NEW COLORIMETRIC APPLICATIONS OF THE COLORED FERRICYANIDE-ORTHO-DIANISIDINE SYSTEM. INDIRECT COLORIMETRIC ESTIMATION OF CYSTEINE

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THE OUTSTANDING importance of cysteine is well-known, as its presence is fundamental in various biochemical processes.

It has been proved that among other actions, cysteine has a beneficial effect on the body as it minimizes the injurious effects produced by radioactivity, x-rays and by the intoxications due to heavy metals. It has also a powerful antihistaminic action. Moreover, the fact that cystine contained in wool, hair and horn materials of human or animal origin yields cysteine when its sulfur bonds are broken, gives interest to the study of the amounts of cystine produced in these breakings, thus allowing a better understanding of these reactions.

All these reasons led us to the study of the possible application to the quantitative estimation of cysteine by a new colorimetric method by means of a reaction we previously developed for the determination of various mercapto derivatives (1).

First of all we undertook a wide bibliographic study of the different methods in existence for its determination. Their number and their diversity is extraordinary; this shows clearly the interest shown in its estimation.

The main methods used for cysteine determination can be classified as:

Volumetric methods (iodometric, generally)

Colorimetric methods (based on oxidation, formation of azo-derivatives, complexes, etc.)

Electrometric methods (potentiometric, amperometric, polarographic)

Colorimetric methods are among the most frequently used. Among them some may be mentioned: those which are based on the reaction between cysteine and ferric ion in presence of different aromatic amines (2, 3) or

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between cysteine itself and sodium nitroprussiate (4). Hazeloop based his method on the colored reaction between cysteine and *o*-benzoquinone in chloroform solution (5); Schöberl based his on the reduction of phosphotungstic acid followed by the colorimetric determination of the blue compound obtained (6).

The Hellerman (7) method must be placed among the iodometric; cysteine is treated with an excess of *o*-iodosobenzoic acid, yielding cystine and iodobenzoic acid; the excess of the former being titrated iodometrically.

Barnstein's method can be listed among the gasometric procedures: cysteine is oxidized to cystine by means of a solution of I_2 in KI. The excess of I_2 is determined by measuring the released N_2 after I_2 has reacted with hydrazine (8).

The amperometric methods using Hg^{+2}, Cu^{+2} and Ag^{+} salts were studied by Kolthoff (9–11) and are among the newest electrical developments.

It appeared to us, having carried out the study of these existing methods, that if our formerly reported method for the determination of mercapto derivatives was applicable to the estimation of cysteine it could be of a greater simplicity and sensitivity.

The Basics of the Method

In previous papers (12, 13) we reported that it is possible to determine colorimetrically some oxidizing agents taking advantage of the fact that they transform *o*-dianisidine into quinonic compounds which possess a strong bluish-green color, this color turning red upon acidification.

Later on we made use of this ferricyanide *o*-dianisidine system for the indirect colorimetric estimation of organic and inorganic reducing agents. We have thus studied the determination of Sn^{+2}, As^{+3}, $S_2O_3^{-2}$, $S_2O_4^{-2}$ and H_2O_2 (14, 15) and of many mercapto derivatives (1).

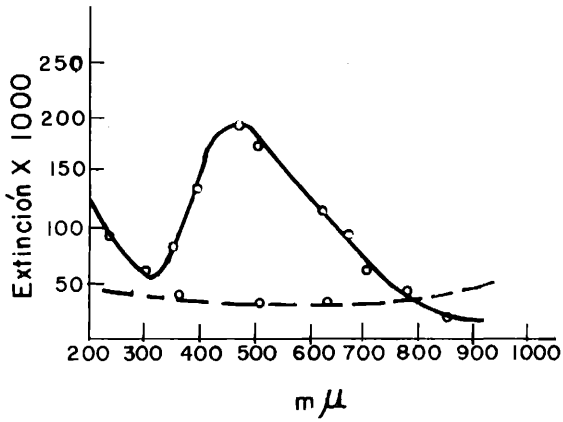

Figure 1.—Absorption spectrum in presence of cystine at pH 1.5 and at pH 3.

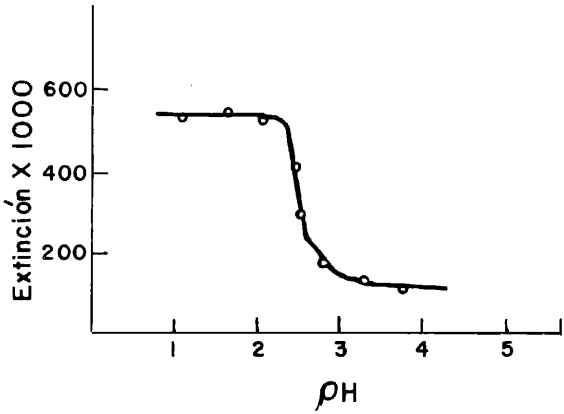

Figure 2.—Variation of the extinction with pH at 470 mμ cystine being present.

To make our method applicable to cysteine we had to study first the best conditions to ensure its oxidation with ferricyanide. It was found that the reaction takes place quantitatively in an alkaline medium (pH 10–11).

Further we studied the absorption spectrum of the red color obtained at pH 1–2 when *o*-dianisidine reacts with an excess of ferricyanide, and after reacting with cysteine; our conclusion was that the presence of the products of oxidation does not modify the spectrum (Fig. 1). The same procedure carried out at pH 4 gave evidence that in the portion of the spectrum considered the extinction of the red color is always greater than the extinction of the bluish-green one.

We examined the pH influence on the red color obtained (Fig. 2), its

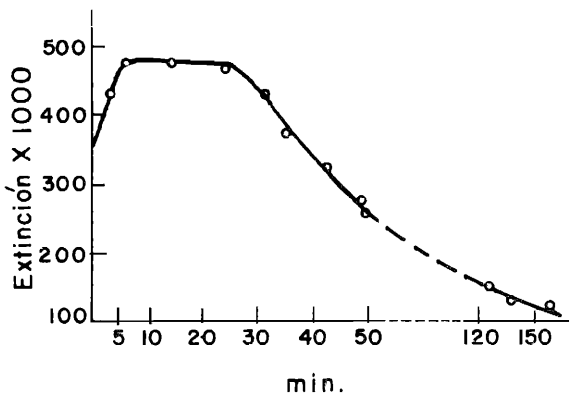

Figure 3.—Variation with time of the extinction at pH 1.5 and 470 mμ.

stability (Fig. 3) and the need to add zinc salts which after precipitating not only the ferrocyanide formed in the reaction but the traces that come as impurities with the added ferricyanide increase the oxidation potential of the latter and the sensitiveness of the reaction as well.

Keeping in mind that in biochemical equilibria, as in every possible determination of cysteine, there is to be taken into account the presence of considerable amounts of cystine, we carried out a second lot of determinations with both compounds being present in a 50:1 cystine/cysteine concentration ratio.

In such conditions we found that the extinctions obtained were identical with those read before the addition of cystine; therefore it may be asserted that the extinction of the red color obtained does not depend on the presence of the products resulting from the oxidation of cysteine.

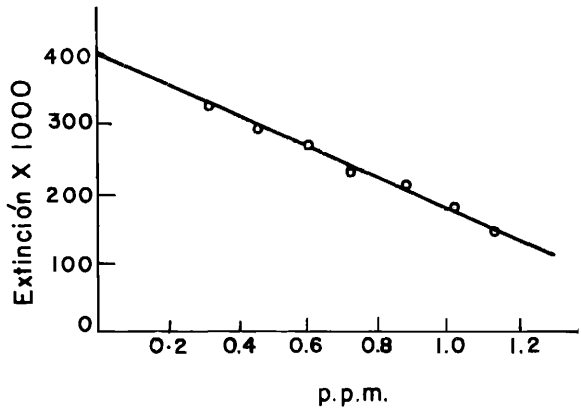

Figure 4.—Variation with cysteine concentration of the extinction at pH 1.5 and 470 mμ.

The results obtained allow the application of a new method for the estimation of cysteine; the procedure is easy even at concentrations as low as 0.8 μgm./ml. for which Beer's law is valid (Fig. 4).

Experimental

Reagents

(a) Use potassium ferricyanide, analytical grade, recrystallized many times from water and dried at 100°C. A solution is made containing 0.310 gm. of this salt per liter, which corresponds to 0.200 gm. of $Fe(CN)_6^{-4}$/l.

(b) Cystine. 100 mg. are dissolved in one liter of water.

(c) Cysteine. 100 mg. are dissolved in one liter of water.

(d) Sulphuric acid, 2 *N*.

(e) Zinc sulphate, 2 *N*.

(f) *o*-Dianisidine; 0.5 gm. of *o*-dianisidine is dissolved in 50 ml. of

acetone and the solution is made up to 100 ml. with distilled water. The solution thus obtained shows a slight coloration but can be used without further purification.

Apparatus

The extinctions were measured with a Beckman DU spectrophotometer provided with a photomultiplier. The cell used had a 10 mm. light path and the slit width chosen was the one giving the maximum sensitivity.

The pH values indicated in the text were taken with a Beckman G. apparatus.

Procedure

1 ml. of ferricyanide solution is measured into a 100 ml. volumetric flask; 20 ml. of distilled water are added, followed by the solution of cysteine to be determined. To ensure a complete reaction between cysteine and ferricyanide, the mixture was allowed to stand for five to ten minutes. Then 0.5 ml. of $ZnSO_4$ solution and finally 0.5 ml. of (freshly prepared) aqueous solution of *o*-dianisidine is added and the volume is made up to 100 ml. with distilled water. Readings are taken at 470 mμ after ten and thirty minutes standing.

When a series of assays are made adding 10 ml. of the cystine solution simultaneously with the cysteine solution the results obtained for the extinctions are identical with those previously obtained.

Conclusion

1. A new indirect colorimetric method for determination of cysteine is presented; the method is based on the reducing action of cysteine on an excess of ferricyanide ion after which this excess is estimated using *o*-dianisidine.

2. For the cysteine concentrations considered, no perceptible variations of extinction values of the obtained colors have been observed even when large amounts of cystine were present reaching as much as 50 times cysteine concentrations.

3. Extinction values remain constant during the interval of five to thirty minutes after the *o*-dianisidine reagent has been added.

4. Beer's law remains valid up to minimal cysteine concentrations of 0.6 μgm./ml. corresponding to a 1:1, 700,000 dilution limit.

Summary

A new indirect colorimetric method for cysteine determination is described. The method depends on the oxidation of cysteine in alkaline solution using a known excess of ferricyanide, the latter being determined by the extinction at 470 mμ of the colored solution produced by adding *o*-dianisidine to the mixture previously acidified at pH 1.5–2. The

proposed method allows a quick and easy estimation of cysteine in solutions containing amounts as little as 0.3 μgm./ml. The presence of cystine even in concentrations reaching 50 times those of cysteine does not interfere with the procedure.

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EFFECT OF VARIOUS METALLIC IONS ON THE GROWTH OF *Penicillium glaucum, Aspergillus niger* and *Achorion quinckeanum*

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IF IT IS true that the growing of microörganisms is dependent on the presence of metal ion traces, it is no less certain that some cations in a specific concentration have the opposite effect. Of the latter, silver is the best known and its antiseptic effect has been used profitably for quite a long time. Nowadays in spite of the synthetic disinfectants and antibiotics, the antiseptic properties of the silver salts are still used.

The process of inhibition by metals has been studied mainly on the growth of bacteria, and on *Staph. aureus* in particular in particular.

Here related researches result from several observations. First: when observing the growth of *Achorion quinckeanum* on pieces of guinea pig

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