Effects of Phosphate and Other Anions on the Reaction between Ferrous Ion and Cytochrome *c**

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ABSTRACT: The reduction of cytochrome *c* by ferrous ion is markedly affected by oxygen, and the nature and concentration of various ions present in the reaction mixture. Also, the autoxidation of the products of the reaction between cytochrome *c* and ferrous ion takes a variable course dependent on the ionic constituents of the medium. These effects have been explored in some detail with reaction mixtures buffered at pH 7.5 with tris(hydroxymethyl)aminomethane or sodium phosphate. In addition, several anions (including inorganic phosphate, arsenate, and adenosine phosphates) have been found to effect an extensive, facile reduction of the cytochrome by an equivalent amount of ferrous ion if the anion concentration is low, comparable with the concentration of the protein. Remarkably, the extent of this facile reduction appears to reach its limit when only about half of the total cytochrome is reduced. At high anion concentrations, the extent of this facile reduction becomes suppressed and extensive reduction can only be achieved if the reductant is also provided in large excess. The course of autoxidation of the reduced cytochrome is also affected by these anions. These effects could be related to biological reactions of cytochrome *c*.

As an extension of earlier studies on the autoxidative dephosphorylation of phosphoprotein–iron complexes (Grant and Taborsky, 1966; Rosenstein and Taborsky, 1970), we have been exploring the possibility that iron complexes of phosphoproteins might react also with components of the mitochondrial respiratory chain. Recently, we described corollary results of this work concerning the nature of the interaction of cytochrome *c* with a phosphoprotein in the absence of iron (Taborsky, 1970). At this time, we report on the reaction between cytochrome *c* and iron in the absence of phosphoprotein, relating observations on the oxidation–reduction state of the heme iron, with emphasis on the sensitive response of the reaction between ferrous ion and ferricytochrome *c* to the presence of phosphate and certain other anions.

Materials and Methods

Cytochrome *c*, prepared from horse heart, was obtained from the Sigma Chemical Co. and was used without further treatment. The type, lot number, and certain of the properties of these preparations will be given in the text. None of them contained more than about 4% reduced form.

ATP was obtained from Sigma (crystalline, equine muscle, Na salt, 99%), ADP from P-L (Na salt), and AMP from Pabst (crystalline). Other reagents were various commercial products of reagent grade quality.

Solutions, except those of simple salts, were prepared freshly. Concentrations were calculated on the basis of weight. The degree of purity of cytochrome *c* after apparently complete chemical reduction was assessed by OD measurement at 550 and 526 nm, an isosbestic point of the oxidized and reduced forms. Based on the extinction coefficients reported by Margoliash and Frohwirt (1959), all preparations except the acid-treated, type XII, cytochrome *c* were pure to an extent of 95% or better. Further comment on the purity of the cytochrome will be made in conjunction with Table I. Buffer solutions were prepared with Tris (Sigma, "Trizma Base") and HCl, or with sodium phosphate. Initial pH values remained constant within about 0.1 pH unit in all experiments.

Reaction mixtures were routinely prepared by the addition to buffered ferricytochrome solutions (usually 3 ml) of small volumes (1–100 μl) of the desired additional components. A solution of $FeSO_4$ in water was added last, at zero time. About 95% or more of the iron salt was in the reduced form as ascertained by analysis according to Sandell (1950). In aerobic experiments, the mixtures were stirred in 10-ml beakers, exposing a relatively large surface of the solution to air. Evaporation was controlled with Parafilm covers. In anaerobic experiments, a Thunberg-type spectrophotometer cell was used and the ferrous salt solution was mixed with the protein solution after evacuation with an oil pump.

Since this study has been concerned to a significant extent with the autoxidizability of the cytochrome (a generally accepted indication of denaturation), the possible effect which mechanical aspects of the experimental procedure such as stirring or evacuation might have on the reactive properties of the protein had been specifically considered. Treatment of cytochrome *c* with reducing agents other than $FeSO_4$, under conditions which are strictly comparable with those underlying the experiments dealt within this paper, showed that the cytochrome was not rendered more autoxidizable merely as a result of the mechanical operations. For example, under these conditions, alternative reductants have been observed to produce ferrocytochrome *c* with relatively much greater stability to autoxidation than the stability of some of the reaction products of concern in this context. Although, in a number of instances, no appreciable difference could be seen in the outcome of experiments involving cytochrome reduction and autoxidation with samples either stirred or at rest, stirring was adopted for the routine procedure because comparisons of certain reaction mixtures kept in stoppered vessels (not evacuated but without air space) with others kept freely

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TABLE I: Comparison of Different Cytochrome Preparations in Terms of Their Reducibility by Ferrous Ion in Tris and Phosphate Buffers.[a]

Cytochrome *c* Preparation[b]	Maximal Reduction (%)		
	Tris	Phosphate	
	$16Fe^{2+}$	$2Fe^{2+}$	$8Fe^{2+}$
Type III, 40C 7310	42	47	92
Type III, 70C 0940	32	26	80
Type III, 99B 7470	48	34	83
Type VI, 65B 7300	47	37	89
Type XII, 104B 7470	20	14	28

[a] For experimental conditions, see legend for Figure 1. [b] All cytochrome *c* preparations were derived from horse heart and are those of the Sigma Chemical Company. The manufacturer identifies them as follows: Type III is 90–100%, Type VI is 95–100% pure, both being free of $(NH_4)_2SO_4$ and NaCl; Type VI has been prepared without the use of trichloroacetic acid; Type XII (about 90% pure) is equivalent to the acid-modified, polymeric cytochrome fraction II described by Margoliash (1954) and Margoliash and Lustgarten (1962). The lot number of the individual preparations used in these experiments is given following the Type number.

exposed to air showed that the dissolved oxygen level has the potential of becoming a limiting factor in at least some of the aerobic experiments. The evacuation procedure (involving slow air removal from rapidly swirled solutions) also appeared to be adequate for the preservation of whatever integrity a given cytochrome preparation had since it could be shown that cytochrome reduced with reagents other than Fe^{2+}, *in vacuo*, could remain in a nearly completely reduced state over periods of time which sufficed for even complete autoxidation of iron–cytochrome mixtures. Of course, any of the observed enhancements of autoxidation could be a reflection of denaturation but it would need to be ascribed to the particular composition of the reaction mixtures rather than to their physical treatment.

The oxidation–reduction state of the heme protein was assessed spectrophotometrically. Absorption spectra were recorded, as a rule, with a Bausch and Lomb Spectronic 505 spectrophotometer, between 500 and 600 nm, in the 0–1 OD range, using 0.5-cm cells. Occasionally, spectra in the Soret region were taken (with 0.1-cm cells). Visible and Soret spectra of the reaction mixtures after completion of the reactions showed, in general, good qualitative and quantitative agreement with the spectra reported by Margoliash and Frohwirt (1959) for the oxidized and reduced forms of cytochrome *c*.

In certain cases, especially with phosphate-containing solutions, a turbidity appeared in the reaction mixtures, increasing gradually over a period of about 10 hr. The insoluble material which sedimented readily upon centrifugation consisted of a ferric complex of undefined composition: it contained the bulk of the added iron and some phosphate (determined according to Sandell (1950) and Sumner (1944), respectively) but only a small fraction of the heme protein. Since the turbidity contributed significantly to the apparent OD, the composition of the reaction mixtures in terms of oxidized and reduced forms of cytochrome *c* could not be calculated directly on the basis of absolute OD values. The OD values at 526 and 550 nm were obtained from the spectra, their difference taken, and the change in the value of this difference adopted as the measure of the change in the state of oxidation of the heme protein.[1]

The overall precision of the results is estimated, on the basis of the standard deviation from the mean of several sets of replicate analyses, to be about 1–5% reduced cytochrome. Indeed, the satisfactory reproducibility of the experiments—when performed using the same lot of cytochrome *c* in any given set—gives further reason to conclude that the procedures employed did not cause nonspecific denaturation of the protein. The essential features of quantitative relationships yielded by experiments of a given set were also reproducible when the set of experiments was repeated with a different lot of cytochrome: data varied from lot to lot only in the absolute sense and such variations (which will be illustrated below) are without significant effect on the interpretation of the findings.

Results

Reduction of Cytochrome c with Ferrous Ion and Autoxidation of the Reaction Product. The maximally attainable level of reduction of cytochrome *c*, at any given concentration of Fe^{2+}, is strikingly dependent on the nature of the buffer, as shown in Figure 1. In Tris-HCl buffer (50 mM, pH 7.5), in air, even a large excess of ferrous ion reduces cytochrome *c* only to a limited extent (Figure 1A, solid circles). When air is excluded, all of the heme protein is readily reduced (open circles). In contrast, when the buffer is Na-phosphate (50 mM, pH 7.5), reduction is much more facile: nearly complete reduction can be observed if Fe^{2+} is present in large excess, even in the presence of air (Figure 1B).

The difference between the two buffer systems is not due to their different ionic strength. Adjustment of the Tris buffer with NaCl to the level of the ionic strength of the phosphate buffer is without consequence.

The autoxidizability of the ferrocytochrome which is produced in these experiments appears to be a function of the extent to which the heme protein had been reduced in the first place. Partial reduction, not exceeding half of the total cytochrome, yields a product in either of the two buffer systems which undergoes oxidation at a slow, monotonous rate. Reduction in excess of about half of the total cytochrome (achieved only in phosphate buffer) results in a biphasic course of autoxidation: an initial, relatively rapid reoxidation is followed by a slow reaction.

Control reaction mixtures without added iron, or with ferric instead of ferrous ion, showed no significant changes in spectra.

Comparison of Various Preparations of Cytochrome c. Since some deviations from strict quantitative reproducibility of results of the type shown in Figure 1 had been encountered

[1] Implicit in this procedure is the assumption that the contribution of turbidity to the OD is the same at the two wavelengths. This assumption rests on the finding that the OD of reaction mixtures in which appreciable turbidity developed changed *in parallel* at the three isosbestic points: 526, 542, and 556 nm. For example, the average OD change at these wavelengths amounted to 0.145, with a standard deviation of only 0.012 ODU (12 measurements). This deviation corresponds to about a negligible 2% reduction of the cytochrome. Occasional OD measurements after removal of the turbidity confirmed the validity of our routine procedure.

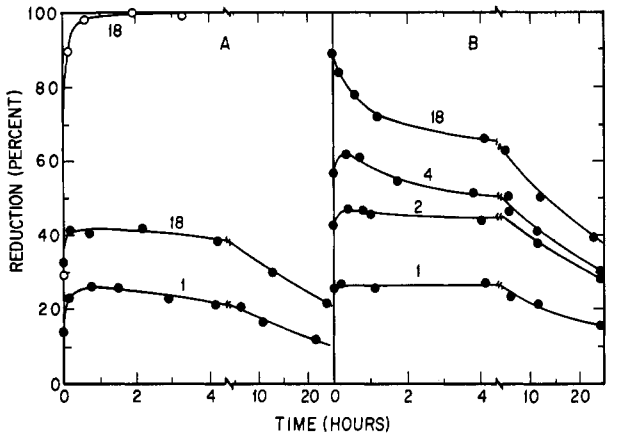

FIGURE 1: Time course of the reduction of cytochrome *c*, with varied amounts of ferrous ion, and of the autoxidation of the reduced heme protein in (A) Tris-HCl and (B) sodium phosphate. Initially, all reaction mixtures contained about 0.05 mM ferricytochrome *c* (Sigma, type III, lot 40C 7310) and the indicated molar equivalents of $FeSO_4$, in 50 mM buffer at pH 7.5. All experiments but one (shown with open circles, performed *in vacuo*) were carried out aerobically at about 21°.

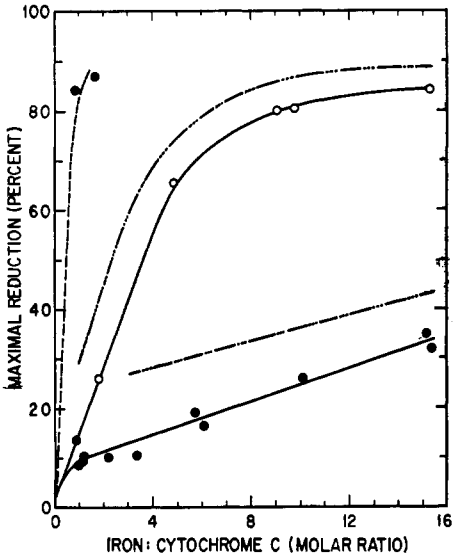

FIGURE 2: Variation of the maximal extent of the reduction of cytochrome *c* with the concentration of ferrous ion, in Tris (●) and phosphate (○) buffers. Solid lines, reactions in air; broken line, reactions *in vacuo*. The lot of ferricytochrome *c* used in these experiments was 70C 0940 (Sigma Type III). For experimental conditions, see the legend for Figure 1. The dotted lines are a schematic representation of a similar set of experiments carried out with a different lot of cytochrome *c* (Sigma Type III, 40C 7310): upper line, phosphate; lower line, Tris. The experimental points have been omitted for the sake of clarity: their scatter was comparable to that in the experimental series shown in detail.

between different lots of cytochrome preparations, a detailed comparison of several samples was made. Table I summarizes the salient features of these experiments. These data illustrate the magnitude of the variability from lot to lot (*cf.* types III and VI, preparations of presumably "native" cytochrome *c*). The data also indicate that the generalization offered in conjunction with the detailed results given in Figure 1 appears to be justified: reduction levels exceeding about one-half of the total cytochrome cannot be attained in Tris buffer even when the reductant is present in relatively large excess (16 Fe^{2+}/cytochrome) although comparable levels of reduction are reached with only a small excess of iron (2 Fe^{2+}/cytochrome) when the buffer is phosphate and in this case nearly complete reduction results when the level of ferrous ion is raised (8 Fe^{2+}/cytochrome). It is of interest that the denatured cytochrome preparation, type XII, shows no appreciable buffer-dependent response. This suggests that the integrity of the cytochrome structure may be of importance for the manifestation of these buffer effects.

The lack of complete identity between the various native preparations could be due to some variation in the amounts of possible artifactual molecular species of cytochrome *c* which may be present in the several lots (for a summary of such species, see Margoliash and Schejter, 1966). However, the presence of sizeable amounts of polymeric species can be excluded for three reasons. Ultracentrifugal analysis of some of these preparations has revealed the possibility of such contamination to a maximal extent of about 10% (*cf.* Taborsky, 1970). The presence of the 695-nm absorption band with an apparent extinction coefficient within about 10% of the value reported for monomeric cytochrome *c* (Schejter *et al.*, 1963) confirms this view and the already mentioned stability of the reduced form when prepared with reductants other than Fe^{2+} reaffirms it.

It must be noted that the contrasting behavior in Tris and phosphate buffers shown by the several cytochrome preparations described in Table I was not parallelled by a presumably native preparation of cytochrome *c* of a different manufacturer. It is highly unfortunate that we have been unable so far to pursue this observation further. We could not obtain delivery for nearly a year of either the same or a different lot of the same manufacture from the original source.

Dependence of the Maximal Reduction of Cytochrome c on the Concentration of Ferrous Ion. Figure 2 shows in detail the dependence of the maximal reducibility of ferricytochrome *c* on the concentration of $FeSO_4$. In Tris buffer (lower solid line), the level of reduction rises slowly in parallel with the concentration of Fe^{2+}. In phosphate buffer (upper solid line), maximal reducibility increases with increasing ferrous ion levels much more steeply. It is likely that the indicated leveling-off at less than complete reduction is a reflection of some autoxidation which had already occurred by the time of the earliest measurement (about 1 min after zero time; *cf.* the time course shown in Figure 1B).

Figure 2 also permits a detailed comparison between two cytochrome preparations (solid and dotted lines). The difference between them can be seen to be limited to the difference in the extent of maximal reduction achieved at low iron–cytochrome ratios. The effect of increasing ratios is essentially invariant between these preparations (and others, not shown here).

Effects of Oxygen. When air is excluded, cytochrome *c* can be completely reduced, even in Tris buffer, whether ferrous ion is present in excess (Figure 1A, open circles) or in stoichiometrically equivalent amount (Figure 2, dashed line).

Other effects of air have also been noted. For example, in Tris buffer, aerobically reduced cytochrome *c* undergoes autoxidation with a half-time of some 20 hr, while anaerobically reduced cytochrome autoxidizes with a half-time of only about 4 hr. In both of these cases, autoxidation occurs at a monotonous rate, in contrast to the biphasic course of the autoxidation observed with ferrocytochrome *c* in phosphate buffer (Figure 1B).

It seems certain that whatever the role of iron might be in

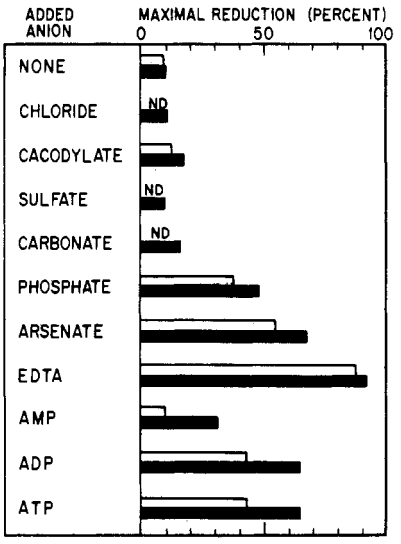

FIGURE 3: Effectiveness of various anions in promoting the reduction of cytochrome *c* by ferrous ion. All reactions were carried out with one reducing equivalent of ferrous ion, in Tris buffer and air, under conditions given in detail in the legend for Figure 1. The lot of ferricytochrome *c* was 70C 0940 (Sigma type III). The molar anion: cytochrome ratios were 1:1 (open bars) or 10:1 (solid bars). ND, not determined. Values for P_i, ADP, and ATP are averages of replicate measurements. The indicated levels of maximal reduction were attained within 2 min in the case of every anion which promoted extensive enhancement of the reduction beyond the control level.

producing these differences, it is not a simple catalytic one. The different rates of autoxidation referred to above bear no relation to the amount of iron present: the solutions contained equal amounts of iron and—during the autoxidative phase of the reaction—all of it in the ferric form. In other experiments, similar to these, the autoxidation rates were noted to be insensitive to the variation of the amount of iron between 1 and 4 equiv.

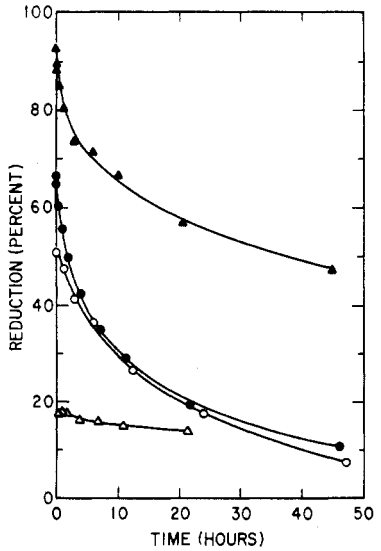

FIGURE 4: Time course of the autoxidation of ferrocytochrome *c* produced with one reducing equivalent of ferrous ion in the presence of 10 molar equivalents of the given anions. For experimental conditions, see the legend for Figure 3. (▲) EDTA, (●) ADP, (○) P_i, (△) cacodylate.

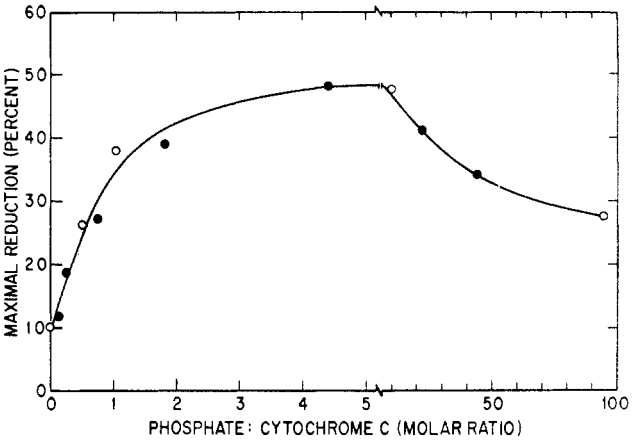

FIGURE 5: Maximal reduction of ferricytochrome *c* by ferrous ion as a function of inorganic phosphate concentration. For experimental conditions see the legend for Figure 3. Open circles represent averaged results of replicate experiments.

Effect of Various Anions. The ferrous ion–cytochrome reaction is markedly influenced by certain anions at very low concentration. The experiments shown in Figure 3 (involving the use of 1 equiv of Fe^{2+}) were carried out with such anions present in amounts of 1 (open bars) or 10 (solid bars) equiv with respect to the cytochrome (and iron). Several of the anions (chloride, cacodylate, sulfate, carbonate) show little or no enhancement of the maximally attainable level of reduction over and above the control level (in the absence of added anion). Others (phosphate, arsenate, EDTA, and the adenosine di- and triphosphates) promote extensive reduction of the cytochrome. Note should be taken of the fact that these anions exert this effect at concentrations of only 0.05 and 0.5 mM, that is 100–1000 times lower than the concentration of the buffer to which they are added. Under somewhat different conditions, similar enhancements of cytochrome reduction (and subsequent autoxidation) have been noted with dinitrophenol being the added anion. In general, it appears that these effective anions promote partial, approximately one-half reduction of the cytochrome with great facility even if their concentration is just at the level of stoichiometric equivalence, without much additional gain being produced when providing them in excess. Indeed, when the excess is very large—as it is the case in the experiments with phosphate buffer in which an anion:cytochrome ratio of 1000:1 prevails—the effectiveness of the anion, instead of being increased, is essentially lost (note the approximately equal, low level of reduction achieved with 1 equiv of Fe^{2+} in Tris and phosphate buffers, shown in Figure 2; this point will be documented in more detail in the following section).

Effective anions also affect the course of autoxidation. Figure 4 illustrates this point for solutions of cytochrome, ferrous ion, and selected anions present in molar ratios of 1:1:10. EDTA effects a biphasic autoxidation reaction, reminiscent of the reaction observable with large excesses of Fe^{2+} and phosphate (in phosphate buffer; see Figure 1B). In contrast, the low concentrations of ADP and P_i are associated with an apparently monotonous autoxidation rate.

Dependence of the Reducibility of Ferricytochrome c by Ferrous Ion on the Concentration of Inorganic Phosphate. Figure 5 depicts the results of experiments by which we sought to explore the relationship between the maximally attainable level of reduction (with 1 equiv of ferrous ion) and the relative concentration of one of the effective anions, phosphate. The

results show that the anion effect tends to become saturating at a relatively low phosphate:cytochrome ratio and this saturation level is achieved at a fractional stoichiometric stage, corresponding to about "half-reduction" of the cytochrome. The figure also shows that the effect of the anion is gradually lost as its relative concentration is increased further. The apparent saturation noted in these experiments is likely to reflect a truly limiting case since it has been observed that at the most effective, low concentrations of phosphate, variation in the amount of reductant (over the range of 1–15 equiv) is not at all paralleled by a corresponding variation in the level of maximally attainable reduction. Maximal reduction was observed in these experiments within the narrow range of about 40–50%, irrespective of the number of Fe^{2+} equivalents used. This is in marked contrast with the highly appreciable effect of the variation of the number of reducing equivalents on the level of reduction when the medium contains a very large excess of anion, *i.e.*, phosphate buffer (*cf.* Figures 1B and 2).

Discussion

The enhancement of the reduction of cytochrome *c* by ferrous ion in phosphate buffer, in comparison with the reaction in Tris-HCl, could be taken as evidence of complex formation providing the thermodynamic driving force behind cytochrome reduction on account of the strong affinity of phosphate for ferric ion (Weber *et al.*, 1954; Van Wazer and Callis, 1958; Kurihara and Sano, 1970).[2] Yet, particular aspects of our experiments require that we envisage the reaction as a more complicated process than the simple establishment of an oxidation–reduction equilibrium. Our comments will be directed at (1) the general nature of the possible complicating features of the interaction between iron and cytochrome *c* in different media, and (2) the specific implications of the effects which are seen with strongly complexing anions.

It seems clear that the aerobic reactions must be governed to a large extent by kinetic features of several component (and competing) processes which lead to the observed end results. (Of course, the initial rapid and extensive reduction of the heme iron—whenever it is noted—could still be the simple manifestation of a mass action effect on the equilibrium position of the ferricytochrome–ferrocytochrome reaction.)

It must be assumed that ferrous ion and cytochrome *c* interact with oxygen in an interdependent manner such that the reduction of the heme iron is not the sole consequence of the interaction with respect to the cytochrome molecule. The differing properties of the aerobically and anaerobically reduced cytochrome (*e.g.*, variable ease of autoxidation) require this assumption. Indeed, additional consequences of the ferrous ion–ferricytochrome *c* interaction, affecting the protein moiety of the cytochrome, have been observed (Taborsky, 1971[3]). It must be assumed further that the precise course of the reaction is determined by the nature of the ligands which are associated with the nonheme iron. This assumption stems from the observed variability of the reaction as the ionic composition of the reaction medium is varied. The observation of monotonously and biphasically proceeding autoxidations in differently constituted solutions is a case in point.

The involvement of an "activated" derivative of oxygen is likely in aerobic reactions in which ferrous complexes participate and the nature of such an involvement may be expected to be affected by the particular ionic ligands that are available for complex formation. Although the identity of the reactive iron complex species cannot be deduced from these experiments, it can be circumscribed. In the case of relatively poor ligands [*e.g.*, sulfate (Whiteker and Davidson, 1953) and chloride (Rabinowitch and Stockmayer, 1942; Carter and Clews, 1924)], hydroxide (Lanford and Quinan, 1948) and the protein itself undoubtedly compete for the iron. When strong complex-forming anions are present—for example, phosphate (Lanford and Kiehl, 1942; Holroyd *et al.*, 1957; Salmon, 1952, 1953), adenosine di- and triphosphates (Goucher and Taylor, 1964), or EDTA (Kolthoff and Auerbach, 1952)—they must be expected to alter the constitution of the reactive iron complex in a major fashion. Significant kinetic consequences may then ensue since it is known that the rate law of the reaction of ferrous ion with oxygen depends on the nature of the iron complex involved (Huffman and Davidson, 1956), and the nature of the transient oxygen derivative may depend as well on the particular iron complex with which the oxygen is associated and may effect the change in the mechanism of the reaction implied by a change in the rate law (Taube, 1965).

The possibility of a highly specific reaction involving the strongly complexing phosphates and related anions is implied in some of the effects noted in this study. Particularly, (1) the ability of these anions *in stoichiometrically equivalent amounts* to affect the reduction and autoxidation of the cytochrome, and (2) the remarkable *fractional stoichiometry of the electron saturation* of the cytochrome (at the level of about half-complete reduction) are noteworthy in this respect.

Cytochrome *c* is known to interact strongly with phosphate (Barlow and Margoliash, 1966), and the effectiveness of phosphate in influencing the rate of cytochrome reduction by various reducing agents is also well documented (Greenwood and Palmer, 1965; Mora *et al.*, 1965; Yates and Nason, 1966; Mora *et al.*, 1967). A recent abstract (Zipper *et al.*, 1971) reports an oxygen effect linked to phosphate. Most of these studies, however, differ from the work we have described in that the phosphate concentrations employed greatly exceeded the cytochrome concentration.[4] The several contrasting features of the phosphate effect at low and at high concentration (*e.g.*, with respect to the maximally attainable level of reduction, the variability of the extent of reduction with the concentration of ferrous ion, and the kinetic course of the autoxidation of the reduced heme iron) indicate to us that the mechanism underlying these effects may be qualitatively different depending on the concentration of phosphate relative to iron and cytochrome.

At low P_i concentrations, relative to iron concentration, predominant species are iron monophosphates of a 2:1 (Holroyd *et al.*, 1957) or 1:1 (Lanford and Kiehl, 1942) composition. Similar, strong complexes may be expected to be formed with ADP and ATP, but not with AMP (Goucher and Taylor, 1964). In all of these cases, additional, *specific co-*

[2] The potential of oxidation–reduction couples of iron salts or complexes varies over a wide range; its value depends on the nature of the complexing agent and pH and it will be higher or lower, relative to the potential of the aquo ion system (E_0 = 0.771 V), depending on whether and to what extent ferrous or ferric complex formation is favored (Michaelis and Friedheim, 1931; Michaelis and Smythe, 1932; George *et al.*, 1966).

[3] Unpublished data.

[4] The experiments of Yates and Nason (1966) are exceptions in this respect. They are most likely reflections of a specific rate enhancement by the various phosphates, but their design was not intended to give insight into the nature of the effect.

ordination with ligands provided by the protein would be possible. In cases of high phosphate concentration, any specific binding to the protein would meet effective competition from the excessively large anion population and the mechanism of electron transfer[5] from such complexes to the cytochrome heme iron could be forced to undergo a major change. The similarity of the effect of a low concentration of EDTA to the effect of a high concentration of P_i supports this view: the EDTA–chelate is very highly coordinated even at low EDTA concentration (Higginson, 1962).

The effect may be a specific *phosphate* effect not just the effect of a specific complex form of iron. Carbonate, which is effective along with other strong complex-forming anions in the promotion of the autoxidation of Fe^{2+} *per se* (Weiss, 1935), is ineffective in the cytochrome–ferrous ion reaction as are the various weak complex formers, such as chloride, although at high concentration they too promote iron autoxidation.

The fractional stoichiometric aspects of some of our observations (most strikingly shown by Figure 5, but implied also in the transition from a rapid to a slow autoxidation rate occurring after a fraction of the total cytochrome had been reoxidized, as shown in Figure 1B) raise the possibility of some form of multimolecularity of the reaction mechanism with respect to the cytochrome. Observations by Shashoua (1965) and by Hommes (1964) have been interpreted by them giving explicit consideration to a dimeric form of cytochrome *c* as the functional unit, a hypothesis which had been advanced previously by Chance and Williams (1956). Under certain conditions, the binding of metal (chromium) to cytochrome *c* in a ratio of 1:2 has also been observed (Kowalsky, 1969). In terms of such a hypothetical dimer (or oligomer), facile reduction or facile autoxidation of only part of the total molecular population could be rationalized.

Any potential biological significance of the reduction of cytochrome *c* by ferrous ion, the ensuing autoxidation, and the effects of certain anions on these reactions is a matter of conjecture at this stage. In the context of this study, recent reports on the reaction of P_i with the respiratory chain (Papa *et al.*, 1970) and on the possible involvement of nonheme iron in oxidative phosphorylation (Butow and Racker, 1965; Boyer, 1968; Racker, 1970) are particularly thought-provoking.

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[5] Electrons are referred to interchangeably with reducing equivalents, without an implied commitment to a particular mechanism of oxidation–reduction.

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Interactions of Phosphatidylcholine Vesicles with 2-*p*-Toluidinylnaphthalene-6-sulfonate†

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ABSTRACT: The interaction of 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) with phosphatidylcholine vesicle, a closed shell-like sphere with a continuous bilayer surrounding a volume of solvent, was studied by the gel filtration method. Thermodynamic parameters were estimated from the temperature dependence of the apparent intrinsic association constant from 5 to 40°. In the presence of spin-label 2,2,6,6-tetramethylpiperidine-1-oxyl the binding affinity of TNS to phosphatidylcholine vesicle was decreased slightly, whereas the maximum number of equivalent binding sites was unaffected. These data suggest that TNS molecules are most likely located in a geometrically restricted region near the interface between the hydrocarbon core and the polar head region of the lipid bilayer and that the phosphatidylcholine vesicle–TNS binding is of the "nonclassical" hydrophobic type. Sedimentation velocity analysis revealed that the sedimentation coefficient of the phosphatidylcholine vesicle–TNS complex increased linearly as a function of $\bar{r}$, the ratio of moles of bound TNS to moles of phosphatidylcholine, over the TNS concentration range studied. These results provide evidence to argue against gross conformational change of the vesicle due to TNS binding.

The fluorescent compounds ANS[1] and TNS have been widely used as hydrophobic probes to investigate the conformational changes in proteins (Edelman and McClure, 1968; Stryer, 1968) and more recently the structural changes in biological membranes (Tasaki *et al.*, 1968; Azzi *et al.*, 1969). In view of the fact that phospholipids are major components of most biomembranes, and that the hydrophobic core of the phospholipid bilayer seems likely to be the binding site for these hydrophobic probes, a study of the phospholipid–probe interaction can provide information of value for the interpretation of data obtained from probe studies on biological membranes.

This communication describes studies of the binding of TNS to phosphatidylcholine vesicles using the gel filtration method of Hummel and Dreyer (1962). The stoichiometry and apparent intrinsic association constant of the phosphatidylcholine vesicle–TNS complex formation were measured at various temperatures, and were also determined in the presence of the electron spin-label Tempo. These binding results were further correlated with hydrodynamic studies of the phosphatidylcholine vesicle–TNS complex in an attempt to reach a consistent picture of the structure of the complex. The advantage of using the spherical phosphatidylcholine vesicle as a membrane model system stems from its well-characterized properties (Huang, 1969; Huang *et al.*, 1970; Huang and Charlton, 1971).

Methods

Materials. The phosphatidylcholine was isolated from hen egg yolk by the column chromatographic method (Huang, 1969). The purity of the preparation was checked by thin-layer chromatography (Skipski *et al.*, 1964). Tempo was prepared according to the method of Rozantzev and Neiman (1964). TNS and Tris were supplied by Sigma Chemical Co., St. Louis, Mo. TNS solutions of desired concentration were prepared in 0.1 M KCl–0.01 M Tris, adjusted to pH 8.0.

Preparation of Phosphatidylcholine Vesicles. The procedure for the preparation of phosphatidylcholine vesicles was a modification of that described previously (Huang, 1969). Lyophilized phosphatidylcholine (300–400 mg) was suspended in 8 ml of buffered 0.1 M KCl solution (0.1 M KCl in 0.01 M Tris buffer) at pH 8.0. The suspension was ultrasonically irradiated (20 kHz) under nitrogen at 2° for 2.5 hr and then centrifuged at 105,000*g*, at 4°, for 60 min. The resulting supernatant was first concentrated to about 2 ml in a Sartorius collodion bag and then was subjected to upward-flow gel filtration, at 4°, on a column (25 × 50 cm) of Sepharose 4B which had been previously equilibrated with the same buffered KCl solution. The elution pattern of the phosphatidylcholine dispersion consists of two distinct peaks (fractions I and II). Those portions of fraction II which show a linear relation between absorbance at 300 nm and lipid phosphorous content and for which the linear regression line passes through the origin were used in all studies. Phosphatidylcholine concentrations in vesicle solutions are expressed in terms of lipid phosphorous (P_i) as determined spectrophotometrically (Huang, 1969). Employing the modified procedure, homogeneous vesicle fractions collected directly from the column effluent were concentrated enough for our purpose so that the final ultrafiltration step reported earlier could be eliminated.

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[1] Abbreviations used are: ANS, 1-anilino-8-naphthalenesulfonate; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; Tempo, 2,2,6,6-tetramethylpiperidine-1-oxyl.