up to approx. 4·5 ml. with water. The micrometer syringe was used to introduce 0·04 ml. of the potassium tri-iodide reagent. The flask was immediately shaken until the contents were homogeneous and the violet was allowed to develop at room temperature for exactly 30 sec., timing being commenced from the moment the reagent had been added. At the end of this period, further colour formation was checked by the addition of 0·5 ml. of 0·02 N-sodium dithionite and the solution was mixed until homogeneous. The volume was adjusted to exactly 5 ml. with water, and, after standing for 5 min., the solution was transferred to a silica spectrophotometer cell of 1 cm. cross-section. The extinction of the solution was measured at a wavelength of 590 mμ within 15 min. of development, against a blank containing all the reagents.

RESULTS

Factors controlling development of colour. In early experiments the reagent used was methyl-pyridinium iodide hexaiodide dissolved in pyridine, prepared by the method of Trowbridge & Diehl (1897). This compound appears to dissociate in pyridine solution with the formation of both Py⁺I₃⁻ and I₄⁻ ions, as shown from spectrographic evidence (Reid & Mulliken, 1954). Later experiments indicated that a solution of elementary iodine in pyridine was more convenient. Unfortunately solutions of iodine in pyridine are rather unstable (Zingaro, VanderWerf & Kleinberg, 1951), and refinement of the method led to the use of potassium tri-iodide in water as a stable reagent. Experiments with various solutions of potassium iodide and iodine proved that maximum colour development was possible if the reagent was prepared by dissolving 0·075 g. of iodine and 0·4 g. of potassium iodide in 10 ml. of water. Excessive quantities of iodide were to be avoided as this led to a diminution of colour intensity. This, however, and the effect of other anions will be discussed later.

Erratic results were obtained when technical-grade pyridine was used but this was remedied by fractionation to a constant-boiling pyridine. The role of pyridine in the reaction is unknown, but its specificity is quite marked. Other pyridine derivatives were tried but only 2·6-lutidine produced any
colour, and this was of low intensity. 4-Chloro-
z-hydroxy-, β-hydroxy-, N-oxy-pyridines, pyridine
carbinol and nicotinamide all gave negative results.

Colour development is also dependent on the pH
of the reaction mixture and thus careful adjustment
is necessary for consistent results. Experiment
proved that the maximum colour intensity was
obtained at pH 11-0-12-0.

Characteristics and stability of colour. The
characteristic violet obtained during the reaction
has the absorption spectrum shown in Fig. 1. There
is a sharp peak with a maximum absorption at
560 m, and a subsidiary shoulder at 450 m,.
The former was used for the estimation of histidine.

The violet pigment was observed to decompose in
the presence of excess of iodine and as this process
took place the main peak diminished and the
shoulder assumed more importance. When a large
excess of iodine was present this process was rapid,
the development of the violet being hardly detect-able
and the absorption of the reaction mixture
showing no peak at 560 m,. The colour of this
solution was orange-brown.

This phenomenon made quantitative application
of the reaction difficult as it was often necessary to
add fairly large excesses of iodine to solutions of
low histidine content to obtain a calibration curve
of useful range. This difficulty was overcome by the
introduction of a technique whereby all excess of
iodine was removed from the system as soon as
development of the violet was complete. Sodium
dithionite proved to be a suitable reagent for the
purpose and a calculated quantity was therefore
added to the reaction mixture as the colour
approached its maximum intensity. An arbitrary
time of 30 sec. was chosen as the period in which

the colour was allowed to develop, as this gave
good colour intensities without appreciable inter-
fERENCE from the brown decomposition product.
A further advantage of this technique was that it
rendered the blank solution completely transparent
at 560 m, and thus possible errors introduced
from this source were eliminated. The dithionite, in
addition, may protect the pigment from oxidation
as its presence considerably increased the colour
stability. Even with the use of dithionite, the
colour was observed to fade slowly in the dark,
though reliable measurements of colour intensity
could be made within 15 min. of development of
the colour.

Specificity and interference. The ability to form
the violet colour with pyridine and iodine appears
to be confined to compounds very closely related to
histidine. Histidine, histamine and histidine
methyl ester are the only compounds so far in-
vestigated which exhibit this colour reaction
readily. N-Acetylhistidine, imidazole and carmo-
line (N-β-alanylhistidine) gave negative results,
whereas histidine amide produced only a very
faint colour, which may merely be due to hydro-
lysis to free histidine under the alkaline conditions
of the reaction. The following compounds gave no
detectable violet colour under the specified condi-
tions: benzimidazole, tryptophan, N-acetyltrypto-
phan, indoleacetic acid, creatinine, β-alanine, β-
phenylalanine, tyrosine, sarcosine, β-mercaptop-
ethylamine, ascorbic acid, 3-amino-1:2:4-triazole
and xanthine.

It would appear therefore that the violet is
formed only by compounds possessing the β-
aminoethyl-4 (or -5)-imidazole structure, with or
without an α-carboxylic substituent. Fig. 2 shows
the effect of modifying the carboxyl group of

![Fig. 1. Ultraviolet-absorption spectra of the final reaction mixture, prepared as described in the text, containing 25 μg. of histidine: (A) normal reaction mixture at pH 11-0-12-0; (B) reaction mixture after addition of excess of 0-01 n-HCl (pH 9-0-9-5).](image1)

![Fig. 2. Calibration curves for the spectrophotometric estimation of histidine (O), histamine (△) and histidine methyl ester (■). Solutions were prepared as described in the text and 0-04 ml. of potassium tri-iodide reagent was used in all cases. Extinctions were measured at 560 m,μ.](image2)
histidine. Calibration curves for the estimation of histidine, histamine and histidine methyl ester were plotted, and from these it may be seen that the dependence of colour intensity on structure follows the order:

$$R \cdot CO_2H > RH > R \cdot CO_2 \cdot Me > R \cdot CO \cdot NH_2,$$

where R is (C\(_3\)H\(_7\)N\(_2\))CH\(_2\)CH(NH\(_2\)). A free \(\alpha\)-amino group appears to be necessary for colour production.

The amino acids tryptophan and tyrosine normally interfere seriously in colorimetric estimations of histidine. These amino acids, at concentrations approaching 30 \(\mu\)g./mL, themselves produced no detectable colour on treatment with the iodine-pyridine reagent, but the presence of either reduced the intensity of the violet formed by histidine. For the microdetermination of histidine and histamine in protein hydrolysates and biological materials it was necessary to remove all interfering substances. This was conveniently achieved by selective absorption of histidine and histamine from samples, by the ion-exchange-resin technique of Voigt (1959). Small amounts of lysine and arginine, which were also absorbed, did not interfere with the estimation. Prior removal of protein by ultrafiltration was found advisable, however.

Heavy metals and some anions were observed to have pronounced effects on colour production. The heavy metals such as copper, zinc and iron probably reduced the availability of free histidine by complex formation (Martell & Calvin, 1952). Copper, in addition, reacts with pyridine, producing a blue complex which makes estimation of the violet difficult. The influence of various anions is summarized in Fig. 3, which indicates that strong nucleophilic anions, e.g. SCN\(^-\), are most effective in reducing colour intensity. In the experiments relating to Fig. 3, 0-05 ml of 0-2\(\text{M}\) sodium hydroxide was used for pH adjustment in all cases. It must be borne in mind therefore that the true effect of some of these anions may be complicated by slight pH differences.

Calibration and reproducibility. Calibration curves for the determination of histidine with various quantities of the potassium tri-iodide reagent are shown in Fig. 4. It was observed that an increase in the quantity of reagent increased the intensity of colour produced. The optimum quantity was found to be 0-04 ml, as this gave good intensities in the range 1-60 \(\mu\)g. (1.5-40 \(\times\) 10\(^{-3}\) \(\mu\)moles) of histidine with minimum interference from the brown decomposition product. It is recommended, however, that over the range 20-40 \(\times\) 10\(^{-3}\) \(\mu\)moles of histidine, 0-05 ml of reagent should be used for greater accuracy. Histidine and histamine may therefore be detected in solutions containing as little as 1 \(\mu\)g./mL and determinations may be made with solutions containing more than 2 \(\mu\)g./mL. Reproducibility under these conditions was good, extinction measurements being consistent within 3%. Following the standard practice of duplicate determination on a given solution, we were able to determine the histidine content within 5%.

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**Fig. 3.** Effect of various anions on the intensity of colour produced by 30 \(\mu\)g. of histidine. Solutions were prepared by the standard method except that the stipulated quantity of anion solution was added immediately before the addition of pyridine. ○, 0-1\(\text{M}\)-Sodium citrate; Δ, 0-1\(\text{M}\)-sodium acetate; ●, 0-1\(\text{M}\)-sodium iodide; ■, 0-1\(\text{M}\)-azide; □, 0-1\(\text{M}\)-potassium thiocyanate.

**Fig. 4.** Calibration curves for the spectrophotometric estimation of histidine with various quantities of potassium tri-iodide reagent. The solutions were prepared as described in the text. Extinctions were measured at 560 \(\mu\)m; ○, with 0-05 ml. of reagent; ●, with 0-04 ml. of reagent; □, with 0-03 ml. of reagent; ■, with 0-02 ml. of reagent.
DISCUSSION

The nature of the violet pigment formed during the reaction is not known. Attempts to isolate the pigment by chromatography and solvent extraction were unsuccessful. It was observed, however, that on addition of mineral acid the violet reaction mixture became orange and this process was reversible. This colour change occurred at pH 9.5-10.1, which may be taken to indicate that the violet product is anionic in nature, possibly involving a keto-enol system.

The foregoing method was developed with the object of estimating combined histidine in the intact protein molecule. With this in view, the proteins chymotrypsin, trypsin, lysozyme, globin, ribonuclease and glucagon were tested, but no colours were obtained. It is particularly surprising that glucagon yielded negative results, as this protein possesses, in its N-terminal histidine (Bromer, Sinn, Staub & Behrens, 1956), the structural features apparently requisite for colour production.

SUMMARY

1. A new colorimetric method for the determination of histidine and histamine is described. The method is of use for solutions containing 1-60 \( \mu \)g./ml.

2. The colour reaction is specific for histidine and closely related compounds, namely, histamine and histidine methyl ester. Imidazole does not react and histidine amide gives only a very faint colour.

3. Tryptophan and tyrosine do not form colours under the conditions of the reaction but interfere with histidine colour formation. The interference of some heavy metals and anions is discussed.

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REFERENCES


Isolation and Structure of a New Factor of the Vitamin B\(_{12}\) Group:
Guanosine Diphosphate Factor B

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The chromatographic and electrophoretic analysis of the substances having vitamin B\(_{12}\)-like activity produced by a strain of *Nocardia rugosa* (Di Marco & Spalla, 1957), and by some mutant strains, indicated the presence of vitamin B\(_{12}\), factor B, and two unknown factors (Di Marco, Alberti, Boretti, Ghione, Migliacci & Spalla, 1957). These two last substances, which we named factors 1 and 2, show a growth-promoting activity only for *Escherichia coli* 113/3 among the vitamin B\(_{12}\)-requiring micro-organisms tested, and are characterized by slow mobility in butanol-acetic acid-water (\(R_f\) values/\(R_p\) of vitamin B\(_{12}\), 0.12 and 0.3 respectively) (Fig. 1). Factor 1 is electronegative at pH values of 2.7 (Fig. 1) and 6.5. The similarity of the chromatographic and electrophoretic behaviour of these substances to that of factors C\(_1\) and C\(_4\) described by Ford & Porter (1952), Ford, Holdsworth & Kon (1955) and Dellweg, Becher & Bernhauer (1956a) prompted us to attempt the isolation and identification of our factors 1 and 2.