NOTES

Spectrophotometric Determination of Micro Amounts of Cysteine

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Oxidation of cysteine by iron(III) has been extensively studied by Leussing *et al.* (*1*). A suitable spectrophotometric method based on the oxidation of cysteine by nitrilotriacetoferrate(III) in the presence of 1,10-phenanthroline has been described by Bydalek and Padolski (*2*). In addition, spectrophotometric methods for the determination of cysteine or sulfhydryl groups have been reported: using Ellman's reagent by Robyt *et al.* (*3*), sodium nitroprusside by Titaev and Balabolkin (*4*), and 2-vinylquinoline by Krull *et al.* (*5*). In a recent paper, Stookey has described a method for the determination of iron(II) using 3-(2-pyridyl)-5,6-bis-(4-phenyl sulfonic acid)-1,2,4-triazine, Ferrozine (*6*). The molar absorptivity of iron(II) Ferrozine chelate is approximately 2.5 times greater than that of iron(II) phenanthroline. Thus, greater sensitivity is obtained for the determination of cysteine, sulfur dioxide (*7, 8*), and ascorbic acid (*9*) by using Fe(II)–Ferrozine rather than Fe(II)–phenanthroline. This note describes the determination of micro amounts of cysteine using iron(III) in the presence of Ferrozine.

EXPERIMENTAL

Apparatus. A Cary Model 14 spectrophotometer was used for the absorbance measurements. The pH of the solution was determined with a Corning Model 12 research pH meter.

Reagents. Ferrozine, 3-(2-pyridyl)-5,6-bis(4-phenyl sulfonic acid)-1,2,4-triazine disodium salt, was purchased from the Hach Chemical Company. The reagent was recrystallized three times from deionized water. The stock solution used was approximately 0.015*M*. Fresh solutions of Ferrozine were used. A stock solution of iron(III), approximately 0.003*M*, was prepared by dissolving iron wire in 10 ml of perchloric acid containing a few drops of 6*M* nitric acid. The solution was heated until perchloric acid fumes appeared and then the contents were diluted to 1 liter. Standard iron solutions were kept out of the sunlight. In the absence of sunlight, standard iron(III) solutions were stable for over a six-month period. Cysteine hydrochloride monohydrate was purchased from Eastman Kodak. Analytical solutions of cysteine were prepared daily by dissolving a desirable amount of cysteine in deaerated water. The purity of cysteine was established using argentometric (*10*) and iodometric (*11*) methods. The average purity of a freshly opened bottle of cysteine was found to be 99.7%. On standing in air, some decomposition was noticed, yielding lower purity values. Stock solutions of acetic acid, monochloroacetic acid–acetate buffers were prepared from the ACS reagent grade chemicals. The desirable pH of the stock solution was obtained by adding 6*M* NaOH to 1*M* acetic or monochloroacetic acid.

Procedure. Solutions for the colorimetric analysis were prepared by the addition of stock reagents to 25-ml volumetric flasks in the following order: 2 ml of a buffer solution, 2 ml of iron(III) perchlorate, 1 ml of Ferrozine, and, finally, varying amounts of cysteine. The contents were diluted to the mark and the absorbance was measured at 562 nm after 20 minutes *vs.* a blank, which contained all the reagents as above except cysteine. Interference by amino acids was checked by adding small amounts of these acids to the reaction mixture prior to the addition of cysteine. The amount of cysteine was determined from a standard curve rather than by calculation, using the expected molar absorptivity of the iron(II)-Ferrozine chelate.

RESULTS AND DISCUSSION

Reduction of iron(III) by cysteine and the resulting absorbance of iron(II)–Ferrozine chelate depends on variables such as the molar ratio of iron(III) to Ferrozine, the pH, the time necessary to reach maximum intensity and the presence of interfering ions. Since iron(III) does not form a Ferrozine chelate, the amount of Ferrozine to be added must be at least in a 3 to 1 ratio with respect to the iron(II) formed in the reaction. The time necessary for the completion of the reaction depends on the pH, the temperature, and the amount of iron(III) added. In all runs, the iron(III) concentration with respect to cysteine has been kept approximately in tenfold excess. Under these conditions, the optimum pH range for the reaction is 3.2 to 4.3. The oxidation of cysteine at room temperature and at pH 3.2 is approximately 95% completed in 5 minutes, and approximately 99% completed in 15 minutes. Thus, all absorbance readings are taken after 20 minutes *vs.* the blank. The absorbance continues changing rather slowly on standing due to the instability of iron(III). The oxidation of cysteine is facilitated by increasing temperature: at 40 °C, 99% of cysteine is oxidized in less than ten minutes. At concentrations of acetate higher than 0.1*M* in the final solution, the reduction of iron(III) by cysteine is somewhat slower than at lower acetate concentrations. Therefore, monochloroacetic acid and monochloroacetate were kept below 0.06*M*. The reduction of iron(III) is also retarded by phosphate or any other chelating agents, if they are present in amounts larger than iron(III) concentration. Chloride and bromide do not interfere up to 800 ppm, while iodine interferes even at 50 ppm. Most of the amino acids do not interfere. Histidine and tryptophan, if present in 500-fold excess over cysteine, yield results somewhat lower than expected. Inorganic cations such as copper(II), zinc(II), and cobalt(II), which form Ferrozine chelates, should be in smaller amounts than iron(III).

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Table I. Absorbance as a Function of Cysteine (for Sample Less Reagent Blank)

Amount of cysteine monohydrate, μg/25 ml[a]	Absorbance at 562 nm	
	Observed[b]	Calculated[c]
10.9	0.070 ± 0.004	0.069
21.8	0.134 ± 0.003	0.137
54.6	0.340 ± 0.003	0.337
109.3	0.690 ± 0.003	0.699
132.1	0.810 ± 0.003	0.817
54.5[d]	0.337 ± 0.003	0.349

[a] The amount of cysteine monohydrate corresponds to the values determined by the other analytical methods. [b] Absorbance was determined on 25 ml of final solution containing 6 μmoles of iron(III) and 15 μmoles of Ferrozine, and 3 ml of monochloroacetate buffer at pH 3.2. [c] Calculated values are based on the molar absorptivity of $28000 M^{-1} cm^{-1}$ for iron(II)-Ferrozine chelate and the assumption that cysteine is oxidized to cystine. [d] Solution contains a mixture of various amino acids in the amount 100 times that of cysteine.

Some of this interference can be overcome by the addition of an additional amount of Ferrozine.

Cysteine in the amounts as low as 10 μg and as high as 150 μg/25 ml have been determined using 1-cm cells as summarized in Table I. The relative precision changes from 4% at 20 μg to 1% at 50-μg levels. A small negative deviation from Beer's law is observed at concentrations higher than 125 μg/25 ml. This may be due to the incompleteness of the oxidation or side reaction of oxygen with cysteine.

Spectrophotometric results indicate that cysteine is oxidized to cystine. The existence of cystine had been confirmed qualitatively.

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Determination of Chromium in Biological Samples Using Chemiluminescence

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The importance of chromium and its function in biological systems have been extensively reviewed by Mertz (*1*). Quantitative measurement of chromium in various animal tissues and in a large variety of foods has been done by Schroeder *et al.* (*2*). They reported that the chromium concentration in human tissue differs with the geographic origin of the samples. Generally, they found higher Cr levels in males than females; for example in human serum: 520 ppb for males and 170 ppb for females. Analysis of chromium in human serum has been investigated by various authors (*3–11*).

Recently, Seitz *et al.* (*12*) reported a chemiluminescence method for the analysis of chromium in water. The method is based on the light emission catalyzed by chromium(III) in the luminol-hydrogen peroxide reaction. The method is sensitive for chromium in the sub-parts-per-billion range, and is specific for chromium when EDTA is added to the sample prior to analysis, in order to mask interference from other metal ions.

This article reports the application of chemiluminescence to the determination of chromium in biological samples as part of a larger program of applying chemiluminescence methods to biological systems. We used NBS standard reference materials: orchard leaves (SRM 1571) and bovine liver (SRM 1577), to check the validity of the method. Both materials have no "certified" value reported for Cr; SRM 1571 has an uncertified value of 2.3 ppm. Chromium analyses were done on human serum samples and compared with literature data. Pooled serum samples were analyzed by chemiluminescence, atomic absorption, and colorimetric methods, to compare the three methods.

EXPERIMENTAL

Apparatus. The apparatus used for chemiluminescence was that described by Seitz *et al.* (*12*). A Cary Model 15 recording spectrophotometer was used for the colorimetric method. A Perkin-Elmer Model 305 B double beam atomic absorption spectrometer equipped with a heated graphite atomizer HGA 70 was used for atomic absorption measurements. The chromium hollow cathode lamp was operated at 25 milliamperes throughout the entire analysis. Wet ashing of samples was carried out using an aluminum heating block. This block had nine holes, each 2½ inches deep, to accommodate 18- × 150-mm Pyrex test tubes. A hole of equal depth but smaller diameter was drilled to fit a 400 °C thermometer so that the temperature during the ashing could be followed. The block was checked for uniformity of heating. Heating uniformity is considered under "Results and Discussion."

Reagents. Ultrex grade sulfuric acid and nitric acid (J. T. Baker Co.), and reagent grade perchloric acid (Matheson, Coleman and Bell) were used for wet ashing. Disodium ethylenediamine tetraacetate, boric acid, potassium hydroxide, and hydrogen peroxide (3%) were Fisher Scientific certified grade. The sodium luminol salt was prepared from luminol (Aldrich Chemical Co.) and reagent grade sodium hydroxide (Fisher Scientific Co.) (*13*). The salt was twice recrystallized from water. Standard

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