Interaction of Cytochrome c, Ferrous Ion, and Phosphate

ELECTRON TRANSFER WITHIN A STOICHIOMETRIC COMPLEX*

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The rate and extent of electron transfer from ferrous ion to ferricytochrome c are enhanced by the presence of inorganic orthophosphate at concentrations comparable to those of reductant and oxidant. Evidence, obtained by the method of continuous variations, shows that the electron transfer occurs within a stoichiometric complex composed of cytochrome c, ferrous ion, and phosphate in molar proportions of about 1:1:1. The incorporation of the anion into this complex appears to result in a modulation of the extent and rate of cytochrome c reduction. The rate of electron transfer obeys a first order rate law, characterized by an apparent first order rate constant of 1.4 min⁻¹. The complex has kinetic significance only; equilibrium dialysis, gel filtration, and sedimentation velocity experiments yielded no evidence for stable binding of phosphate and iron, or of aggregation, on a significant scale. The extent of reduction is limited (for reasons not yet known) to about one-half of the available cytochrome molecules. Reduction in excess of 50% can be achieved only when both, ferrous ion and phosphate, are present in excess of the cytochrome concentration. Kinetic data indicate that reduction to extents over and below 50% occurs by different mechanisms.

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Phosphates can interact with cytochrome c and affect its functional properties. For example, P₀ markedly enhances the reducibility of cytochrome c by ferrous salts (1, 2). The effect is appreciable even when the P₀ concentration is a mere fraction of the cytochrome concentration (on a molar basis) and is far below buffer ion concentrations which can be several orders of magnitude higher (1).

The suggestion of specificity, implicit in this kind of sensitivity, invites considerations of possible functional significance. Indeed, there are many, frequently specific ion effects on cytochrome c, here recorded and suggestions abound concerning their physiological relevance (3). For instance, an ion carrier function has been suggested for cytochrome c on the basis of differential ion binding by its ferric and ferrous forms (4, 5). Nucleotide-cytochrome c interactions have been viewed as reflections of possible regulatory devices (6, 7). Ionic bonds are believed to provide the principal link of cytochrome c to neighboring members of membrane-bound electron transport chains (8–14). Anion effects on the oxidation-reduction potential (15–17), the structure of the protein and its heme conjugate (18–20), and the interaction of the protein with diverse oxidation-reduction reagents (21–28) have received sustained attention for years and the apparently specific binding of an anion to ferricytochrome c crystals has been noted (29, 30)—all of these in vitro studies of anion-cytochrome c interactions having produced insights into the properties of the heme protein as an electron carrier.

Against such a background, it seemed desirable to extend our earlier study (1), with a view to the particular possibility that the observed effects of P₀ at low concentrations, on the reducibility of cytochrome c, may be reflections of the formation of a stoichiometric complex composed of oxidant (ferricytochrome c), reductant (Fe²⁺), and anionic effector (P₀). Schematically, this possibility may be depicted by the following reactions:

\[
\begin{align*}
\text{Fe}^{2+} + \text{Pi} + \text{Cyt}^+ & \rightarrow [\text{Fe}^{2+}-\text{Pi}-\text{Cyt}^+] \quad (1) \\
[\text{Fe}^{2+}-\text{Pi}-\text{Cyt}^+] & \rightarrow \text{Fe}^{3+} + \text{Pi} + \text{Cyt}^+ \quad (2) \\
[\text{Fe}^{2+}-\text{Pi}-\text{Cyt}^+] & \rightarrow \text{Fe}^{2+} + \text{Pi} + \text{Cyt}^+ \quad (3)
\end{align*}
\]

This scheme implies that there is direct interaction between P₀ and cytochrome c (Cyt) (Reaction 1) and it postulates that electron transfer is an intracomplex process (Reaction 2). The scheme implies no commitment regarding the arrangement of ligands in the iron's coordination sphere except that P₀ and protein are considered as possible ligand contributors. Alternatives to Reaction 1 are not excluded. Indeed, as we shall see, some partitioning of the initial reactants (particularly Fe²⁺ and P₀) among competing reactions is likely. Such alternatives could include the formation of other complexes (involving the same constituents in different proportions, or others which are present in the reaction mixture) which may, but need not, be productive of cytochrome reduction via reaction alternatives to Reaction 2. For example, some Fe³⁺ may donate electrons to an acceptor other than the cytochrome (e.g. O₂) or it may reduce cytochrome c without prior complex formation. Finally, the scheme, as shown, is intended to leave the question open for the moment concerning the reversibility of the reactions.

In the following, we present evidence for 1) the formation of a stoichiometric complex composed of equimolar proportions of cytochrome c, Fe²⁺, and P₀; and 2) electron transfer being, in this case, an intramolecular (intracomplex) process.

EXPERIMENTAL PROCEDURES

Materials—Cytochrome c (horse heart) was obtained from Sigma. Type III preparations were used without further purification. The purity and the responsiveness of various cytochrome c preparations to the presence of P₀ were described earlier (1). The phosphorus content of various cytochrome preparations was found to be about 0.03, or fewer, mol/mol of cytochrome c. All other materials were commercial products of reagent or chromatographic grade quality. Solutions were prepared with deionized, glass-distilled water. Concentrations were routinely calculated on the basis of solute weight, but
they were confirmed by spectral or chemical analyses as before (1).

**Cytochrome c Reduction**—Solutions of cytochrome c (in 50 mM Tris-HCl buffer, pH 7.5) were prepared daily and stored in the cold until used. Before an experiment, the solution was equilibrated in a jacketed reaction vessel at 25°C. Additions were made several minutes before “zero time” when the reductant ferrous salt was added. Solutions of FeSO₄·7H₂O in water, were prepared minutes before use.

To avoid significant contamination by ferric iron, it was useful to select ferrous sulfate crystals without visible surface deterioration.) Reaction mixtures were stirred continuously, in air. This was to ensure the relevance of these experiments to earlier work. Much of what we know about the phosphate effect applies to aerobic systems (1, 31), including the particularly interesting fact that oxygen enhances this effect (2). By keeping the reaction mixtures saturated by air, the mixture was kept from any possible kinetic complications which could arise otherwise from any gradual depletion of dissolved oxygen by oxygen-consuming side reactions such as the autoxidation of Fe³⁺.

**Spectrophotometric Measurements**—The course of cytochrome c reduction was followed spectrophotometrically. The reaction mixture was circulated through a flow cell, in a Gilford 2400 spectrophotometer, with a peristaltic pump. Absorbance changes were recorded at 542 nm, where ferricytochrome c shows an absorption maximum in the visible region. Thus, we could correct for a sometimes significant turbidity contribution to the absorbance, made presumably by precipitating iron hydroxides. The degree of cytochrome reduction was calculated on the basis of the absorbance difference at the two wavelengths (A₅₄₂ - A₄₀₀) and the appropriate extinction coefficients taken from the literature (32). The validity of this procedure, resting on the assumption that any turbidity contribution to the absorbance will be identical at the two wavelengths, has been established earlier (1).

Complete spectrophotometric characterization of the untreated, oxidized protein and of its reaction products was obtained with a Cary 14 spectrophotometer.

**Calculations of the Extent and of the Rate of Cytochrome c Reduction**—Typically, reactions were followed for about 15 min. Under most conditions, the reactions were completed within the first few minutes of the reaction period as signaled by the constancy of the A₅₄₂/A₄₀₀ difference. The reaction rate was, in most cases, a simple logarithmic one for three or more half-lives. Apparent first order rate constants were calculated from the linear regression of log (a/a - x) versus t (fitted by the method of least squares) where a = the final amount of available oxidant, and x = the extent of reduction at time t.

As a rule, cytochrome c reduction ceased “prematurely,” before conversion of all of the initially provided ferricytochrome c, even when the reducing agent was present in excess. For purposes of kinetic calculations, we equated the finally attained ferricytochrome c concentration (a measured quantity) with the ferricytochrome c concentration which we assumed was available to react initially. This practice is tantamount to the designation of any unreacted portion of the complex as “inert.” The following is the rationale for this practice. A straightforward description of the progress of cytochrome c reduction became possible in this way. Under initially provided ferricytochrome as “inert.” The following is the rationale for this practice. A straightforward description of the progress of cytochrome c reduction became possible in this way. Under initially provided ferricytochrome as “inert.”

**Sedimentation Velocity Experiments**—The sedimentation rate of ferricytochrome c and of its various reaction products was determined in a Spinco, model E, ultracentrifuge in a manner described earlier (33).

**Binding Studies**—Various solutions containing cytochrome c, P₀, and iron, were subjected to equilibrium dialysis or gel filtration. All solutions were buffered with 50 mM Tris-HCl, pH 7.5. A Bel-Art model 299 cellulose membrane (nominal pore size, 48 Å; retention limit, Mₐ > 12,000) was used in the dialysis experiments which were conducted at room temperature, with mechanical agitation of the two-compartment Plexiglas cell. Gel filtration experiments were done with Sephadex G-10 (Pharmacia) columns (2 × 30 cm), equilibrated with buffer, charged with 5-ml aliquots, and eluted (80 ml/h) and the effluent was analyzed. Cytochrome c was determined spectrophotometrically, phosphorus and iron, by chemical analysis (1). Gel-filtered solutions also contained ³²p (ICN Pharmaceuticals) as tracer which was counted in a xylene-based scintillation fluid containing Triton X-114 (Rohm and Haas) (34) with a Beckman model LS-190 liquid scintillation counter.

**Method of Continuous Variations**—The composition of coordination complexes may be revealed by the method of continuous variations (35). Any measurable and distinctive characteristic (e.g. a spectral feature) of a complex will be, in simple cases, maximal (or minimal) whenever the complex constituents (metal and ligand) are provided in proportions which correspond to their stoichiometry in the complex. In practice, measurements are made on solutions in which the concentrations of the putative complex constituents are varied “continuously” so that the sum of their concentrations is held constant. We applied this method in our search for evidence for the possible formation of a complex involving cytochrome c, Fe²⁺, and P₀. We assumed that complex formation may be revealed not only in terms of some physical or chemical characteristic of the complex itself but also in terms of some consequence of complex formation, such as any change in absorption characteristics of the complex itself but also in terms of some consequence of complex formation, such as complex-dependent electron transfer. Accordingly, cytochrome solutions (0.05 and 0.10 mM, respectively, in two series of experiments) were supplemented with FeSO₄ and P₀, varying the concentrations of these two constituents so that their combined concentrations were constant at a level twice that of the cytochrome concentration (0.10 and 0.20 mM, respectively). The course of cytochrome c reduction was followed as described above. The procedure can be expected to reveal the formation of a “productive” complex even if it is unstable and dissociates upon completion of the electron transfer. The procedure will not reveal the formation of products of side reactions (alternatives to Reaction 1) which are “nonproductive” so far as cytochrome c reduction is concerned, unless they make distinctive contributions to absorbance at the two monitored wavelengths. Analysis of complete spectra of reaction mixtures at various stages of the reaction gave no evidence of such alternative products (or intermediates).

**RESULTS AND DISCUSSION**

**Cytochrome c Reduction as a Function of “Continuously Varied” Concentrations of Phosphate and Ferrous Ion**—Fig. 1 shows the final extent of cytochrome c reduction which was attained as a function of continuously varied concentrations of P₀ and Fe²⁺. Clearly, there is an optimal pair of P₀ and Fe²⁺ concentrations at which heme reduction is maximal. These concentrations, relative to that of the cytochrome, are inde-
dependent of the cytochrome concentration (0.05 mM, lower curve; 0.10 mM, upper curve). As noted before (see "Experimental Procedures"), the finding of such an optimum is consistent with the notion that the reducibility of cytochrome depends on the formation of a cytochrome-Pi-Fe²⁺ complex.

The complex constituents appear to be bound in the molar proportions of about 1:0.8:1.2. There is reason to propose, however, that the actual stoichiometry is simpler than that. There is significant cytochrome reduction even in the absence of Pi. This must occur by way of some alternative to Reactions 1 and 2 (see introduction). A contribution to the overall reduction by a phosphate-free reaction must, therefore, be expected, to a maximal extent when P_i is absent and approaching insignificance as we progress from left to right in Fig. 1. A phosphate-free reaction should cause the experimental curves to be skewed. Were the curves in Fig. 1 to depict only the phosphate-dependent reaction, they would be more symmetrical and their maxima more to the right than they are in fact. It seems reasonable, therefore, to postulate that we are dealing with a ternary complex of which the constituents are equimolar.

Reaction Rates in Continuous Variation Experiments—The experiments on which Fig. 1 is based were subjected to kinetic analysis. All reactions in which the P_i:cytochrome ratio was 0.5 or larger obeyed a first order rate law, through four or more half-lives. The value of the apparent first order rate constant showed no significant dependence on P_i (and Fe²⁺) concentration over the indicated concentration range and was found to be 0.62 ± 0.16 min⁻¹ at 0.05 mM, and 0.66 ± 0.11 min⁻¹ at 0.10 mM cytochrome c. In other words, the rate constant appeared to be insensitive to the cytochrome c concentration as well, within the indicated standard deviations. Reactions in the complete absence of P_i also obeyed first order kinetics. The apparent rate constant was 0.27 ± 0.04 min⁻¹ and was independent of Fe²⁺ and cytochrome c concentrations.

The kinetic course of the reactions within the transition range of molar ratios from 0 to 0.5 (P_i/cytochrome) was less clear cut. Some kinetic complexity was indicated by occasionally observed, small but significant, initial bursts of cytochrome c reduction, followed by a slower process by which the reduction reached its final level in an apparently first order fashion.

It is of special interest to find that most of these reactions proceed by first order kinetics and are characterized by a rate constant which appears to be independent of the concentrations of the constituents of the postulated ternary complex. This finding reinforces our view that electron transfer from ferrous ion to ferricytochrome c occurs after a non-rate-limiting association of reductant and oxidant, accounting thereby for the apparently unimolecular reaction mechanism. In the presence of P_i, the complex incorporates this anion. A consequence is the modulation of electron transfer; it becomes more extensive and more rapid. In the absence of P_i, the first order rate law implies a reaction which is similarly unimolecular. As we shall see in the following section, the suggestion that P_i is, in this case, replaced by another anionic constituent of the reaction medium has an experimental basis.

Effects of Other Constituents of Fe²⁺/Ferricytochrome c Solutions—In addition to reductant, oxidant, and phosphate, the reaction mixtures included the components of the Tris-HCl buffer system. Chloride ion is known to interact with cytochrome c (e.g. Refs. 4 and 28). Tris does not interact with the heme protein (4) but it chelates iron, as suggested, for example, by Tris inhibition of the reduction of cytochrome c by the xanthine oxidase system in which an iron atom appears to be essentially involved at the enzyme's cytochrome-reducing site (36). It was, therefore, indicated that we establish what influence, if any, Cl⁻ and Tris may have on the interaction of cytochrome c, Fe²⁺, and P_i.

Tris-HCl buffer itself provides an appreciable concentration of Cl⁻: about 40 mM in a 50 mM buffer at pH 7.5. It is clear, however, that Cl⁻ exerts no major effect, through competition with phosphate, because the effect of P_i is marked even when its concentration is as low as 0.02 mM. This is about 2000 times lower than the concentration of Cl⁻ in the buffer. Nevertheless, Cl⁻ has some effect. When a typical, 1:1:1 mixture of cytochrome c, Fe²⁺, and P_i was supplemented by an additional 50 mM CI⁻ (as NaCl), the extent of cytochrome reduction was depressed to about 80% of the level noted in the absence of the added Cl⁻. Stimulation by P_i, at 1/1800th of the Cl⁻ concentration, remained appreciable even if somewhat depressed.

Chloride has no appreciable inhibitory or stimulating effect by itself. With 1:1 mixtures of cytochrome and iron, in the absence of P_i, the extent of cytochrome c reduction was the same (9 to 10%) whether the solution was, or was not, augmented by 50 mM NaCl.

The possibility that the Tris component of the buffer had an influence on the reaction was tested by replacement of Tris-HCl with sodium cacodylate. Cacodylate is a nonbinding ion (4). In this buffer system, in the absence of P_i, cytochrome c was reduced to a somewhat greater extent than in Tris buffer. Since P_i was not present, we attribute this enhancement to the absence of Tris, a known iron chelator, rather than to the absence of CI⁻, the effect of which we have already attributed to its ability to provide anionic competition, when present in massive excess over P_i. In the presence of P_i, the buffer replacement was of barely significant consequence.

The relatively small amounts of sulfate ion, introduced as the counterion of Fe²⁺, can be safely ignored. Up to 10 times greater amounts of sulfate had been shown to be without effect (1), consistent with the report that sulfate affects the electrophoretic mobility of cytochrome c just ahead of cacodylate, the last member of a series of ions in order of their decreasing effectiveness (37).

We conclude that both buffer constituents may exert a small influence without affecting the principal thrust of our interpretation of Fig. 1. The weak competition which CI⁻ appears to be capable of, in the ratio P_i, makes the first order reaction rates in the absence of P_i explicable. Chloride may take the place of P_i as the anionic component of the complex. In a Cl⁻-containing complex, modulation of electron transfer may be less effective, as suggested by the small observed rate of reduction (0.27 min⁻¹) in the absence of P_i. Tris may provide a relatively weak competition for iron, possibly by one of the alternatives to Reaction 1 considered in the introduction. This competition appears to suffice to make a portion of the Fe²⁺ unavailable for cytochrome reduction, but it has no effect on the enhancement of cytochrome c reduction by P_i.

Consideration of Alternatives to the Complex Hypothesis—Although the postulation of a ternary complex offers the most direct and simple way to account for Fig. 1, and for the rates of the underlying experiments, an alternative interpretation not invoking a complex should be considered. To find that the reduction becomes increasingly effective as the relative concentration of Fe²⁺ is raised accords with normal expectations; there is more reduction with more reductant. A problem arises as we note that the reaction extent, after it passed through an optimum, falls even while the Fe²⁺ concentration continues to rise. We might suppose that a nonreductive, Fe²⁺-consuming reaction is promoted at high Fe²⁺ concentrations. Such a
reaction would cut down on the availability of Fe²⁺ for cytochrome reduction and produce results like those in Fig. 1. For this to be a realistic alternative, however, it would require that the nonreductive Fe²⁺-consuming process be switched from a secondary role (at low Fe²⁺ concentrations) to a dominant role (at high Fe²⁺ concentrations). For such a switch, we need to assume that the nonreductive reaction proceeds with a higher order of dependence on Fe²⁺ concentration than the reductive process. Although we can speculate reasonably about the occurrence of higher order reactions of iron (e.g. the formation of some polynuclear iron complex), we can rule out its applicability in this case on experimental grounds. We showed earlier (1) that cytochrome reduction proceeds to monotonously increasing extents as the concentration of Fe²⁺ is raised, even to levels many times higher than the maximal levels reported in this paper. We conclude, therefore, that the postulated ternary complex remains the simplest hypothesis by which the relatively efficient reduction of cytochrome c, at particular Pi, and Fe²⁺ concentrations, can be accounted for.

Saturation of P, Effect—The experiments depicted in Fig. 1 appear to have occurred under suboptimal conditions. The incompleteness of the reduction and the greater reaction extent at the higher cytochrome concentration suggest this. They invite consideration of the question whether, and under what conditions, the P, effect might be "saturating," that is produce a true maximum of extent and possibility even of rate of reduction.

We might suppose that Reaction 1 is an equilibrium process which fails to ensure complete association of the complex constituents because K_reduction is unfavorable. Alternatively, we might suppose that Reaction 1, as a practically irreversible process, may occur at a rate which is slow enough to permit alternative reactions to occur on a significant scale (cf. introduction). In either case, the implied limitations on the reductive success of Reactions 1 and 2 could be overcome by concurrent concentration increases of all of the reactants of which the complex is constituted. This prediction presupposes that Reaction 1 is the only process which involves all three of the complex constituents and no other reactant. Alternatives to Reaction 1 presumably depend, in part, on other components of the reaction mixture (e.g. Tris, Cl⁻, O₂) of which the concentrations would be left unchanged. (Given the inferences drawn from Fig. 1, the originally considered possibility of complexes which differed only in the molar ratios of identical constituents can be ignored.)

Fig. 2A shows results of experiments performed with the above rationale; the absolute concentrations of ferricytochrome c, P, and Fe²⁺ were varied but equimolar in every case. It is clear that the extent of the reaction can be made to show the effect of saturation although only at the level of about half-reduction of the available cytochrome c. While puzzling, this apparent "half-of-the-sites" reactivity was not unexpected. Fig. 2B (lower curve) shows, confirming earlier findings (1), that when a 1:1 mixture of oxidant and reductant is supplemented by P, in increasing proportions, the reaction extent increases sharply at first, approaching half-reduction and then drops gradually as the P, concentration rises to great molar excesses. Fig. 2B also shows the results of a parallel experiment (upper curve) in which the Fe²⁺:cytochrome ratio was fixed at 2:1 (i.e. a 100% molar excess of Fe²⁺). This experiment makes it clear that the barrier to complete cytochrome reduction can be breached when both P, and Fe²⁺ are provided in excess. However, it is clear, too, that reduction beyond the 50% level is achieved with greater difficulty in the sense that the reaction extent becomes a less emphatic function of P, concentration beyond the 50% reduction level.

It is noteworthy that the facility with which low P, concentra-
trations can promote up to about 50% cytochrome reduction is associated with equimolar mixtures of cytochrome c and ferrous ion (cf. Fig. 2, A and B). Reduction beyond the 50% level, promoted with lesser ease by higher concentrations of P, and only when Fe²⁺ is present in excess (ironcytochrome, >1), must proceed by a different reaction mechanism.

It is instructive to consider the kinetics of the reactions depicted in Fig. 2. Reactions which involve equimolar reactants at different absolute concentrations (cf. Fig. 2A) obey first order kinetics at concentrations up to 0.2 mM. Beyond this concentration, the kinetics become somewhat complex, coincident with the increasingly depressed reaction extent as...
the reactant concentrations rise above the 0.2 mM level. (Both 
kinetic complexity and depressed reaction rate are likely to 
be manifestations of some aggregation phenomenon since the 
specificity of the protein-Pi-iron interaction may lose its dom-
inant significance.) Reactions which involve iron and cyto-
ochrome in 1:1 and 2:1 molar ratios and varying relative 
amounts of Pi (cf. Fig. 2B) also obey first order kinetics so 
long as the reaction extent stays below 50%. Beyond 50% 
reduction, the kinetics becomes emphatically complex, as one 
might expect in view of the speculation (cf. preceding par-
agraph) that at excessive Pi and Fe3+ concentrations some new 
reaction mechanism becomes operative, superimposed on the 
intracomplex mechanism.

First order rate constants are summarized in Table I. The 
“saturation effect” which was noted in terms of the reaction 
extent can also be recognized in terms of reaction rates. Before 
kinetic complications are allowed to obscure the picture (at 
high concentrations of all reactants, and at high concentra-
tions of Pi, when Fe3+ is also present in excess), the first order 
rate constant approaches a maximal value of about 1.3 to 1.5 
min\(^{-1}\). This appears to be the true rate of the reaction within 
the ternary complex. From the rates calculated for the reac-
tions in the continuous variation study which we had reason 
to believe occurred under less than optimal conditions, it is 
clear that the apparent first order rate constants calculated 
for those reactions must have represented a sum of first order 
reactions including electron transfer within the cytochrome-
Pi-iron complex and also the less facile reaction within a less 
effectively modulated complex containing Cl\(^-\) as the anionic 
component.

**Stability of Cytochrome-Pi-Iron Complex**—The postulated 
complex appears to have only kinetic significance. Dialysis 
equilibrium experiments conducted with 0.25 mM ferricyto-
chrome solutions and with equimolar concentrations of Pi (in 
the usual buffer) showed that phosphorus concentrations on 
the two sides of the membrane were identical within analytical 
error. We estimate that an association reaction with K 
of the order of 10\(^5\) M\(^{-1}\) would have been detected. This is not incon-
sistent with reported binding constants (5, 38). Iron was not 
used in these experiments; precipitation of, presumably, iron 
hydroxides was inevitable during the long periods required. 
However, typical reaction mixtures including iron were gel-
filtered. In experiments in which the Pi:cytochrome ratio 
ranged between 2 and 200, less than 0.1% of the phosphate 
was eluted in fractions containing the protein. The resolution 
of protein and anion was similarly complete when the column 
was charged with a cytochrome/Pi-iron mixture (in equimolar 
amounts). Sedimentation analysis was carried out primarily 
for the purpose of detecting any aggregation of the protein as 
a consequence of its interaction with Pi, iron, or both. The 
sedimentation coefficients (in six experiments) were identical 
within 0.05 S with the sedimentation coefficient of the protein 
under the same conditions. Also, the areas under schlieren 
“peaks” were identical, within the limits of planimetric inte-
gration, throughout an experiment. This indicated that no 
significant amount of protein was removed in the form of 
rapidly sedimenting aggregates.

**Remaining Questions**—The evidence presented and dis-
cussed so far appears to establish that the phosphate-dependent 
enhancement of electron transfer from ferrous iron to ferricytochrome c is a consequence of the formation of an 
unstable but kinetically significant complex constituted of 
cytochrome c, ferrous iron, and inorganic phosphate in the 
molar proportions of 1:1:1. The anion appears to modulate the 
reaction by increasing its efficiency and facility.

Several unanswered questions remain. It is not known in 
what way, if any, mediation may be involved in the electron 
transfer. Under the conditions of these experiments, the initial 
transfer of electrons from Fe\(^{2+}\) to O\(_2\) must be considered, in 
view of the stimulating effect of O\(_2\) (2) and the well docu-
tmented reducibility of cytochrome c by the superoxide anion 
(36, 39-42). However, the reported lack of inhibition of cyto-
ochrome reduction by Fe\(^{2+}\) in the presence of O\(_2\) scavengers 
makes the involvement of superoxide unlikely (2). In addition, 
the possibility of direct O\(_2\) binding to the postulated 1:1:1 
complex would also argue against superoxide involvement in 
view of the proposal that superoxide generation is associated 
with aerobic transition metal systems when direct coordina-
tion of O\(_2\) to the metal is not feasible (43). We suspect that 
the autooxidation of ferricytochrome c, in systems in which 
iron and phosphate are present in excess (1), may involve 
superoxide which has been suggested to be generated as a 
product of cytochrome autooxidation, causing its apparent 
deceleration (44).

A second unanswered question relates to the apparent 
saturation of the Pi effect when the cytochrome is only half-
reduced. An experimental accounting for this phenomenon is 
yet to be accomplished. One clue may be provided by our 
earlier observation that cytochrome c reduction exceeding 
50%, by excess Fe3+ (in Pi buffer), is followed by relatively 
rapid autooxidation, in contrast to cytochrome c reduced to 
extents below 50%. We suggested above that the mechanisms 
of cytochrome reduction by Fe3+ to extents below and above 
50% differ. Exploration of the Pi effect under anaerobic con-
ditions may be useful in an effort to elucidate the mechanism 
of reduction beyond the half-reduced stage.

Finally, it remains to be shown where on the cytochrome 
surface the interaction with Fe\(^{2+}\) and Pi occurs. In view of the 
apparent specificity of the interaction, there must be a particu-
lar binding site associated with it. Locating the site is desir-
able because 1) it would confirm the specificity of the inter-
action, 2) it would permit useful comparisons with other 
known or suspected anion binding sites, and 3) it could assist 
in the definition of the electron transfer mechanism. We plan 
to report shortly on the results of experiments which bear on 
the identity of this anion binding site.

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Complex of Cytochrome c, Ferrous Ion, and Phosphate 5251

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