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Redox Interactions between Cytochrome Components and Transition Ions and Metals†

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ABSTRACT: Factors influencing phosphate activation of ferricytochrome *c* reduction by Fe^{2+} have been investigated. Rates of ferricytochrome *c* reduction by Fe^{2+} in phosphate-containing system are further increased by (1) high pH, (2) high O_2 tension, and (3) the presence of Fe^{3+} in the medium. In the presence of both phosphate and Fe^{3+}, reduction of cytochrome *c* by Fe^{2+} is activated in synergistic fashion. Involvement of superoxide anion (O_2^-) in the oxygen enhancement of reduction rates is unlikely, based upon lack of inhibitory effect by the following superoxide anion scavengers; superoxide dismutase (erythrocuprein), 3,4-dihydroxybenzoic acid and 1,2-dihydroxybenzene-3,5-disulfonic acid. The effect of O_2 upon reduction of ferricytochrome *c* by Fe^{2+} is also strongly influenced by the anion species in the medium. Thus, in the presence of phosphate, 100% O_2 markedly activates reduction rates, while in the presence of bicarbonate, 100% O_2 markedly inhibits reduction rates. In Tris systems, 100% O_2 exerts a negligible to very slight activation of reduction rates. Ferrocytochrome *c* produced by reduction by Fe^{2+} is reoxidizable by Cu^{2+}, ferricyanide, and by cytochrome oxidase obtained from yeast and beef heart mitochondria, but not by Fe^{3+}. Visible and Soret region absorption spectrum tracings show that yeast and beef heart mitochondria are also reduced by Fe^{2+}, with the apparent difference that in yeast mitochondria, cytochrome *b* and flavoprotein components are significantly less reduced than in heart mitochondria. The direct electrolytic reduction of cytochrome *c* by Zn^0 and Cu^0 dusts and powders is also reported.

A selective role of phosphate in the reduction of cytochrome *c* by the transition ions Cr^{2+} and Fe^{2+} has been reported (Yates and Nason, 1966; Kowalsky, 1969; Zipper *et al.*, 1971; Taborsky, 1972). Yates and Nason (1966), Zipper *et al.* (1971), and Taborsky (1972) found that the reduction of ferricytochrome *c* by Fe^{2+} is activated by phosphate, and that this activation is sensitive to the presence of other anionic species and O_2 in the medium. We now report additional detailed investigations of these phenomena, and also observations of other redox interactions between transition ions and metals (M^0), and cytochrome components.

Materials and Methods

Tris-HCl, Tris base, and cytochrome *c*, type III, horse heart, were obtained from Sigma Chemical Co. (St. Louis, Mo.). N_2, prepurified grade, and O_2, extra dry grade, were from Matheson Gas Products (East Rutherford, N. J.). Bovine erythrocyte erythrocuprein was obtained from Miles Laboratories, Inc., Elkhart, Ind. Pure metals from ALFA Inorganics, Inc. (Beverly, Mass) were: Cu powder, 1 μ, 99.95%; Cu foil, 5 mil, 99.000%; Fe powder, 325 mesh, 99.9%; Fe foil, 5 mil, 99.998%; Co foil, 5 mil, 99.7%; Ni foil, 5 mil, 99.998%; Zn powder, 200 mesh, 99.999%; Zn foil, 10 mil, 99.9%. Foils and strips were stored in 95% ethanol. For experiments, they were cut, dried, weighed, and used immediately. All other chemicals were of reagent grade. To obtain ferrous chloride free from ferric ion, reagent grade $FeCl_2 \cdot 4H_2O$ crystals were rinsed with distilled water and dried rapidly with filter paper under a stream of nitrogen. Other transition metal salts used were $FeCl_3 \cdot 6H_2O$ and $CuSO_4 \cdot 5H_2O$. All solutions were prepared immediately prior to use. Water was triple distilled, once from a tin-lined container and twice from glass.

Absorption spectra were obtained in a Perkin-Elmer Model 450 recording spectrophotometer or in a Process and Instruments Co. Model RS3 recording spectrophotometer. Fe and Cu were determined with a Perkin-Elmer Model 290 atomic absorption spectrophotometer. pH measurements were made with a Radiometer pH meter Model TTT 3C, using a Corning semimicro combination electrode (Type 476020). Rates of cytochrome *c* reduction or oxidation were determined in Thunberg-type cuvets with a 1.0-cm light path by measuring absorbance changes at 549 nm. 4 ml of cytochrome *c* solution was placed in the lower portion of the cuvet. Next, the native metal sample or 0.08 ml of solution containing the transition metal ion was placed in the upper side arm of the Thunberg

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TABLE I: Influence of Buffer Species and pH on Rate of Reduction of Ferricytochrome *c* by Fe^{2+}.[a]

Buffer (M)	Tris, pH 7.4	Tris, pH 8.3	Phosphate, pH 7.1
1×10^{-4}	0.63	1.30	
1×10^{-3}	0.81	2.69	16.4
1×10^{-2}	0.75	9.70	80.0
1×10^{-1}	1.90	22.9	66.4
0.2	2.40	39.9	37.3
0.4	4.15	45.0	*b*

[a] Reactions carried out under nitrogen. Final concentrations were: ferricytochrome *c*, 3.2×10^{-5} M; Fe^{2+}, 1×10^{-3} M. Temperature was 25°. Initial reduction rates are expressed as nmol ml^{-1} of ferricytochrome *c* reduced per min. [b] Experiment not performed because of poor solubility of buffer component at this molarity.

vessel. To exclude air, N_2 was flushed through the system for 10 min. The system was then closed to the atmosphere, and an initial zero-time reading taken. Cuvet contents were then mixed several times by rapid inversion, and absorbance was measured at varying time intervals for total periods of from 3 to 30 min, keeping the reacting system at 25 ± 0.5°. For very rapid reactions, measurements were made at 10- to 15-sec intervals during the first 60 sec following mixing of reactants. Absorbance changes were plotted *vs.* time. Initial reaction rates were determined by extrapolation from the graphs, and expressed as nmol ml^{-1} cytochrome *c* reduced or oxidized per min. pH values and turbidities were checked prior to, and following experiments, to make certain that significant changes involving these variables had not occurred during the experiment.

Interactions between transition metal ions and suspensions of beef heart and yeast mitochondria and solubilized mitochondrial cytochrome oxidase (Person *et al.*, 1965) were followed by recording visible absorption spectra (400–700 nm) at sequential time intervals at room temperature (18–22°). A "multiple-reflectance filter paper technique" devised in this laboratory for working with turbid suspensions was used (Person and Fine, 1964). Mitochondria were isolated from water according to methods previously described (Person *et al.*, 1965).

TABLE II: Influence of pH Variation in 0.01 M Phosphate Buffer upon Ferricytochrome *c* Reduction by Fe^{2+}.[a]

pH	Initial Reduction Rate
5.6	2.4
6.1	5.4
6.5	10.9
7.0	117.7
7.5	185.0
8.0	Too fast

[a] Final concentration of Fe^{2+} was 1×10^{-3} M; temperature, 25°; reaction carried out under N_2.

TABLE III: Effect of Ionic Strength on the Rate of Reduction of Ferricytochrome *c* by Fe^{2+} in Presence of Buffers.[a]

Buffer	Salt Added	Γ/2	Initial Reduction Rate
0.1 M Tris, pH 7.4		0.08	1.97
0.1 M Tris, pH 7.4 plus	0.3 M NaCl	0.38	3.06
0.1 M Tris, pH 7.4 plus	0.5 M NaCl	0.58	3.08
0.1 M Tris, pH 7.4 plus	0.9 M NaCl	0.98	4.70
0.1 M Tris, pH 7.4 plus	0.3 M KCl	0.38	3.21
0.1 M Tris, pH 7.4 plus	0.5 M KCl	0.58	3.59
0.1 M Tris, pH 7.4 plus	0.9 M KCl	0.98	5.02
0.01 M PO_4, pH 7.1		0.06	17.10
0.01 M PO_4, pH 7.1 plus	0.05 M NaCl	0.11	7.67
0.01 M PO_4, pH 7.1 plus	0.1 M NaCl	0.16	5.29
0.01 M PO_4, pH 7.1 plus	0.2 M NaCl	0.26	2.97
0.01 M PO_4, pH 7.1 plus	0.05 M KCl	0.11	4.81
0.01 M PO_4, pH 7.1 plus	0.1 M KCl	0.16	2.97
0.01 M PO_4, pH 7.1 plus	0.2 M KCl	0.26	2.48

[a] In Tris systems, final concentration of Fe^{2+} is 1×10^{-3} M; in PO_4 systems final concentration of Fe^{2+} is 1×10^{-4} M. Reactions under N_2; temperature, 25°.

Results

Influence of Buffer Species, pH, and Ionic Strength on the Reduction of Ferricytochrome c by Fe^{2+} under Nitrogen. In both Tris and phosphate buffers (Table I), increasing molarity results in an increase in rate of ferricytochrome *c* reduction by Fe^{2+}. In phosphate systems a maximum at 10^{-2} M is present; while in Tris systems, as buffer molarity increases, reduction rates increase with no evident maximum. It is also

TABLE IV: Effects of Variation in Reactant Concentrations upon Reduction Rate of Ferricytochrome *c*.[a]

Fe^{2+} (M)	Ferricyt *c* (M × 10^5)	Molar Ratios of Fe^{2+}: Ferricyt *c*	Initial Reduction Rates
	Part a		
1×10^{-6}	3.2	0.03	0.09
1×10^{-5}	3.2	0.3	0.76
1×10^{-4}	3.2	3.1	7.6
1×10^{-3}	3.2	31.0	91.8
	Part b		
1×10^{-4}	0.64	15.6	1.6
1×10^{-4}	3.2	3.1	7.6
1×10^{-4}	6.4	1.6	14.1
1×10^{-4}	12.8	0.8	25.6

[a] In part a, ferricytochrome *c* concentration was held constant at 3.2×10^{-5} M and Fe^{2+} was varied. In part b, final Fe^{2+} concentration was held constant at 1×10^{-4} M and ferricytochrome *c* concentration was varied. Buffer was 0.01 M phosphate (pH 7.1); temperature, 25°; reaction carried out under N_2.

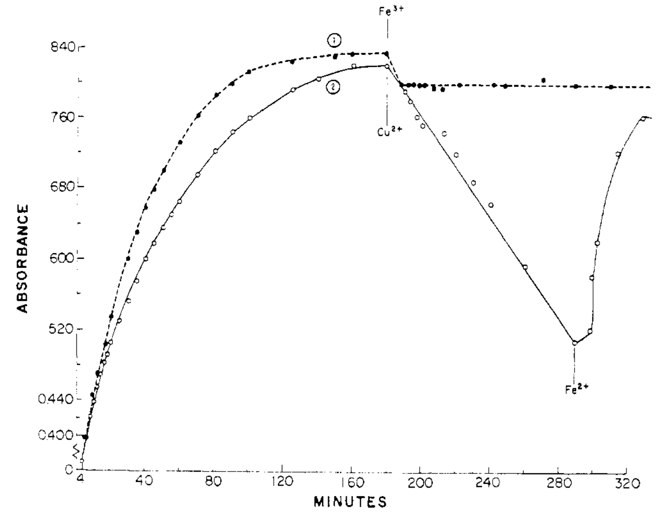

FIGURE 1: Graph showing aerobic reduction of ferricytochrome *c* by Fe^{2+} and reoxidation by Cu^{2+}, but not by Fe^{3+}. Ferricytochrome *c* (4.0 ml), concentration 3.2×10^{-5} M in 0.01 M Tris buffer (pH 7.4), was added to each of two cuvets, 1 and 2 (1.0-cm light paths). The contents of tube 2 were very slightly diluted to avoid overlap of points. At time zero, to each cuvet was added 0.6 ml of 4×10^{-3} M Fe^{2+}, with mixing by rapid inversions and absorbance readings were commenced as shown. When reduction of cytochrome *c* leveled off, to cuvet 1 was added 0.03 ml of 4×10^{-2} M Fe^{3+}; and to cuvet 2 was added 0.03 ml of 4×10^{-2} M Cu^{2+} as indicated on the graph; 110 min after addition of Cu^{2+} to cuvet 2, 0.1 ml of 4×10^{-3} M Fe^{2+} was added, and reduction of the cytochrome *c* commenced. Subsequent additions of as much as 0.45 ml of 4×10^{-3} M Fe^{3+} to systems similar to those in cuvet 1 (not shown in graph) could not reoxidize the Fe^{2+}-reduced ferrocytochrome *c*. Note that because the reactions were carried out in Tris buffer systems, reduction rates are relatively slow as compared to rates in systems in which phosphate buffer was used (see Figure 2).

seen (Tables I and II) that in both Tris and phosphate buffers, increasing pH produces marked increases in reduction rates.

The influence of increasing ionic strength upon reduction rates in Tris and phosphate buffers was studied by means of NaCl and KCl additions (Table III). In Tris systems, increasing ionic strength produced increases in reduction rates, while in phosphate systems, the reverse was true and increase in ionic strength caused decrease in reduction rates.

Effect of Change in Reactant Concentration on the Rate of of Ferricytochrome c Reduction by Fe^{2+}. When either ferricytochrome *c* or Fe^{2+} concentrations were increased in the presence of fixed concentrations of the other, reduction rates increased proportionately (Table IV). In part a, as the concentration of Fe^{2+} was increased a thousand-fold in the presence of fixed ferricytochrome *c* concentration, so did reduction rates increase by a factor of 10^3. In part b, as ferricytochrome c concentration was increased 20-fold in the presence of fixed Fe^{2+} concentration, reduction rates increased by a factor of 16.

TABLE V: Influence of O_2 on Reduction of Ferricytochrome *c* by Fe^{2+} in Different Media.[a]

	Initial Reduction Rate		
Atmosphere	Phosphate	Bicarbonate	Tris
N_2	7.3	32.1	2.8
Air	23.3	4.1	4.4
O_2	36.1	2.0	3.0

[a] Reactions were carried out at 25° in 0.01 M PO_4 buffer (pH 7.0), 0.03 M $NaHCO_3$ (pH 7.2), and 0.05 M Tris buffer (pH 7.4). Ferricytochrome *c* concentration was 3.2×10^{-5} M; final Fe^{2+} concentration was 1×10^{-4} M.

TABLE VI: Reduction of Ferricytochrome *c* by Fe^{2+} in the Presence of Varying Amounts of Fe^{3+}.[a]

Fe^{3+} (M × 10^4), Final	Gas Atmosphere	Reduction Rate
0 (control)	N_2	8.3
1.2	N_2	16.2
2.5	N_2	31.1
5.0	N_2	61.0
0 (control)	O_2	22.1
2.5	O_2	50.9
5.0	O_2	52.7

[a] Concentration of ferricytochrome *c* was 3.2×10^{-5} M, in body of Thunberg-type cuvet. Final concentration of Fe^{2+} was 1×10^{-4} M. Varying concentrations of Fe^{3+} were added to the cytochrome prior to mixing by inversion. Reactions were carried out in 0.01 M phosphate buffer (pH 7.0) at 25°.

Reoxidation of (Fe^{2+}*-Reduced*) *Ferrocytochrome c*. CYTOCHROME *c* OXIDASE. Aliquots (0.05–0.1 ml) of a solubilized beef heart mitochondrial oxidase preparation (Person *et al.*, 1965) to yield 0.06 mg of protein/ml (final concentration) were added to the Fe^{2+}-reduced cytochrome *c* solutions in 0.01 M PO_4 (pH 7.0) and the ferrocytochrome *c* oxidation rate was determined spectrophotometrically. As a control, the rate of oxidation of dithionite-reduced cytochrome *c* by the same oxidase preparation was also measured. Rates for Fe^{2+} and dithionite-reduced cytochrome *c* were, respectively, 1.73 and 2.32. Fe^{2+}-reduced cytochrome *c* was also oxidized by yeast and heart mitochondria.

Cu^{2+}. Aerobic addition of Cu^{2+} (SO_4^{2-} or Cl^-) to Fe^{2+}-reduced cytochrome *c* reoxidized the cytochrome. However, molar ratios of Cu^{2+}:ferrocytochrome *c* required for the oxidation were higher than the molar ratios of Fe^{2+}:ferricytochrome *c* required for reduction of the cytochrome. For example, a Cu^{2+}:ferrocytochrome *c* ratio of 468 gave an oxidation rate of 81, whereas a Fe^{2+}:ferricytochrome *c* ratio of 31 gave a reduction rate of 91.

Fe^{3+}. It is not possible to reoxidize Fe^{2+}-reduced cytochrome *c* by means of Fe^{3+} as shown in Figure 1.

$Fe(CN)_6^{3-}$. $Fe(CN)_6^{3-}$ very rapidly oxidized Fe^{2+}-reduced cytochrome *c*.

Influence of O_2 *on Reduction of Ferricytochrome c by* Fe^{2+}. O_2 can either enhance or reduce the rate of ferricytochrome reduction, depending upon the nature of the medium in which the reaction is carried out. In Table V results are given for ferricytochrome *c* reduction by Fe^{2+} in phosphate, bicarbonate, and Tris media, under N_2, air (20% O_2), and 100% O_2 atmospheres. In the Tris medium, reduction rate is relatively unaffected by the gas phase. In the phosphate medium, O_2 causes a marked enhancement in reduction rate. In the bicarbonate medium, O_2 markedly decreases the reduction rate, while under N_2, reduction of ferricytochrome *c* by Fe^{2+} is enhanced.

TABLE VII: Influence of Fe^{3+} and of Phosphate upon Reduction of Ferricytochrome *c* by Fe^{2+}.[a]

System	Reduction Rate
Control	5.1
Control + Fe^{3+}	13.1
Control + PO_4^{3-}	36.7
Control + PO_4^{3-} + Fe^{3+}	75.1

[a] A 3.2×10^{-5} M ferricytochrome *c* solution in 0.05 M Tris buffer (pH 7.4) was the control solution. Fe^{2+} was added to give a final concentration of 1×10^{-3} M, and 0.1 M phosphate buffer at pH 7.3 was added to give a final phosphate concentration of 0.001 M. Final Fe^{2+} concentration was 1 × × 10^{-3} M. Temperature was 25° and the reaction took place under nitrogen.

Although in a phosphate system under O_2, initial reduction rates for a given concentration of Fe^{2+} were always significantly greater than under N_2, the *degree or extent* of reduction was less under O_2 than under N_2 (Figure 2).

Possible Influence of Fe^{3+} and Superoxide Anion Scavengers on the Rate of Reduction of Ferricytochrome c by Fe^{2+}. INFLUENCE OF Fe^{3+} ADDITIONS. Because of the known oxidation of Fe^{2+} to Fe^{3+} in the presence of O_2, the possible influence of prior addition of varying amounts of Fe^{3+} to the reducing mixtures was investigated (Table VI). Under either N_2 or O_2, reduction rate increased markedly when Fe^{3+} is present. Phosphate further enhances the Fe^{3+} stimulated reduction of cytochrome *c* by Fe^{2+} (Table VII). Of other di- and trivalent cations (Ca^{2+}, Mg^{2+} and Al^{3+}) similarly tested for possible catalytic influence on Fe^{2+} reduction of ferricytochrome *c*, only Al^{3+} gave a relatively slight increase in reduction rate.

POSSIBLE INVOLVEMENT OF SUPEROXIDE ANION, O_2^-. Activation of the reduction of cytochrome *c* by Fe^{2+} in the presence of O_2, suggests possible participation of superoxide anion, O_2^- (Fridovich and Handler, 1962; Handler *et al.*, 1964; Massey *et al.*, 1969; McCord and Fridovich, 1969; Miller, 1970). That such an involvement of O_2^- in the present experiments is very unlikely is shown by the fact that in the presence of the superoxide scavengers 3,4-dihydroxybenzoic acid ($(HO)_2$-Bz)[1] and erythrocuprein (Table VIII), noi nhibition of aerobic reduction of ferricytochrome *c* was noted. Tiron, also a superoxide scavenger, gave results similar to those of 3,4-dihydroxybenzoic acid. Both Tiron and $(HO)_2Bz$ actually stimulated reduction rates, as also noted by Miller (1970), presumably on the basis of their complexation with Fe^{2+}, and resulting enhanced catalysis of the reduction.

It was also found that addition of catalase alone, and catalase plus ethanol are without effect on the reduction of cytochrome *c* plus Fe^{2+}.[2]

Reduction of Yeast and Beef and Beef Heart Mitochondria by Fe^{2+}. It is known that when Fe^{2+} is added to mitochondrial suspensions, lipoperoxide formation occurs and mitochondrial ghosts are formed (McKnight *et al.*, 1965; McKnight and Hunter, 1966). Because the redox state of cytochrome components in such systems have not been intensively investigated, suspensions of isolated yeast (*Torula utilis*) and of beef heart mitochondria were treated with Fe^{2+}. Soret and visible region absorption tracings show that the addition of Fe^{2+} to either yeast or heart mitochondria reduces flavoprotein, and cytochrome *a*, *b*, and *c* components (Figures 3 and 4). However there was a consistently lesser degree of reduction of flavoprotein and cytochrome *b* components of the yeast mitochondria, as compared with flavoprotein and cytochrome *b* components of heart mitochondria.

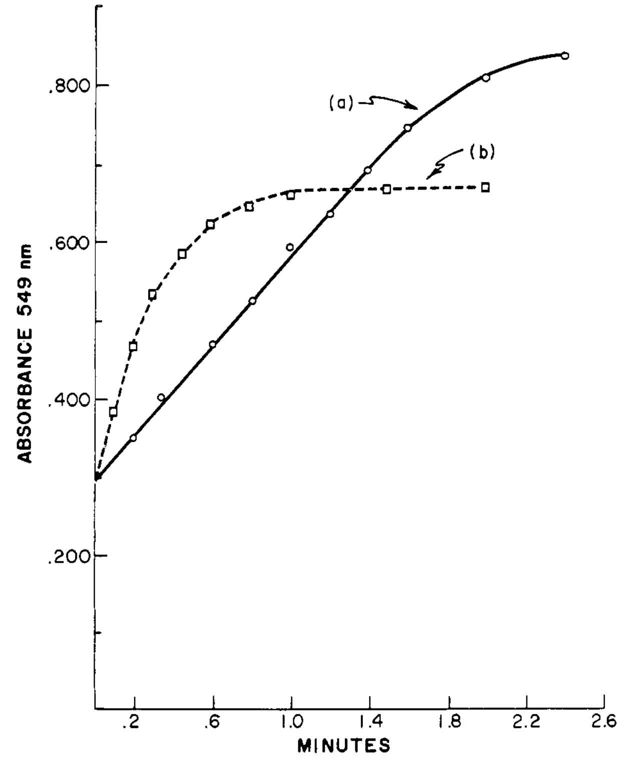

FIGURE 2: Difference in reaction kinetics for reduction of ferricytochrome *c* (3.2×10^{-5} M) by 1×10^{-4} M Fe^{2+} in 0.01 M phosphate buffer (pH 7.1) under 100% N_2 (curve a) and under 100% O_2 (curve b). Note that in a phosphate system under 100% O_2, the initial reduction rate is always more rapid than under 100% N_2 (42.5 *vs.* 12.2); while under 100% N_2 the degree of reduction is always greater than under 100% O_2 (79% *vs.* 53%).

TABLE VIII: Reduction of Ferricytochrome *c* by Fe^{2+} in the Presence of 3,4-Dihydroxybenzoic acid ($(OH_2)Bz$) and Erythrocuprein (EC).[a]

System	Reduction Rates O_2
Fe^{2+}	20.9
Fe^{2+} + $(OH_2)Bz$ (0.006 mg/ml)	85.6
Fe^{2+} + $(OH_2)Bz$ (0.012 mg/ml)	119.6
Fe^{2+} + EC (0.006 mg/ml)	22.6
Fe^{2+} + EC (0.012 mg/ml)	21.6

[a] The reactions were carried out at 25° under 100% O_2 atmosphere. Final ferricytochrome *c* concentration was 3.2×10^{-5} M and final Fe^{2+} concentration was 1×10^{-4} M.

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[1] Abbreviations used are: $(HO)_2Bz$, 1,2-dihydroxybenzoic acid; Tiron, 1,2-dihydroxybenzene-3,5-disulfonic acid; EC, erythrocuprein.

[2] Unpublished data.

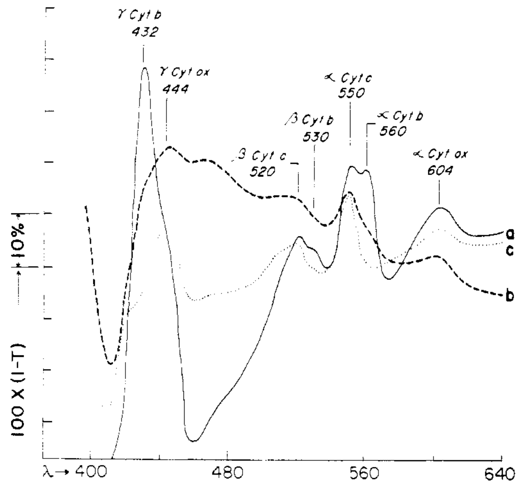

FIGURE 3: Direct tracings of ratio recorded absorption spectra of suspensions of *Torula utilis* mitochondria containing 8 mg of protein/ml in 0.1 M Tris buffer (pH 7.4) (1.0-cm light path). The ordinate scale for this figure, and for Figure 4 is 100 × (1 − *T*), where *T* = transmittance. This recording mode is best suited for absorption studies of turbid suspensions with the spectrophotometer used in this particular experiment (Process and Instruments Co., Model RS3). Curve a (reference, dithionite *vs.* nondithionite-treated samples) displays typical maxima of reduced cytochrome *c*, *b*, and oxidase components as indicated by the markers; and also of oxidized flavoprotein components in the 460-nm region. Curves b and c show tracings, 15 min and 4 hr, respectively, after addition of 1.8×10^{-3} M Fe^{2+} (final concentrations to each of two separate mitochondrial suspensions, which were run against equivalent optical density suspensions of nontreated mitochondria). Curves b and c show that cytochrome *c* and oxidase components are approximately 59–70% reduced as compared to the degree of reduction effected by dithionite, while cytochrome *b* components are either nonreduced or only slightly so (5–10%). (The above figures are semiquantitative estimates based upon peak–trough heights of the respective maxima.) The lesser reduction of cytochrome *b* and flavoprotein components by Fe^{2+} was seen consistently with *Torula* mitochondrial suspensions and subsequent additions of Fe^{2+}, or prolonged incubations, did not increase the degree of cytochrome *b* or flavoprotein reductions.

Reduction of Ferricytochrome c in the Presence of Transition Metals (M^0). In view of the preceeding results, it was of interest to determine whether or not cytochrome *c* would be reduced in the presence of native transition metals (M^0) without addition of metal ions as such. Metals studied were Cu, Zn, Ni, Fe, Co, and Au. The native metal as strip (7 × 3 mm), powder or fine dust (0.5–1 g) was added to 5–10 ml of a ferricytochrome *c* solution in a glass-stoppered test tube or in a Thunberg-type cuvet, under air or N_2 atmospheres. Using concentrations of cytochrome *c* similar to those given previously (3×10^{-5} M), Cu powder and Zn dust effected better than 80% reduction of the cytochrome in less than 1 min (aerobic). Reduction rates in presence of Fe powder or strip were much slower, *i.e.*, 20–30% reduction in 2 hr. Co, Ni, and Au were completely ineffective during similar time intervals. Preliminary Cu^{2+} and Fe^{3+} determinations by atomic absorption spectrophotometry showed that cytochrome reduction was accompanied by release of ions from the native metals. Thus, using Fe strip, with a reduction rate of 1.29 nmol ml^{-1} per min, an Fe^{3+} concentration of 4.3×10^{-5} M was found. With Cu powder, concomitant with cytochrome reduction, the faint blue color of Cu^{2+} ion was immediately visible. Although very preliminary, the above experiments are reported here because they establish the direct electrolytic reduction of cytochrome *c* by transition metals without the necessity for use of H_2.

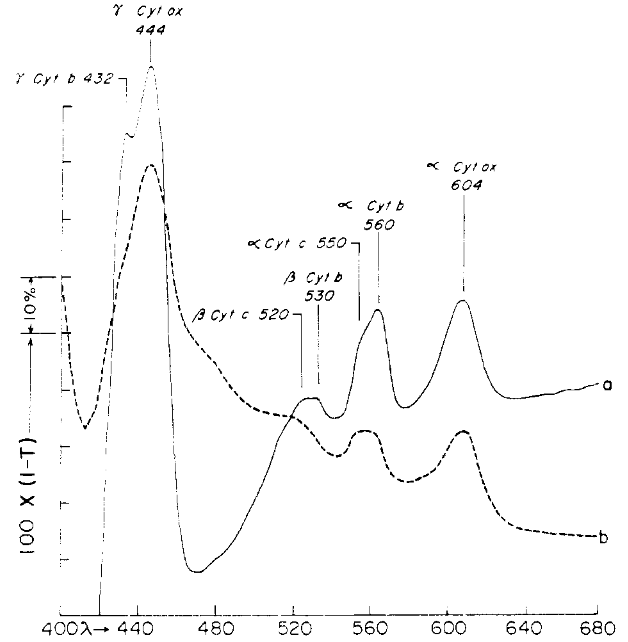

FIGURE 4: Direct tracings of ratio recorded absorption spectra of beef heart muscle mitochondria. Curve a, reference curve of dithionite *vs.* non-dithionite-treated samples containing 8 mg of protein/ml in 0.1 M Tris buffer (pH 7.4) (1.0-cm light path). Curve b, obtained 15 min after adding 1.8×10^{-3} M Fe^{2+} (final concentration) to a mitochondrial suspension, which was run against a nontreated mitochondrial suspension of equivalent optical density. Note that with heart mitochondria, not only are the cytochrome *c* and oxidase components reduced, but also that the *b* and flavoprotein components are relatively more reduced by Fe^{2+}, than in the case of the *b* and flavoprotein components of yeast mitochondria, as illustrated in Figure 3.

Discussion

A selective influence of phosphate upon transition ion (Cr^{2+} and Fe^{2+}) reductions of cytochrome *c* was reported by Yates and Nason (1966), Kowalsky (1969), ourselves (Zipper *et al.*, 1971), and Taborsky (1972). The present studies complement and extend those mentioned above. Taborsky (1972) pointed out that although the strong affinity of phosphate for Fe^{3+} may provide a thermodynamic driving force for reduction of the cytochrome by Fe^{2+}, kinetic parameters involving some form of ligand or complex formation may be of greater significance. The present data of Tables VI and VII showing that Fe^{3+} alone activates, and that Fe^{3+} and phosphate, together, synergistically activate the reductions, support Taborsky's suggestion. These data also raise the possibility that a cytochrome *c*–phosphate–ferric ion complex may be involved, because phosphate is strongly bound by both oxidized and reduced cytochrome *c* (Margoliash *et al.*, 1970; Schejter and Margalit, 1970). Also relevant in this context is the fact that while $Fe(CN)_6{}^{3-}$ and Cu^{2+} can oxidize dithionite- or Fe^{2+}-reduced cytochrome *c*, Fe^{3+} cannot do so. On the basis of standard E_0 values ($Fe(CN)_6{}^{3-}/Fe(CN)_6{}^{4-}$ = +0.36 V; Cu^{2+}/Cu^+ = +0.153 V; Fe^{3+}/Fe^{2+} = +0.771 V; ferricytochrome *c*/ferrocytochrome *c* = +0.254 V). Fe^{3+} should be a better oxidizing agent for ferrocytochrome *c* than either $Fe(CN)_6{}^{3-}$ or Cu^{2+}, in the absence of complicating factors. Thus, the inability of Fe^{3+} to oxidize the reduced cytochrome remains an intriguing but as yet unanswered problem. Coulombic interactions would undoubtedly be involved in such complexes as have been postulated above, and the inhibitory influence (*via* electrostatic screening) of increasing ionic strength on the phosphate-activated reductions (Table III) also is compatible with this concept. (However, it must be noted that while compatible with the existence of complex formation, the ionic strength inhibitions cannot be offered as proof of occurrence.) Additional compatible evidence comes from the contrasting behavior of Tris, which can complex Fe^{3+}, but which is a nonbinding species for cytochrome *c* (Margoliash *et al*, 1970; Schejter and Margalit, 1970). Tris promotes relatively little activation of the reductions; and increasing ionic strength exerts a relatively weak activation rather than inhibition of the reductions occurring in Tris media.

The sensitivity of cytochrome *c* reduction by Fe^{2+} to different anions is further exemplified by the relative lack, aerobically, of an activating influence by carbonate (Taborsky, 1972) or bicarbonate (present studies). On the other hand, anaerobically, bicarbonate is seen to promote a considerable activation of cytochrome *c* reduction by Fe^{2+} (Table V). This leads next, to the question of a possible involvement of superoxide anion (O_2^-) in the aerobic reduction of cytochrome *c* by Fe^{2+}. The question must be answered at present in the negative, because the known superoxide scavengers, erythrocuprein (superoxide dismutase; McCord and Fridovich, 1969), $(HO)_2Bz$, and Tiron (Miller, 1970), are without inhibitory influence on the reductions, However, a role for O_2^- cannot be ruled out unequivocally, since Massey *et al.* (1969) have pointed out in the case of flavoprotein-generated O_2^-, that it is possible for known superoxide scavengers or inhibitors *not* to exert an inhibiting effect in O_2^--mediated reactions under certain conditions. They point out that this may occur when, as superoxide anion is generated, it remains firmly bound in the solvent cage of the flavoprotein and is thereby made inaccessible to the action of the inhibitor. It is more likely (at least in part) in the present experiments, that the activating influence of high O_2 tension (and also of high pH) stem from their favoring conversion of Fe^{2+} to Fe^{3+}, the latter ion having been shown to possess an activating influence on the reduction process. But while promoting activation of the reductions in this way, O_2 will also compete with cytochrome *c* for electrons. This is shown in Figure 2, in which it is seen that in the presence of O_2, electron flow to cytochrome *c* starts faster, but also stops sooner and at a significantly lower degree of reduction (53%) than is the case under N_2 (79%) reduction.

The differential reduction of yeast and heart mitochondrial components by Fe^{2+} (Figures 3 and 4) serve to extend our considerations to systems at higher levels of organization. Previous studies of Fe^{2+}–mitochondrial interactions have dealt primarily with lipid peroxidation, mitochondrial ghost formation, and related phenomena (McKnight *et al.*, 1965; McKnight and Hunter, 1966). It is now evident that these peroxidation-linked changes are accompanied by reductions in the cytochrome and flavoprotein mitochondrial systems; and the degree of reduction of individual molecular species may vary with the mitochondrial source.

The demonstrated reduction of cytochrome *c* by Cu^0 and Zn^0 provides evidence for the first time, of a direct electrolytic reduction of cytochrome *c* by native metals. Recent studies (Land and Swallow, 1971; Lichtin *et al.*, 1971) have provided some evidence in favor of primary electron transfer to cytochrome *c* apoprotein rather than to its heme during pulse-radiolysis generated reductions, in which hydrated electrons are the reducing species. Such experiments suggest that electron transfer to apoprotein should also be considered when surveying possible mechanisms of transition metal–cytochrome *c* reduction. This is now under study in our laboratory.

Acknowledgments

We thank Dr. Albert S. Fine of the Veterans Administration Hospital, New York, for his cooperation in the early phases of this study, and also Dr. Norman Sutin of the Brookhaven National Laboratory, Upon, N. Y., for informative and helpful discussions.

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