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THE EFFECT OF OXYGEN UPON THE MICRO DETERMINATION OF HISTIDINE WITH THE AID OF THE PAULY REACTION

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SUMMARY

The colour obtained upon the addition of diazosulphanilic acid to histidine in alkaline medium (Pauly reaction) is bleached rather suddenly after a certain lag. This lag is shorter the more oxygen is present in the alkaline reaction medium.

A method of determining histidine on a micro scale, consisting of an improved Pauly reaction after paperchromatographic separation, is described in detail.

INTRODUCTION

Although accurate chromatographic methods for determining the amino acid content of protein hydrolysates are available, methods requiring but simple equipment are still useful, especially where only a limited number of amino acids need to be determined. The determination of histidine by a combination of paper chromatography and Pauly reaction, as described by FRAENKEL-CONRAT[1], is an example of such a method.

The present paper deals with a complication that may arise with the latter method and gives directions to avoid it.

In the Pauly reaction the red compound resulting from the reaction of histidine and diazo sulphanilic acid is measured photometrically. First sulphanilic acid and $NaNO_2$ are added to the histidine solution to be tested, whereupon the solution is made alkaline with soda solution. A red colour is then developed. Possibly because of the known instability of the colour, FRAENKEL-CONRAT[1] recommends that the extinction be read 5 min after the addition of the soda. This precaution, however, besides being rather impractical when several readings are to be made simultaneously was found by the present author to be insufficient for reproducible results owing to variability in the rate of bleaching. Individual determinations sometimes deviated as much as 50 % from the mean value.

A search for the cause of the instability of the dye revealed that the presence of oxygen after the addition of soda is one of the factors involved in bleaching. This is illustrated in Fig. 1. A reasonably stable colour is produced if the Na_2CO_3 solution is degassed *in vacuo* shortly before mixing with the diazo solution (Fig. 1 A). The latter solution is already oxygen-free due to the great excess of HNO_2 (15-fold) over the sulphanilic acid as usually recommended[2]. The soda and diazo solutions were mixed by gentle swirling.

A sudden decrease of extinction is observed if the soda solution is not degassed and mixing is carried out by gentle swirling (Fig. 1 B). The colour is even less stable if the solutions are mixed by shaking (Fig. 1 C). A very fleeting colour is observed if a current of oxygen is passed through the diazo mixture for 1 min before and 15 sec after the addition of soda (Fig. 1 D).

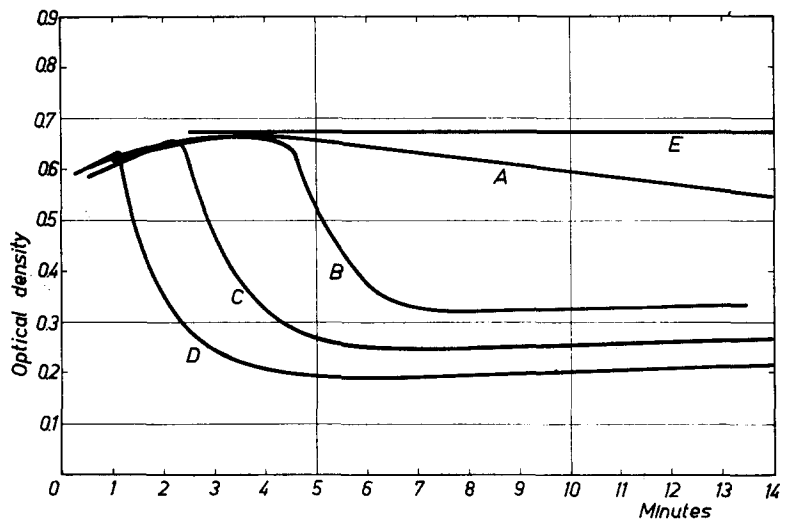

Fig. 1. Optical density of the diazo dye of histidine as a function of time. For explanation see text.

The form of the curves is very remarkable and suggests an autocatalytic reaction. Matters, however, are not quite as simple as that, since the addition of 10 % of a bleached reaction mixture to a fresh reaction mixture immediately after the addition of the non-degassed soda solution does not decrease the stability of the colour. This

would have been the case if the bleaching reaction was catalyzed by the products of the bleached dye. An explanation must await further experimental data.

Curves B and C of Fig. 1 demonstrate in particular how variable the results can be if the diazo and soda solutions are inadvertently mixed.

However, even if an oxygen-free soda solution is used, the colour is not entirely stable (Fig. 1 A). Complete stability could be obtained by the addition of ethanol as recommended by MCPHERSON[3] (Fig. 1 E). Methanol and dioxane proved to have the same effect, but acetone cannot be used because it produces a strong red colour itself.

The method of MCPHERSON has the additional advantage that, instead of 5 % Na_2CO_3, one adds only 0.25 volume 20 % Na_2CO_3, which reduces the amount of oxygen to less than 25 %, gases being less soluble in concentrated salt solutions than in diluted ones. The data of Fig. 1 indicate that it is advisable to stir gently when the soda and diazo solutions are mixed and to add alcohol after 2 min. These precautions lead to the directions given in the experimental part.

The results of the determination of histidine in a few proteins of known amino acid content by the present method are presented in Table I. Each determination was carried out in 4-fold and individual determinations deviated by no more than 2 % from the mean value. The values obtained compare favourably with those reported in the literature.

TABLE I

HISTIDINE CONTENT OF VARIOUS PROTEINS

Protein	*N content assumed* %	*Histidine content g/100 g protein*	
		calculated	*found*
Insulin[4]	15.9	5.4	5.3
Bovine serum albumin[5]	16.1	4.0	3.9
β-Lactoglobulin[5]	15.6	1.65	1.8
Casein[6]	15.6	3.1	3.1

EXPERIMENTAL

The determination of histidine in proteins

About 8 mg of protein is hydrolyzed in 1 ml of twice distilled 6 *N* HCl *in vacuo* at 110° for 24 h (insulin 32 h). The hydrolyzate is evaporated *in vacuo* over KOH, redissolved in water and again evaporated *in vacuo*. Dissolution and evaporation are carried out once more. Finally the residue is dissolved in 0.4 ml of 10 % isopropanol and centrifuged. From a microsyringe two aliquots of 0.1 ml are delivered to be used for *N* determination in duplo according to MCKENZIE's modification of the Kjeldahl method[7].

0.025-ml portions of the hydrolyzate are applied as bands on strips of Whatman paper 3 MM (width 2 cm, length 35 cm), 5 cm from the bottom. Ten of these strips, 5 strips containing the histidine standard (1 mg of histidinehydrochloride·1 H_2O/ml) and 5 strips containing the unknown, are developed for 22 h by the ascending-descending method of BLOCK[8], using 80 % *n*-butanol as a solvent.

The chromatograms are dried and two of them (one standard and one unknown) are stained with a diazo spray. Using these chromatograms as guides, strips 2.5 cm

long, containing the histidine bands, are cut from the other eight chromatograms. From the same chromatograms blank strips of 2.5 cm length are cut out from below the point of application. Each strip is tightly folded zigzagwise in two directions and introduced into a testtube (length 10 cm, width 1.1 cm), 2.5 ml of water, containing 0.25 % ethylene diamine tetraacetate is added. The cutting now partly unfolds such that it does not stick to the walls of the vessel and such that water can circulate freely. The tube is stoppered and rotated slowly head over heels for 1 h.

2 ml of the extract are pipetted into a small erlenmeyer, after which 0.2 ml of 1 % sulphanilic acid in 1.4 *N* HCl and 0.2 ml of 5 % $NaNO_2$ are added. After 10 min 0.6 ml of 20 % Na_2CO_3 is added whilst swirling gently. Exactly 2 min later 2 ml of 75 % alcohol is added and mixed by swirling without special caution. The solution is brought into a 1 cm cuvet and the extinction is read at 530 mμ.

A standard, an unknown and the two corresponding blanks are treated simultaneously. Blank strips yield an optical density of about 0.06. The blanks of the standard and of the unknown should not differ by more than 0.01 in optical density. This can be attained if the filter-paper is of good quality and the strips are handled carefully, preferably wearing rubber gloves.

Experimental conditions of Fig. 1.

Curves A–D of Fig. 1 were obtained at 20–22° under similar conditions, using 2.6 ml of 5 % Na_2CO_3 instead of 0.6 ml of 20 % Na_2CO_3 and 2.0 ml of alcohol. The concentration of histidine after the addition of all reagents was 5.10^{-5} *M*. All curves except D were measured at intervals of 30 sec. For curve D the reactions were carried out directly in the cuvet and the extinctions were measured with the aid of a recording instrument.

MATERIALS

All reagents were of analytical grade. The proteins were commercial preparations, used without further purification. They were obtained from the following sources: crystalline insulin, "Philips-Roxane", The Netherlands, batch 04/05 A5-V; bovine serumalbumin, "Poviet Producten N.V.", Amsterdam, The Netherlands batch 1954-36; caseine, "Amsterdamse Chininefabriek N.V.", The Netherlands; β-lactoglobulin, L. Light & Co. Ltd.", Great Britain (no batch mentioned).

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