[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF OREGON]

The Oxidation of Cysteine by Iron and Hydrogen Peroxide

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RECEIVED DECEMBER 13, 1956

The conditions of formation of the labile purple iron(III) cysteine complex in oxygen-free acid and basic solution have been studied. In acid solution the complex decomposes in less than 0.5 sec., but under basic conditions persists for approximately 40 min., the color fading according to a roughly exponential law. The absorption spectrum of the residual color possesses maxima at 445 and 570 mμ, the extinction values being 15.0 and 19.0, respectively. In presence of hydrogen peroxide the cysteine complex of iron is rapidly oxidized from the II to the III state which, being unstable, decomposes yielding cystine and iron(II) ion, the process continuing until all peroxide has reacted. The rates of formation of the iron(III) cysteine complex, its oxidation by peroxide at *p*H 8, and the reaction of iron(II) ion, cysteine and peroxide, have been studied using a rapid-flow technique.

Introduction

In mildly acid solution cysteine reacts with iron(III) ion to produce a deep-purple color[3] which fades in a short time and cannot be restored by oxygen, but is restored by hydrogen peroxide. The color forms again on adding iron(III) ion as long as unoxidized cysteine is present.

As early as 1909 it was discovered that the rate of oxidation of cysteine by gaseous oxygen is greatly increased by the addition of trace amounts of iron.[4] Harris[5] put forward a theory of the iron-catalyzed oxidation of cysteine in alkaline solution which was later stated more precisely by Michaelis[6] on the basis of analogy with the cysteine complexes of cobalt. According to Michaelis, iron(II) tris-cysteinate is first formed which is oxidized first to iron(III) tris-cysteinate then to a complex of cysteine and cystine with iron(III). The latter complex reacts with two molecules of free cysteine yielding free cystine and iron(II) tris-cysteinate. This cyclic process results in the oxidation of all cysteine present to cystine.

When gaseous oxygen is the oxidant it is reduced to water, hydrogen peroxide being an intermediate. Pirie[7] showed that addition of peroxide to an iron-containing cysteine solution increased the rate of oxidation, but no direct evidence for the existence of hydrogen peroxide in this reaction was available until Schales[8] carried out the reaction in presence of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), when a very pronounced chemiluminescence resulted. This luminescent reaction is known to occur only in presence of peroxide ion which may thus be detected in concentrations down to 1 part in 10^7.

Working in absence of oxygen, Schubert[9] isolated a number of complexes of iron with cysteine, and presented a theory similar to that of Michaelis but differing in the constitution of the complexes assumed as intermediates. According to Schubert, at *p*H 8–10, the first formed complex is iron(II) bis-cysteinate which is oxidized by oxygen to a binucleate complex of iron(III). The third step consists in the reduction of the iron(III) complex to iron(II) bis-cysteinate, iron(II) hydroxide and cystine.

The purpose of the present work was to reinvestigate the iron–cysteine system. Accordingly, the conditions of formation of the labile iron(III) cysteine complex have been studied in the presence and absence of peroxide ion, and the views of Michaelis and Schubert have been substantiated.

Experimental

Chemicals.—All reagents were of C.P. grade. Cysteine hydrochloride was supplied by Eastman Organic Chemicals, Rochester, New York, and was used without further purification. Assay of fresh samples by three independent methods showed it to contain about 94% -SH group.[10] A portion of cysteine oxidized with iron-free nitric acid gave no color with thiocyanate and thus may be considered free from iron.

Equipment.—Spectral determinations were carried out using a Beckman spectrophotometer, model DU, and 1 cm. quartz cells fitted with ground-glass stoppers.

Cysteine Complex of Iron(III). Formation in Acid Solution.—Experiments were conducted under nitrogen in which 0.1 ml. of 0.01 *M* iron(III) ammonium sulfate was added to eight oxygen-free solutions of 0.01 *M* hydrochloric acid (10 ml. each) containing cysteine ranging in concentration from 10^{-1} to 5×10^{-5} *M*. Water used to prepare solutions was freed from dissolved oxygen by boiling, sweeping out with oxygen-free nitrogen and storing under nitrogen. In all cases a transient purple coloration was produced which persisted for approximately 0.5 sec.

Cysteine Complex of Iron(III). Formation in Alkaline Solution.—Repeating the above experiments in 0.01 *M* ammonia solution the same purple color formed. This faded gradually on standing, usually persisting for about 20 to 40 minutes depending on the concentration of cysteine.

Cysteine Complex of Iron(III). From Iron(II) Cysteine and Hydrogen Peroxide.—To six oxygen-free solutions of 0.01 *M* hydrochloric acid, containing cysteine ranging in concentration from 10^{-1} to 5×10^{-4} *M*, was added 0.1 ml. of 0.01 *M* iron(II) ammonium sulfate, free from iron(III) ion. Reactions were conducted under nitrogen. To each solution 3.0 ml. of 3% hydrogen peroxide was added. No color was produced, but positive reaction with thiocyanate ion indicated the presence of iron(III). On standing cysteic acid precipitated.

A similar series of experiments at *p*H 8 likewise gave no coloration with peroxide, but within a few seconds cysteic acid precipitated. That all cysteine had reacted was shown by a negative reaction with sodium nitroprusside.

Spectrum of Iron(III) Cysteine Complex.—Absorption spectra of 9×10^{-3} *M* solutions of complex were determined between 400 and 600 mμ. Solutions were prepared by mixing 10.0 ml. of 0.01 *M* cysteine in ammonia at *p*H 9 with 1.0-ml. aliquots of either 0.01 *M* iron(II) ammonium sulfate or iron(III) ammonium sulfate. The initially intense purple of the iron(III) cysteine was allowed to fade until the intensity of color remained sensibly constant, as deter-

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(1) Adapted in part from a portion of the Ph.D. thesis of Roy G. Neville placed in the Library, University of Oregon, Eugene, Oregon, September, 1954.

(2) For reprints: 3013 63rd Avenue S. W., Seattle 16, Washington.

(3) E. Baumann, *Z. physiol. Chem.*, **8**, 299 (1884).

(4) A. P. Mathews and S. Walker, *J. Biol. Chem.*, **6**, 299 (1909).

(5) L. J. Harris, *Biochem. J.*, **16**, 739 (1922).

(6) L. Michaelis, *J. Biol. Chem.*, **84**, 777 (1929).

(7) N. W. Pirie, *Biochem. J.*, **25**, 1565 (1931).

(8) O. Schales, *Ber.*, **71B**, 447 (1938).

(9) M. P. Schubert, THIS JOURNAL, **54**, 4077 (1932).

(10) R. G. Neville and G. Gorin, *ibid.*, **78**, 4891 (1956).

mined by plotting the optical density at a given wave length (445 mμ) *versus* time. The spectrum was then determined. Absorption maxima occur at 445 and 570 mμ, the extinction values being 15.0 and 19.0, respectively. The spectra obtained from either iron(II) or iron(III) ion were identical.

Apparatus for Determining Rates of Fast Reactions.—A simplified apparatus, based on the principle used by Roughton and Millikan,[11] was employed to obtain semi-quantitative measurements on the rates of reaction of cysteine with iron.

The apparatus consisted of two 250-ml. tap-funnels (serving as reservoirs for the reactants) which were connected to a T-tube, one arm of which was 150 cm. in length (internal diameter, 0.36 cm.) and was graduated in centimeters. Pressure could be applied simultaneously to both solutions by means of a mercury head, and a rate of flow of approximately 1 meter per second could be obtained. One centimeter on the arm of the T-tube corresponded, therefore, to 0.01 second. For greatest accuracy complete mixing must occur by turbulent flow, but in these experiments the flow was largely streamline.

Rate of Formation of Iron(III) Cysteine from Iron(III) Ion and Cysteine.—Two hundred and fifty milliliters of 0.01 *M* cysteine at *p*H 8 was placed in one reservoir, and 250 ml. of 0.01 *M* iron(III) ammonium sulfate was placed in the other reservoir of the above apparatus. By means of stopcocks, the iron solution was allowed to flow, then the cysteine was run in. The deep-purple iron(III) complex formed immediately, the maximum intensity of color being within 1 cm. of the mixing chamber.

Rate of Formation of Iron(III) Cysteine from Iron(II) Ion, Cysteine and Hydrogen Peroxide.—Two hundred and fifty milliliters of 0.01 *M* cysteine in dilute ammonia at *p*H 8 (containing 1.0 ml. of 0.01 *M* iron(II) ion per 100 ml. of cysteine solution) were allowed to react with 0.01 *M* hydrogen peroxide (iron-free) in the mixing chamber of the apparatus. The rates of flow of the two solutions were equal. The cysteine solution was purple due to iron(III) complex formed by oxidation of iron(II) ion by the oxygen in solution. This color served to show the course of the reaction. On mixing the two solutions the color faded within 1 cm. of the mixing chamber.

Rate of Oxidation of Cysteine by Hydrogen Peroxide.—Two hundred and fifty milliliters of 0.01 *M* cysteine (iron-free) in dilute ammonia at *p*H 8, containing a trace of nitroprusside ion, was allowed to react with 0.01 *M* hydrogen peroxide in the mixing chamber of the apparatus. Most of the sulfhydryl-nitroprusside color faded within 1 cm. of the mixing chamber, but a faint color persisted to a length of 60 cm.

Results and Discussion

Under oxygen-free conditions, addition of small amounts of iron(III) ion to acid solutions 10^{-1} to 5×10^{-5} *M* in cysteine produces a transient purple complex which persists for about 0.5 second or less. Similar experiments at *p*H 8–9 result in a purple complex which fades gradually but persists for 20–40 minutes depending on the cysteine concentration. Decomposition of this complex is thus strongly catalyzed by hydrogen ions. Addition of hydrogen peroxide to oxygen-free dilute acid or alkaline solutions of cysteine containing iron(II) ion produces no colored complex. That a very rapid oxidation occurs is demonstrated by the precipitation of crystalline cysteic acid a few seconds after peroxide addition.

These oxidation phenomena may be interpreted in the following manner. Hydrogen peroxide oxidizes the iron(II) cysteine complex to the iron(III) state which, being unstable, decomposes immediately yielding cystine and iron(II) ion, in accordance with the observations of Michaelis and Schubert. The cycle of oxidation and reduction of the iron continues until all the peroxide has reacted and then ceases, the iron returning to the divalent state.

At *p*H 8, the semi-quantitative rate data show that mixing iron(II) ion and cysteine with hydrogen peroxide results in complete oxidation of the purple iron(III) complex within 0.01 second. Under the same *p*H conditions the purple complex is formed within 0.01 second on mixing cysteine with iron(III). In absence of iron 0.01 *M* cysteine at *p*H 8 is completely oxidized within 0.6 second by 0.01 *M* hydrogen peroxide. These data show the very powerful catalytic effect of iron on the rate of oxidation of cysteine by oxygen or hydrogen peroxide.

At *p*H 9, the absorption spectrum of the purple complex shows maxima at 445 and 570 mμ. By comparison, at the same *p*H, the spectra of the bis- and green tris-complexes of cobalt(III) with cysteine and 2-mercaptoethylamine (in which amino and sulfhydryl coördination occur) possess maxima at 440 and 570–580 mμ.[12] Coördination in the iron(III) complex may also occur *via* the amino and sulfhydryl groups of cysteine, but at the present time there is no evidence to substantiate this hypothesis.

Acknowledgment.—The author wishes to express his thanks to the Research Corporation for financial assistance throughout this investigation.

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[11] F. J. W. Roughton and G. A. Millikan, *Proc. Roy. Soc.* (*London*), **A155**, 258 (1936).

[12] R. G. Neville and G. Gorin, THIS JOURNAL, **78**, 4893 (1956).