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Metal-Mediated Reduction of Cytochrome c by Thioglycolic Acid[1]

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The reduction of cytochrome *c* by thioglycolic acid was found to be extremely sensitive to metal catalysis. The rate of the uncatalyzed reaction was negligible in comparison with rates obtained from reactions supplemented with catalytic amounts of copper or iron. Both the catalyzed and uncatalyzed reactions were independent of pH (near neutrality) but when *o*-phenanthroline was included in the reaction mixture, a pH dependence was induced. This pH dependence is the result of an interference of oxygen with the metal complexes. A comparison of the rate constants at zero ionic strength, which were obtained from the application of the Debye–Hückel theory for the ionic strength dependence, demonstrated that copper complexes are superior catalysts as compared with iron complexes. Our results suggest that in the copper-mediated reaction, the catalyst is a cupric thioglycolate complex with a net charge of −2. The addition of *o*-phenanthroline to the reaction mixture results in a tenfold decrease in the catalytic activity and in a change in the net charge of the catalyst to −1. At pH 8 the iron-mediated reduction is catalyzed by a ferric thioglycolate complex, whereas at pH 7 a ferrothioglycolate complex provides the catalytic activity. Both complexes have a net negative charge of −2. At both pH's the catalytic activity is completely abolished by the addition of *o*-phenanthroline. The results demonstrate the effectiveness by which metal–sulfur complexes can facilitate one-electron transfer reactions and could therefore serve as a model in the study of various biological oxidations.

Cytochrome *c*, a component of the mitochondrial electron transport chain, has been the subject of active investigations following the elucidation of its tertiary structure by X-ray crystallography (*1*). Information concerning the mechanism of electron transfer to and within cytochrome *c* is mainly derived from studies on the reduction of this molecule by metal complexes such as $Fe(EDTA)^{2-}$ (*2*), $Fe(CN)_6^{4-}$ (*3*), $Ru(NH_3)_6^{2+}$ (*4*), and Cr^{2+} (*5*); free radicals such as superoxide (*6*) and CO_2^- (*7*), as well as by other diverse reductants such as dithionite (*8*), ascorbate (*9*), catechol (*10*), and glutathione (*11*). The possible involvement of nonheme iron proteins as bridges between some heme iron components of the electron transport chain (*12*) brings into attention another class of possible reductants of cytochrome *c*, namely, the nonheme iron center analogs. This type of agent, which in its

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simplest form could be represented by metal complexes of sulfhydryl reagents (*13*) has not been given serious consideration as a reductant of cytochrome *c*.

In a previous communication we examined the effects of a number of reagents as catalysts for the reduction of cytochrome *c* by thioglycolic acid in an effort to underline the importance of chemical contamination in the properties and kinetic behavior of such a system (*14*). During that study it became apparent that the mechanism of the reduction of cytochrome *c* by sulfhydryl reagents and the nature of the reducing species varied with the catalyst employed. Furthermore, evidence was obtained to support the hypothesis that in the presence of ferric or cupric ions the species responsible for the reduction of cytochrome *c* were the complexes of these ions with thioglycolic acid. Evidence for a direct interaction between the metal complexes of thioglycolic acid and cytochrome *c* as well as information concerning the nature of these complexes is presented in the present paper.

MATERIALS AND METHODS

Horse heart ferricytochrome *c* (Sigma Chemical Co., Type III) was used without further purification. Ferricytochrome *c* concentrations were determined by measuring the change in absorbance at 550 nm that results from the full reduction of cytochrome *c* with sodium ascorbate (ε_{550} = 18,500 M^{-1} cm^{-1}) (*15*).

Thioglycolic acid (Sigma Chemical Co., grade I) was distilled twice under vacuum. The distilled acid could be stored at −20°C for as long as 2 months without any significant loss of sulfhydryl group activity as demonstrated by the dithiodipyridine test (*16*).

All buffers were prepared and treated to remove metal contaminants as previously described (*14*).

Ionic Strength and pH

Tris–sulfate buffer solutions ranging from 2.5 to 50 m*M* in anion concentrations and from 6.5 to 8.5 in pH were prepared by mixing stock solutions of 0.5 *M* Tris, pH 10.4, and 0.5 *M* tris–sulfate, pH 6.0, and appropriately diluting them with triple distilled water. The addition of Tris base to a standard quantity of acid was found to be most suitable for the preparation of a series of buffers having the same ionic strength but varying pH. The measure of ionic strength employed in this study was the conductivity which was found to be independent of pH in buffers prepared according to the above method. Conductivity was found to have a linear relationship with molarity for Tris–sulfate buffers up to 0.04 *M*. Deviations from this linear relationship were observed at higher concentrations, possibly due to associations between $Tris^+$ and SO_4 to yield $[Tris–SO_4]^-$.

Assay Methods

The reduction of cytochrome *c* was studied by mixing 1 ml of 2.94 m*M* thioglycolic acid with 0.1 ml of the metal catalyst, immediately followed by the addition of 1 ml of 100 μM cytochrome *c*. Solutions of cytochrome *c* and thioglycolic acid

were prepared in the same buffer shortly before mixing, while solutions of metal catalysts were prepared in slightly acidic water to avoid precipitation. Reduction of cytochrome *c* was followed at 550 nm. Semilogarithmic plots of the declining concentrations of cytochrome c^{3+} versus time were made for each case and pseudo-first-order rate constants were calculated from the half-times of the reactions whenever plots were found to be linear.

Oxygen concentrations during the course of the reaction and rates of oxygen uptake for thiol autoxidation were monitored with a YSI Model 53 oxygraph and recorder.

Formation and decomposition of ferric thioglycolate complexes were monitored at 525.6 nm (isosbestic point between the reduced and oxidized cytochrome *c*). The concentration of ferric thioglycolate complexes was estimated from their absorption at the same wavelength using an extinction coefficient of $3.7 \times 10^3\ M^{-1}$ cm^{-1} (*17, 18*).

THEORY

An important manifestation of electrostatic interactions is the ionic strength dependence of reactions between ions. The ionic strength dependence, according to the transition state formulation, is treated as the result of changing activity coefficients (*19*). The Debye–Hückel treatment for the activities yields the equation

$$\ln k = \ln k_{I\to 0} - \frac{Z_1^2 aI^{1/2}}{1 + KR_1} - \frac{Z_2^2 aI^{1/2}}{1 + KR_2} + \frac{(Z_1 + Z_2)^2 aI^{1/2}}{1 + KR_{\neq}} \qquad [1]$$

where Z_1 and Z_2 are the charges of the reactants, R_1 and R_2 are the radii of the reactants, $R_{\neq}$ is the radius of the transition state, a is a constant with a value of 1.17, and K is a function of ionic strength equal to $0.329I^{1/2}$ $Å^{-1}$ (*20*).

Assuming in our study that the radius of the activated metal–thioglycolate–cytochrome *c* complex is equal to that of the cytochrome *c* alone Eq. [1] becomes

$$\ln k = \ln k_{I\to 0} + \frac{aI^{1/2}}{1 + KR_1}\left[2Z_1Z_2 + Z_2^2 - Z_2^2\frac{1 + KR_1}{1 + KR_2}\right]. \qquad [2]$$

The radius of cytochrome *c*, as estimated from the formula $R_1 = 0.717(MW)^{1/3}$ (*21*) is 16.6 Å. Its charge is $+6.4e$ to $7e$ around neutral pH (*6, 22*). Quantities that are treated as unknowns are the charge and the radius of the reducing species. Assuming that the reducing species are complexes of metals with thiols (*14*) an average radius of 6 Å or less is assigned to them. At an ionic strength of 0.1 this yields values for KR_1 and KR_2 of 1.66 and 0.6, respectively. These values are rather large, and therefore the application of the simplified expression,

$$\ln k = \ln k_{I\to 0} + aI^{1/2}(2Z_1Z_2), \qquad [3]$$

which is derived from Eq. [2] by assuming that $KR \ll 1$, is not appropriate in this case.

Equation [2] is rather complex for a convenient interprolation of data; however, it can be simplified to Eq. [3] by assuming that the factor $2Z_1Z_2$ is much larger than the difference $Z_2 - Z_2(1 + KR_1/1 + KR_2)$:

$$\ln k = \ln k_{I\to 0} + \frac{aI^{1/2}}{1 + KR_1}(2Z_1Z_2). \quad [4]$$

The assumption leading to Eq. [4] becomes valid only at low ionic strengths and at values of Z_1 larger than Z_2. In the case of cytochrome *c* the relatively large surface charge of cytochrome *c* assures that Eq. [4] can be applied with minimal error (less than 10%) provided that the ionic strength is kept lower than 0.1.

Replacement of symbols with numerical values results in the final formulation of the ionic strength dependence for the reactions by cytochrome *c* under the given limitations:

$$\log k = \log k_{I\to 0} + \frac{I^{1/2}}{1 + 5.2I^{1/2}} Z_1Z_2. \quad [5]$$

Expressions similar to Eq. [5] have been derived with the aid of the Brønsted equation (*23*). The application of these expressions for the reaction between cytochrome *c* and hydrated electrons (*22*) or superoxide anion (*6*) was successful in the respect that the values of the surface charges that were determined with these reactions (*7e* and *6.3e*) were close to those estimated from the sequence data of the protein (*1*). It is therefore appropriate to assume that the product Z_1Z_2 (Eq. [5]) contains a factor of the magnitude of the surface charge of cytochrome *c*, the rest being contributed to the charge of the reducing species.

RESULTS

Copper-Mediated Reduction of Cytochrome c

The copper-mediated reduction of cytochrome *c* by thioglycolic acid is first order to the concentration of ferricytochrome *c* under all conditions tested and is practically independent of pH (within the tested range) or the presence of oxygen (*14*). The k_{obs} calculated from the first-order plots is also a linear function of the cupric ion concentration (Fig. 1). Therefore the following rate law appears to apply in this case:[3]

$$d[\text{cyt } c^{3+}]/dt = k_2[\text{Cu complexes}][\text{cyt } c^{3+}]. \quad [6]$$

Iron-Mediated Reduction of Cytochrome c

The iron-mediated reduction of cytochrome *c* by thioglycolic acid is first order to the concentration of cytochrome *c* when excess thioglycolic acid is used under alkaline conditions. At acid or neutral pH, however, the reaction becomes auto-

[3] Abbreviations used: cyt c^{3+}, ferricytochrome *c*; cyt c^{2+}, ferrocytochrome *c*; *o*-phen, *ortho*-phenanthroline; RSH^-, thioglycolic acid; $RSSR^{2-}$, dithiodiglycolic acid.

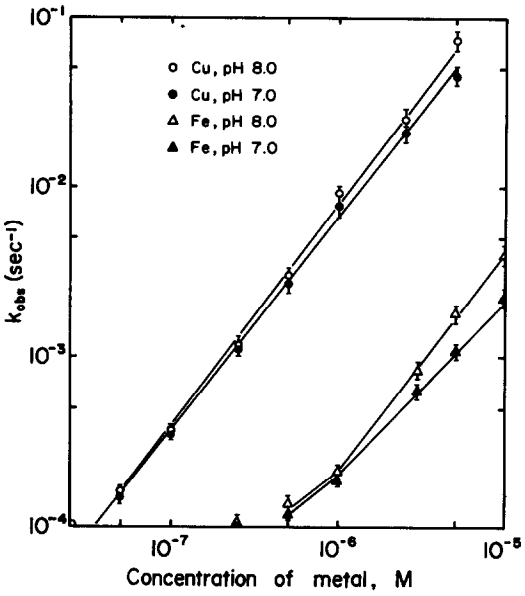

FIG. 1. Effect of metal concentration on the rate of reduction of cytochrome *c* by thioglycolic acid. Reaction mixtures contained 1400 μM thioglycolic acid, 40 μM cytochrome *c*, and $Fe(NH_4)_2(SO_4)_2$ or $CuSO_4$ at the concentrations indicated. Solutions were buffered with 0.02 *M* Tris–sulfate at the pH values indicated.

catalytic and in some instances approaches zero-order kinetics. Deviation from first-order kinetics is intensified by increasing the concentration of the buffer (Fig. 2) and also by using high concentrations of the catalyst. This behavior of the iron-catalyzed reaction, which is not observed with the copper–cytochrome *c* system, suggests that at acid or neutral pH the concentration and/or the activity of the reducing species does not remain constant throughout the reaction.

Addition of ferrous iron to excess thioglycolic acid under aerobic conditions results in the formation of a red complex that was identified from its absorption spectrum ($\varepsilon_{525} = 3.7 \times 10^3\ M^{-1}\ cm^{-1}$) as ferric thioglycolate (*17, 18*). The ferric thioglycolate complex is slowly reduced to yield ferro complexes which, in turn, are rapidly reoxidized by molecular oxygen or other oxidants. Oxygen is consumed during this redox cycle of iron and the thiol is oxidized to its disulfide form in a process known as autoxidation. The oxygen uptake by a solution of iron in thioglycolic acid is related to the bleaching of the red ferric thioglycolate complex but the former reaction is always faster than the net reduction of ferric to ferrous iron due to its amplification by free radical chain reactions (*18, 24*). At pH 8.0 the breakdown of the ferric thioglycolate complex in the presence or absence of cytochrome *c*, as measured from the absorption at 525.6 nm (isosbestic point between the reduced and oxidized cytochrome *c*), and the rate of oxygen uptake by the system are very slow compared with the rate of reduction of cytochrome *c* (Fig. 3). Under these conditions, therefore, the concentration of the iron complexes is constant throughout the reaction. This could justify the following rate

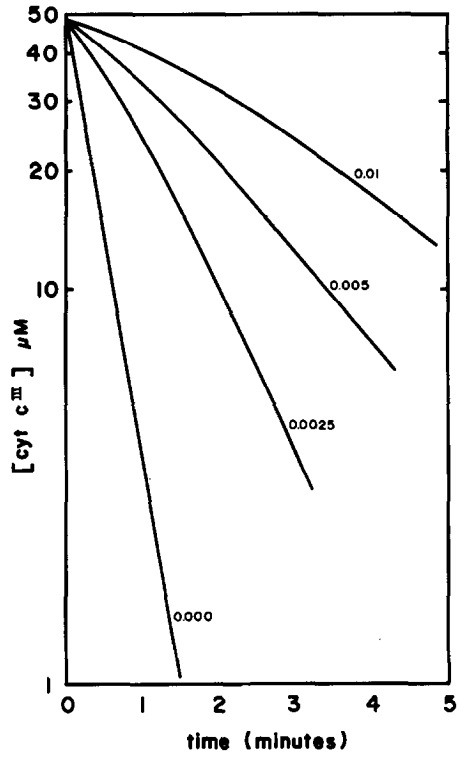

FIG. 2. First-order plots of the iron-catalyzed reduction of cytochrome *c* with thioglycolic acid as a function of the buffer concentration. Cyt c^{3+}: 50 μM; $Fe(NH_4)_2(SO_4)_2$: 8 μM; RSH^-: 1400 μM; buffer: tris–sulfate, pH 7.5, at the concentrations indicated (*M*).

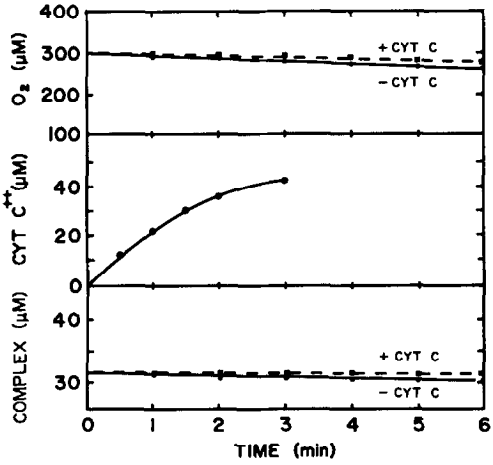

FIG. 3. Time courses of the reduction of cytochrome *c*, bleaching of ferric thioglycolate complexes, and the oxygen uptake taking place in a solution containing 50 μM cytochrome *c*, 1400 μM thioglycolic acid, 32 μM $Fe(NH_4)_2(SO_4)_2$, and 0.02 *M* Tris–sulfate at pH 8.0. Time courses of the bleaching of ferric thioglycolate complexes and oxygen uptake in the absence of cytochrome *c* are also shown for comparison.

law for the iron-mediated reduction of cytochrome *c* at alkaline pH:

$$d[\text{cyt } c^{3+}]/dt = k_2[\text{Fe complexes}][\text{cyt } c^{3+}]. \quad [7]$$

A different situation exists at pH 7.0. In the absence of cytochrome *c* the rate of bleaching of the ferric thioglycolate complex and especially the rate of oxygen uptake become comparable to the rate of reduction of cytochrome *c* under similar conditions (Fig. 4). Cytochrome *c* slightly inhibits the rate of reduction of the ferric thioglycolate complex. It has a more pronounced effect in that it reduces the rate of oxygen uptake to negligible levels. Both the reduction of ferric thioglycolate and the oxygen uptake resume their expected rates after the reduction of cytochrome *c* has been completed. These results indicate that cytochrome *c* affects the rate of reduction of the ferric complex only slightly, but it apparently completely neutralizes the species responsible for the autoxidation of the thiol.

Since at pH 7.0 the concentration of the ferric thioglycolate (and therefore that of the ferro complexes) does not remain constant during the reduction of cytochrome *c*, Eq. [2] cannot be applied. Autocatalytic reactions are expected if the ferro complexes, or the superoxide ions generated through the interaction of these complexes with oxygen, are capable of reducing cytochrome *c* more effectively than the ferric thioglycolate complex does.

Table 1 represents a detailed study of the reactions involved in the iron-mediated reduction of cytochrome *c*. Four sets of rates represent the oxygen uptake in the presence and absence of reduced cytochrome *c*, the bleaching of the ferric thioglycolate complex, and the reduction of cytochrome *c*. The rate of bleaching at all pH values tested cannot by itself account for the remarkable increase in the rate of the reduction of cytochrome *c* at neutral pH. An amplification of the rate of the reduction of the ferric thioglycolate complex similar to that resulting in the oxygen uptake reaction must be considered to explain the pH effect. Evidence for the diversion of electron transfer from oxygen to cytochrome *c* is offered from the data in Table 1. Note that the rate of oxygen uptake at pH 7.3 or 7.0 equals the rate

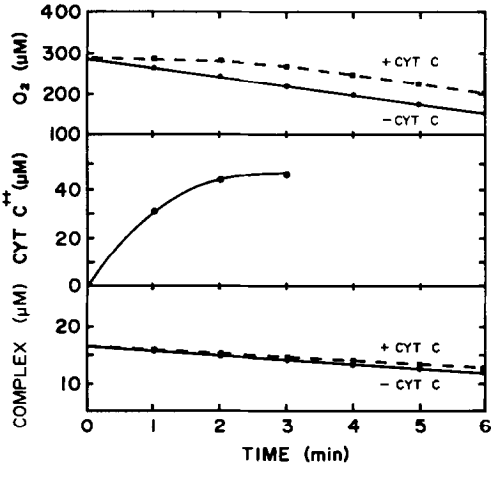

FIG. 4. Same as Fig. 3 except that the pH was reduced to 7.0.

TABLE 1

A COMPARISON BETWEEN THE RATES OF REDUCTION OF CYTOCHROME *c* AND THE SECONDARY REACTIONS RESULTING FROM THE AUTOXIDATION OF THIOGLYCOLIC ACID IN THE PRESENCE OF IRON CATALYSTS

[Fe] (μM)	pH	Rate of reduction of cyt *c* (μM/min)	Rate of oxygen uptake (μM/min)		Rate of reduction of ferric iron[b] (μM/min)
			+cyt *c*[a]	−cyt *c*	
10	7.0	14.6 ± 0.8	7.2 ± 0.2	7.3 ± 0.1	0.21
10	7.3	11.9 ± 0.5	3.2 ± 0.4	2.7 ± 0.2	0.14
10	8.0	7.1 ± 0.3	0.3 ± 0.1	0.3 ± 0.1	0.10
20	7.0	31.9 ± 1.5	16.0 ± 1.6	18.0 ± 1.2	1.20
20	7.3	22.1 ± 1.2	6.7 ± 0.8	6.7 ± 0.5	0.47
20	8.0	15.2 ± 0.6	1.1 ± 0.5	1.8 ± 0.6	0.29
30	7.0	56.3 ± 2.1	28.0 ± 2.1	30.8 ± 1.5	3.04
30	7.3	36.0 ± 1.3	13.7 ± 2.4	16.3 ± 1.8	0.76
30	8.0	22.7 ± 0.8	2.5 ± 0.5	3.1 ± 0.3	0.35
40	7.0	78.2 ± 2.5	39.2 ± 4.1	44.8 ± 2.8	6.20
40	7.3	51.8 ± 2.3	23.0 ± 2.1	25.2 ± 2.0	1.06
40	8.0	29.8 ± 1.2	4.0 ± 0.3	5.1 ± 0.4	0.53
50	7.0	110.6 ± 4.8	56.0 ± 5.6	61.6 ± 5.8	9.40
50	7.3	67.4 ± 2.3	28.1 ± 2.8	30.0 ± 3.0	1.76
50	8.0	38.0 ± 1.6	5.9 ± 0.8	6.7 ± 0.5	0.60

Note. Reaction mixtures in 0.02 *M* Tris–sulfate buffer of the appropriate pH contained 1400 μM thioglycolic acid, ferric iron as indicated, and 50 μM cytochrome *c*, when this component was included.

[a] Rates were measured after all the cytochrome *c* was reduced and the autoxidation of the thiol reached a maximum value (Figs. 3 and 4).

[b] These values were obtained in the absence of cytochrome *c*.

of reduction of cytochrome *c* at the respective pH if the rates of reduction of cytochrome *c* at pH 8.0 are subtracted from them.

Effect of pH on the Iron-Catalyzed Reaction

The small pH effect observed for the copper- and iron-catalyzed reduction of cytochrome *c* by thioglycolic acid has led to the conclusion that metal catalysis for this reaction is pH insensitive as compared with other types of catalysis (*14*). This observation has been confirmed in the present study using a different approach (Table 2). In the case of iron catalysis, however, the conclusion is valid only at low concentrations of the catalyst ($< 5\ \mu M$). A pH dependence becomes increasingly significant with increasing concentrations of iron in the reaction mixture (Table 1). Such behavior, however, is in accordance with the hypothesis that ferrothioglycolate complexes and/or intermediates of the autoxidation of thioglycolic acid interfere with the reduction of cytochrome *c*. At pH 8.0 the rate of bleaching of the ferric thioglycolate complex and the consequent events leading to the reduction of oxygen are insignificant compared with the rate of cytochrome *c*

TABLE 2

CHARGE PRODUCT AND RATE CONSTANTS FOR THE REDUCTION OF CYTOCHROME *c* BY THIOGLYCOLIC ACID UNDER VARIOUS CONDITIONS

	pH	Z_1Z_2	$k_{I\to 0}$ (sec^{-1})
Uncatalyzed	6.6	6.8 ± 1.1	7.9×10^{-4}
reaction	7.8	8.0 ± 2.4	10.5×10^{-4}
Cu-catalyzed	6.6	12.6	1.6×10^{-1}
reaction	7.8	11.5	1.6×10^{-1}
Fe-catalyzed	6.6	12.9	7.9×10^{-3}
reaction	7.8	14.6	7.5×10^{-3}
Cu/*o*-phen	6.6	7.5	1.8×10^{-2}
system	7.8	7.3	5.9×10^{-2}
Fe/*o*-phen	6.6	6.7	2.3×10^{-4}
system	7.8	7.0	9.8×10^{-4}

reduction under these conditions. At this pH the expected pseudo-first-order behavior of the system suggests that the concentration of the reducing species remains constant and that ferro complexes, if formed, have a small contribution to the overall reduction of cytochrome *c*. At pH 7.0, however, the more rapid formation of ferrothioglycolates results in kinetic abnormalities. Since the formation of ferrothioglycolate complexes is second order to the concentration of ferric iron (*17*) and the undisturbed reduction of cytochrome *c* is first order to that concentration (Fig. 5) the rate of the interfering reaction is expected to decrease faster than the rate of the undisturbed reduction of cytochrome *c*. It follows that below a certain concentration of iron the reduction of cytochrome *c* must be much faster than the bleaching of ferric thioglycolate. Figure 5 demonstrates that at iron concentrations lower than 5 μM the rates of bleaching for ferric thioglycolate become similar at all pH values tested and much slower than the rate of the undisturbed reaction. At those iron concentrations the pH effect on the rate of cytochrome *c* reduction and the deviations from first-order kinetics were observed to be negligible.

Effect of Ionic Strength

The rate of reduction of cytochrome *c* by thioglycolic acid is greatly affected by the ionic strength of the medium. Such an effect is illustrated in Fig. 2 for the iron-mediated reaction; similar patterns are observed for the noncatalyzed and the copper-catalyzed reactions. Plots of the log of the rate constants of a certain reaction at various buffer concentrations versus the proper function of the ionic strength of the medium (see Theory) could provide information about the approximate charge of the reducing species and the thermodynamically significant rate constant of the reaction, and could also reveal changes in the nature of the reduc-

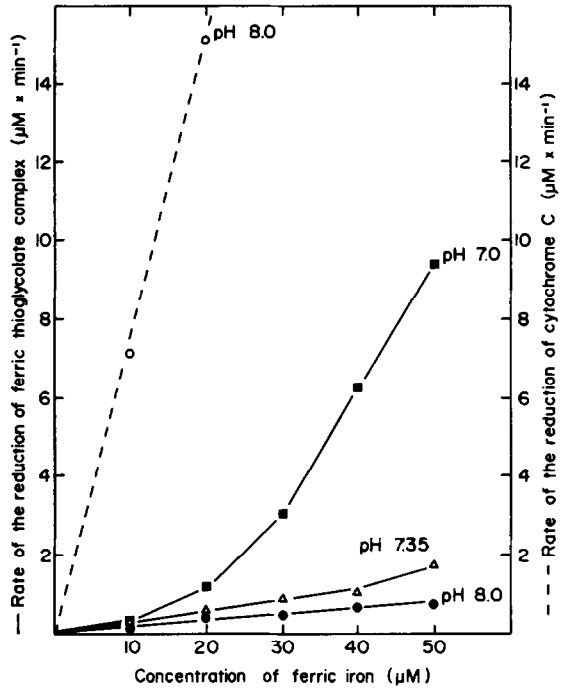

FIG. 5. Rate of reduction of ferric thioglycolate complexes as a function of pH and concentration of ferric iron. Reaction mixtures contained 1400 μM thioglycolic acid, $Fe(NH_4)_2(SO_4)_2$ as indicated, and 0.02 M Tris–sulfate at the pH values indicated. The rate of reduction of cytochrome c (50 μM) by thioglycolic acid (1400 μM) in 0.02 M Tris–sulfate buffer, pH 8.0, as a function of ferric iron concentrations is also given for comparison.

ing species when chelating agents, such as *o*-phenanthroline, are added to the reaction mixtures.

Plots of this type are presented in Figs. 6 and 7 for the copper- and iron-mediated reduction of cytochrome *c*, respectively. Similar plots can theoretically be obtained for the uncatalyzed reaction, but experimentally this proved to be extremely difficult due to the low rates and the extreme sensitivity of this reaction to contaminating metals. It was necessary, therefore, to derive the values for $k_{I\to 0}$ and Z_1Z_2 from a statistical manipulation of a number of values for these parameters obtained from linear regression analyses of several sets of experiments performed at pH 6.6 and 7.8 (Table 2). Values of $k_{I\to 0}$ and Z_1Z_2 for the copper- and iron-catalyzed reactions were derived from Debye–Hückel-type plots similar to those described in Figs. 6 and 7. All obtained values are listed in Table 2.

The following conclusions may be drawn from the data presented in Figs. 6 and 7 and in Table 2:

(a) The uncatalyzed as well as the copper- and iron-mediated reactions are practically pH independent. This supports our previous conclusion that the thiolate ion cannot be the major reducing species under the experimental conditions employed in this work (*14*).

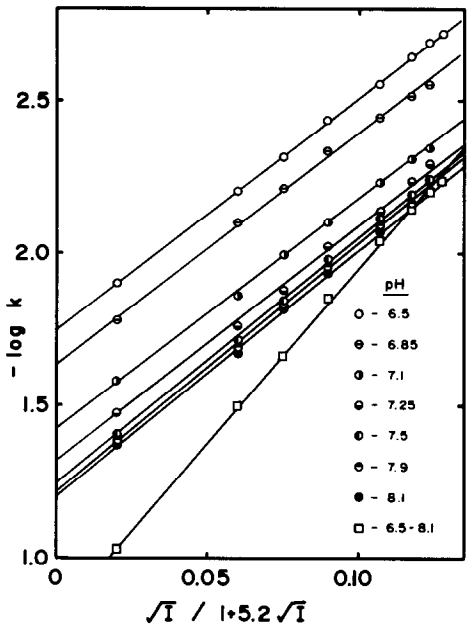

FIG. 6. Debye–Hückel-type plots for the copper (□)- and copper–phenanthroline (○)-catalyzed reduction of cytochrome *c* by thioglycolic acid at various pH values. Pseudo-first-order rate constants were determined from semilogarithmic plots of the concentration of ferricytochrome *c* vs time. Mixtures of cytochrome and thioglycolic acid were supplemented with 1 μM $CuSO_4$ for the copper-catalyzed reactions and with an additional 10 μM *o*-phenanthroline for the copper–phenanthroline-catalyzed reaction.

(b) The reducing species are always negatively charged, even in the presence of *o*-phenanthroline. This observation rules out the participation of any positively charged complexes of *o*-phenanthroline with metal ions in the reduction of cytochrome *c*.

(c) Both iron and copper are powerful catalysts for the reduction of cytochrome *c* but copper is superior to iron in catalyzing this reaction.

(d) *o*-Phenanthroline completely reverses the effect of iron which suggests that iron–phenanthroline complexes fail to act as electron transporters between the thiol and cytochrome *c*.

(e) Copper is active as a catalyst even in the presence of chelating agents, although complexes of cupric or cuprous copper with these agents are not responsible for the direct reduction of the cytochrome.

(f) Although the values for Z_1Z_2 presented in Table 2 are not very accurate due to simplifications of the Debye–Hückel theory, they definitely suggest that the charge of the reducing species is reduced when *o*-phenanthroline is included in the reaction mixtures of both the iron- and copper-catalyzed reactions. This observation is again consistent with our previous conclusion that the reducing species are complexes of the metals with thioglycolic acid and not thioglycolic acid itself.

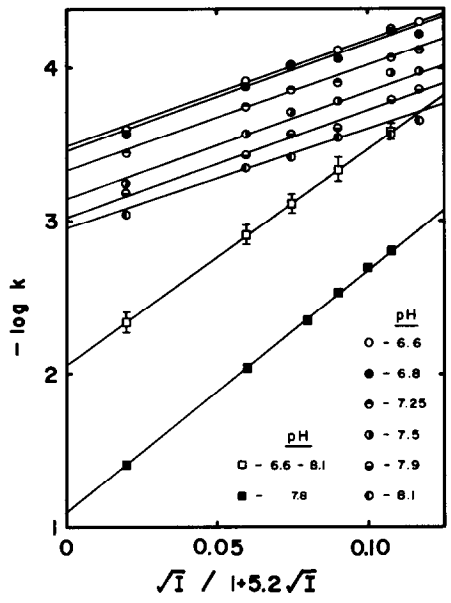

FIG. 7. Debye–Hückel-type plots for the iron (□)- and iron–phenanthroline (○)-mediated reduction of cytochrome *c* by thioglycolic acid at various pH values. Pseudo-first-order rate constants were determined as described under Materials and Methods. Mixtures of cytochrome *c* and thioglycolic acid were supplemented with 1 μM (□) and 5 μM (■) $Fe(NH_4)_2(SO_4)_2$ for the iron-catalyzed reactions and with 10 μM *o*-phenanthroline followed by 1 μM $Fe(NH_4)_2(SO_4)_2$ for the iron–phenanthroline-catalyzed reactions.

The Copper/o-Phenanthroline System

The capacity of copper to catalyze the reduction of cytochrome *c* by thioglycolic acid even in the presence of chelating agents and the pH effect induced by the addition of such agents (Table 2, Fig. 8) place these types of reactions in a special category of metal catalysis. A comparison between the iron-mediated reaction (Table 1) and the copper/phenanthroline system (Fig. 8) reveals that these two reactions, although both subject to pH changes, differ regarding the direction of this effect; while rates in the iron-mediated reaction tend to rise with decreasing pH, the opposite is true for the copper/phenanthroline system. In this respect, the conclusions derived for the former reaction cannot be applied to the latter case and therefore an evaluation of the events leading to the reduction of cytochrome *c* by thioglycolic acid in the presence of copper/phenanthroline is appropriate at this time.

The addition of cupric copper to a solution of thioglycolic acid triggers the autoxidation of this thiol as becomes apparent from the reduction of molecular oxygen (Fig. 9). The rate of oxygen reduction is not significant compared with the rate of the reduction of cytochrome *c* under the same conditions. However, when *o*-phenanthroline is added to a thioglycolate solution along with the copper the rate of oxygen reduction is stimulated to the extent that this reaction becomes

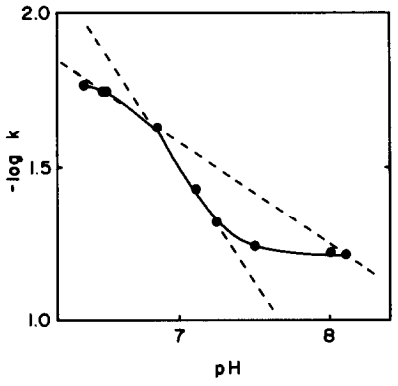

FIG. 8. Effect of pH on the rate (k_{obs} at $I \rightarrow 0$) of the reduction of cytochrome *c* as catalyzed by the copper–phenanthroline complex.

comparable with the reduction of cytochrome *c* (Fig. 9). Furthermore, intermediates participating in the reduction of oxygen are scavenged and participate in the reduction of cytochrome *c* when this component is added to the copper–phenanthroline system.

A comparison between the pH patterns which are induced by copper–phenanthroline catalysis on the reduction of cytochrome *c* (Fig. 8) and the autoxidation of thioglycolic acid (Fig. 10) reveals a significant difference between the two reactions. While the reduction of oxygen is favored by lowering the pH, the reduction of cytochrome *c* follows the opposite pattern. This along with the observation that cytochrome *c* retards the reduction of oxygen suggests that the autoxidation of thioglycolic acid by the copper–phenanthroline system, like in the iron-catalyzed reaction, results in the formation of species capable of reducing cytochrome *c*;

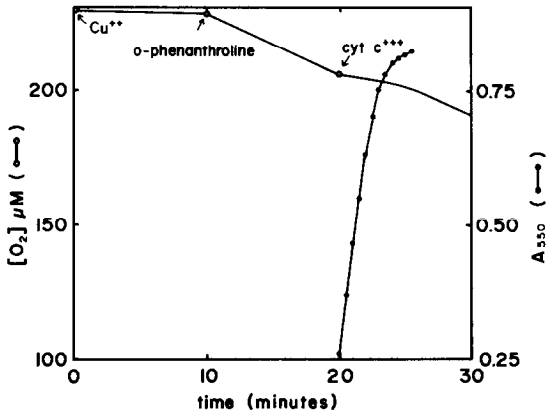

FIG. 9. Course of oxygen uptake during the autoxidation of thioglycolic acid in the presence of cytochrome *c* and/or the copper–phenanthroline complex. Additions of $CuSO_4$, *o*-phenanthroline, and cytochrome *c* to 1400 μM RSH^- in 3 ml 0.02 *M* Tris–sulfate buffer, pH 6.5, were made at the times indicated to final concentrations of 1, 10 and 50 μM, respectively.

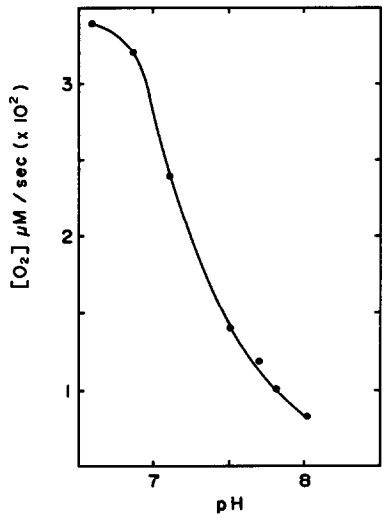

FIG. 10. The pH effect on the autoxidation of thioglycolic acid as catalyzed by the copper–phenanthroline complex. RSH^-: 2800 μM; $CuSO_4$: 1 μM; *o*-phenanthroline: 10 μM; buffer: Tris–sulfate, 0.02 *M*.

however, these species, unlike in the iron-catalyzed reaction, do not reduce cytochrome *c* as efficiently as their immediate precursors.

DISCUSSION

The reduction of cytochrome *c* by metal complexes of thioglycolic acid appears to be complicated by the presence of species participating in the autoxidation of the thiol. Complications appear in the form of nonlinear semilogarithmic plots of the cytochrome *c* concentration vs time and unpredictable relations between the rates of cytochrome *c* reduction and the concentration of the metal mediating the reaction. Since such complications appear frequently in the case of iron-catalyzed reactions it is of interest to examine the reactions involved in the autoxidation of thioglycolic acid in the presence of ferric salts.

Reactions leading to the autoxidation of thiols can be classified into at least two groups. The first group of reactions does not involve oxygen and therefore can proceed under anaerobic conditions. The oxidation of the thiol occurs at the expense of the metal catalyst, leading to the formation of a reduced form of the catalyst. In the case of iron the sequence of events is depicted by Eqs. [8] to [10] (*18*):

$$[Fe^{3+}(OH)(RS)_2]^{2-} + H^- \rightarrow [Fe^{3+}(OH)(RS)]^0 + RSH^- \quad [8]$$

$$[Fe^{3+}(OH)(RS)_2]^{2-} + [Fe^{3+}(OH)(RS)]^0 \rightarrow [Fe_2{}^{3+}(OH)_2(RS)_3]^{2-} \quad [9]$$

$$[Fe_2^{3+}(OH)_2(RS)_3]^{2-} \rightarrow [Fe^{2+}\ \text{complex}] + RSSR^{2-} \quad [10]$$

The structure of the red ferric thioglycolate complex has been determined (*17*) to be $[Fe^{3+}(OH)(RS)_2]^{2-}$; the structure of the blue complex is still uncertain, but presumably contains two iron atoms per molecule (*18*) whereas the structure of the ferrous complex is still unknown.

The rate of reduction of the red ferric thioglycolate complex has been determined by Lamfrom and Nielsen (*18*) to be directly proportional to the hydrogen ion concentration and to be second order to the concentration of iron:

$$-d[Fe^{3+}\text{ complex}]/dt = 3.3 \times 10^6 \frac{[H^+][Fe^{3+}]^2}{[RSH]} M^{-1}\,\text{sec}^{-1}. \qquad [11]$$

The reaction rate therefore increases with decreasing pH and increases more sharply than the rate of cytochrome *c* reduction with increasing concentrations of iron.

The second group of reactions participating in the autoxidation of thiols in the presence of metal catalysts involves molecular oxygen and leads to the reoxidation of the reduced metal catalyst. The exact nature of these reactions is obscure; however, there is a general agreement that a superoxide ion is first generated from the interaction of the reduced metal complex with molecular oxygen (reaction [12]).

$$2Fe^{2+}(RS)\text{ complex} + 2O_2 \rightarrow 2Fe^{3+}(RS)\text{ complex} + 2O_2^- \qquad [12]$$

$$2H^+ + 2O_2^- \rightarrow H_2O_2 + O_2 \qquad [13]$$

$$H_2O_2 + RSH^- \rightarrow RSOH^- + H_2O \qquad [14]$$

$$RSOH^- + RSH^- \rightarrow RSSR^{2-} + H_2O \qquad [15]$$

$$2Fe^{2+}(RS)\text{ complex} + O_2 + 2H^+ + 2RSH^- \rightarrow 2Fe^{3+}(RS)\text{ complex} + RSSR^{2-} + 2H_2O \qquad [16]$$

Presumably, the generated superoxide ion can dismutate to H_2O_2 (Eq. [13]), which in turn can oxidize a sulfhydryl to a sulfenic acid group (Eq. [14]). The sulfenic acid group readily reacts with another sulfhydryl group to form dithiodiglycolate (Eq. [15]). This sequence of events is consistent with the observation of Lamfrom and Nielsen (*18*) that the oxygen utilized in the autoxidation reaction is ultimately reduced to water (Eq. [16]), and that the amount of oxygen utilized is stoichiometric according to the equation

$$4RSH^- + O_2 \rightarrow 2RSSR^{2-} + 2H_2O. \qquad [17]$$

Although the sequence depicted in Eqs. [12]–[16] is largely hypothetical, it is consistent with previous observations regarding the second half of the autoxidation cycle (*25, 26*).

In the case of the iron-mediated reduction of cytochrome *c* at pH 8.0 the rate of bleaching of the ferric thioglycolate complex is too slow compared with the reduction of cytochrome *c*. At the same pH amplification of the reducing power of the ferrothioglycolate complexes through free radical chain reactions, as judged from the rate of oxygen uptake, cannot be responsible for the reduction of cytochrome

c. Therefore, at pH 8.0 the reduction of cytochrome *c* must be attributed to a direct interaction between cytochrome *c* and the ferric thioglycolate complexes which must recycle in excess thioglycolic acid:

$$\text{cyt } c^{3+} + [Fe^{3+}(OH)(RS)_2]^{2-} \xrightarrow{\text{RSH}} Fe^{3+} \text{ complex} + RS\cdot + \text{cyt } c^{2+} \qquad [18]$$

The two sets of reactions [8]–[10] and [12]–[15] take place in the absence of cytochrome *c*. The presence of cytochrome *c* in the reaction mixture does not affect the rate of reduction of the ferric thioglycolate complexes; however, it has a pronounced effect in retarding the reduction of molecular oxygen. The inhibition of oxygen uptake and therefore of the autoxidation of the thiol by the presence of ferricytochrome *c* suggests that this molecule can replace oxygen as an electron acceptor in Eq. [12]. Since the reduced cytochrome *c*, unlike the superoxide ion, cannot promote the oxidation of sulfhydryl groups, the extent of the sequence [12]–[15] is greatly minimized in the presence of cytochrome *c*. The nonparticipation of the oxygen–superoxide couple in the mediation of electrons from ferrothioglycolate to cytochrome *c* is also suggested by the insensitivity of the rate of cytochrome reduction to the presence of oxygen (*14*). However, our observations indicate that the reduction of cytochrome *c* at pH 7.0 accelerates as a function of time. At this pH the reduction of the cytochrome proceeds according to Eq. [18] to the extent that the iron is present as the ferric thioglycolate complex. The rate of reduction would be expected to diminish as the concentration of $[Fe^{3+}(OH)(RS)_2]^+$ diminishes according to Eqs. [8]–[10]. Suggesting that cytochrome *c* can replace oxygen as an electron acceptor in Eq. [12], i.e.,

$$Fe^{2+}(RS) \text{ complex} + \text{cyt } c^{3+} \rightarrow Fe^{3+}(RS) \text{ complex} + \text{cyt } c^{2+}, \qquad [19]$$

is unsatisfactory in that the sequence fails to account for the observed rate acceleration since the bleaching of the ferric complex is very slow.

The kinetic data presented in Fig. 5, as well as elsewhere (*14*), clearly indicate the slow formation of a catalyst that is considerably more effective in reducing cytochrome *c* at pH 7.0 than the ferric complex. These observations are consistent with a catalytic mechanism by the ferrous complex, which is similar to but more effective than the ferric complex:

$$\text{cyt } c^{3+} + Fe^{2+}(RS)_2 \text{ complex} \xrightarrow{\text{RSH}} Fe^{2+}(RS) \text{ complex} + RS\cdot + \text{cyt } c^{2+}. \qquad [20]$$

Complete reduction of cytochrome *c* by ferric thioglycolate complexes can take place at acid or neutral pH, if the concentration of iron in the reaction mixture is kept low enough to avoid reduction of the metal catalyst at a measurable rate. The requirement for low concentrations of iron in avoiding extensive participation of Eq. [20] becomes obvious upon the examination of Eq. [11]. Since the rates of cytochrome *c* reduction (Eq. [18]) and ferric thioglycolate reduction are first and second order, respectively, to the concentration of iron, they increase disproportionally with increasing iron concentrations. The ratios of the two rates are 10, 2,

and 1 at iron concentrations of 5, 28, and 50 μM, respectively. As a result 75, 40, and 34% of the initial rates of the reduction of cytochrome *c* are attributed to Eq. [18], the rest being the result of Eq. [20]. Experimental results indicate that complications due to the participation of Eq. [20] can be avoided completely if the concentration of iron is kept lower than 5 μM. Such conditions were employed for the development of the Debye–Hückel plots, which therefore describe only the interaction between ferric thioglycolate and cytochrome *c*.

Unlike the iron-catalyzed reduction of cytochrome *c* the copper-mediated reaction does not seem to be affected by autoxidation reactions. This is probably due to a higher capacity of cupric thioglycolate complexes to reduce cytochrome *c* than to undergo reduction themselves. Indeed the rate of oxygen uptake by the copper–thioglycolic acid system is several orders of magnitude lower than the reduction of cytochrome *c* by the system. The following equation is suggested to describe the reduction of cytochrome *c* by cupric thioglycolate complexes:

$$[Cu^{2+}(RS)_n]^{2-2n} + \text{cyt } c^{3+} \rightarrow [Cu^{2+}(RS)_{n-1}(RS\cdot)]^{3-2n} + \text{cyt } c^{2+}. \qquad [21]$$

RSH

Although the exact structure of the cupric thioglycolate complexes has not been established, previous reports on the coordination of cupric copper and the nature of its complexes with other sulfhydryl reagents (*27, 28*) advocate two molecules of the thiol complexed to each cupric ion in a square planar configuration. Such a complex would carry a formal charge of $-2e$.

The conclusion that cytochrome *c* is reduced by the oxidized forms of the metal–thioglycolate complexes in systems containing thioglycolic acid in excess of cytochrome *c* and the metal catalyst only in traces is further supported by the application of the Debye–Hückel theory to such systems. The lack of a pH effect on the rate constants of both the iron- and copper-catalyzed reactions demonstrates that the autoxidation of the thiol and the reduction of the metal complexes, both pH-dependent processes, do not interfere with the reduction of cytochrome *c*. In addition the charges of the reducing species as calculated from the slopes of the Debye–Hückel plots agree with the charges of the species already assigned to reduce cytochrome *c* (Eqs. [18] and [21]).

The rate constant (k_{obs}) for the reduction of cytochrome *c*, as derived from first-order plots, should contain a second-order rate constant (k_2) for the reaction between the cytochrome and the reducing species and also a term representing the concentration of these species. Although a linear relationship was found to exist between k_{obs} and the metal catalyst concentration, an accurate derivation of the second-order rate constant was not possible because the concentration of the metal does not necessarily represent the concentration of the reducing species. However, assuming that metal complexes are directly involved in the reaction and that the upper limit of the concentration of the reducing species is equal to the metal concentration, minimum values for the second-order rate constants can be calculated:

$$k_2 = k_{obs}[\text{metal}]^{-1}. \qquad [22]$$

Such calculations yield a minimum second-order rate constant of $1–1.6 \times 10^4$ M^{-1} sec^{-1} for the iron-catalyzed reaction and 1.6×10^5 M^{-1} sec^{-1} for the copper-catalyzed reaction. These values are comparable with those reported for the reduction of cytochrome *c* by ascorbate (*9*), by $[Fe^{2+}(EDTA)]^{2-}$ complexes (*2*), and by $Ru(NH_3)_6^{2+}$ (*4*). This suggests a remote (outer sphere) pathway for the electron transfer between cytochrome *c* and the thiol complexes.

Addition of *o*-phenanthroline to the copper-mediated reduction of cytochrome *c* results in two interesting modifications of the behavior of this system. First, the charge of the reducing species changes from $-2e$ to $-1e$ and second, the rate becomes pH dependent. Previous studies (*29*) have shown that *o*-phenanthroline induces a rapid reduction of cupric to cuprous copper which either accumulates under anaerobic conditions or is reoxidized by molecular oxygen according to the following reactions:

$$2RSH^- + 2(o\text{-phen})_nCu^{2+} \rightarrow RSSR^{2-} + (2n)o\text{-phen} + 2Cu^+ + 2H^+ \quad [23]$$

$$(2n)o\text{-phen} + 2Cu^+ + \tfrac{1}{2}O_2 \rightarrow 2(o\text{-phen})_nCu^{2+} + H_2O. \quad [24]$$

Linear complexes of cuprous thioglycolate $(Cu–RS)^-$ and the superoxide ion, generated during the autoxidation of the thiol, are also considered capable of reducing cytochrome *c*. However, the uncertainty about the nature of the reactions and of the complexes participating in the autoxidation of thioglycolic acid under such conditions and also the dismutase activity of the copper salts does not permit any further speculation on this particular system.

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