Radical Decay Kinetics in Ferrocytochrome *c* Model Membranes. A Spin Label Study[1a,b]

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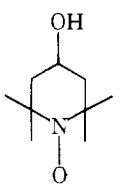
Abstract. The radical decay kinetics of the interaction of the small nitroxide spin label Tempol with model membranes composed of ferrocytochrome *c*, various phospholipids, and water, and with the complementary binary systems composed of protein–H_2O and lipid–H_2O have been investigated by electron spin resonance. Studies involving ferricytochrome *c*, porphyrin cytochrome *c*, and pure and impure lipids have served as controls and show that in pure systems an electron from the ferro iron atom is the source of the radical decay. The observed reactions in both the model membrane and binary ferrocytochrome *c*–H_2O systems are second order and yield rate constants ($k = 1.9 \times 10^{-2}$ and 1.7×10^{-2} M^{-1} sec^{-1}, respectively) and activation energies (7.2 and 7.9 kcal mol^{-1}, respectively) which are equal within experimental error for each system, suggesting that the mechanism of oxidation of ferrocytochrome *c* is the same in both cases. In addition, the results of a study involving the radical decay kinetics of Tempol with ferrous phenanthroline suggest that the electron transfer reaction occurs by an outer-sphere process. From a consideration of the structural and chemical characteristics of cytochrome *c* our results favor a proposed mechanism of oxidation of ferrocytochrome *c* in which the electron leaves the protein through the heme crevice area. To the extent that these results apply as well in mitochondrial membranes the same mechanism is favored for the *in vivo* mechanism of this important protein.

Recently a spin label study of the model membrane system composed of horse heart cytochrome *c*, bovine lecithin, and water was reported by Mukai, Lang, and Chesnut[2] in which it was noted that there was a time-dependent decay of the paramagnetism of the spin label. The source of the decay was not known then but it was noted that in the model membranes made with reduced cytochrome *c* the decay rate was considerably faster than that in those made with ferricytochrome *c*. The iron atom was clearly implicated. It was thought that this phenomenon was worth further study, not only because the physical chemical system is interesting, but also because oxidation-reduction processes are very important in the role that cytochrome *c* plays in the mitochondrial membrane. It was hoped that information about this role of reduced cytochrome *c* in a model membrane might be gained by a kinetic study of the radical decay as observed by esr and that this information might then be useful in the elucidation of the oxidative mechanism of cytochrome *c in vivo*. Accordingly, we have carried out a kinetic study of the interaction of the small paramagnetic nitroxide radical 2,2,6,6-tetramethylpiperidin-1-oxyl-4-ol (Tempol) with model membranes composed of ferrocytochrome *c*, various phospholipids, and water, and with the complementary binary systems composed of protein and H_2O. Our results indicate that the radical reduction (ferrocytochrome *c* oxidation) mechanism is the same in both the model membrane and aqueous systems and that the route of the electron transfer probably involves a direct attack by the radical through the heme area.



Tempol

Materials and Methods

Materials. Horse heart cytochrome *c*, type VI, was obtained from Sigma Chemical Co. while the porphyrin cytochrome *c* was a generous gift of Dr. R. W. Henkens. Reduced cytochrome *c* was either obtained from Nutritional Biochemicals Corp. or was prepared from type VI ferricytochrome *c* by reduction with solid sodium dithionite followed by dialysis from Spectrapor No. 1 dialysis tubing against deionized water at 4°; care was taken to dilute the original dithionite concentration to a negligible amount. The phospholipids were obtained from several sources. Solid phosphatidylserine and pure synthetic L-α-lecithin (dipalmitoylphosphatidylcholine)(PC) were obtained from Nutritional Biochemicals Corp., while pure bovine phosphatidylserine (PS) was obtained from Applied Science Laboratories. The synthetic L-α-lecithin and bovine phosphatidylserine were shown to be pure by thin layer chromatography. The tlc of the former lipid was kindly performed by Dr. C. S. Kim while that of the latter lipid was performed by the supplier. The perchlorate salt of ferrous phenanthroline (high purity) was obtained from the G. Fredrick Smith Co. All of the above materials were used without further purification. Tempol was prepared according to the method of Briere, *et al.*,[3] by Dr. John F. Hower.

Methods. A. Model Membrane Systems. Spin label investigations using Tempol were carried out on model membranes having ferrocytochrome *c*, ferricytochrome *c*, or porphyrin cytochrome *c* as the protein constituent. A mixture of synthetic L-α-lecithin (PC) and solid phosphatidylserine, or a mixture of PC and bovine phosphatidylserine (PS) served as the phospholipid constituent. In addition, water was present. When mixtures of lipids were used, the ratio of phosphatidylcholine to phosphatidylserine was 2:1 (w/w). To prepare the membranes 15 mg of lipid was dissolved in chloroform and the solvent was evaporated by nitrogen leaving a thin film of lipid. The last traces of chloroform were removed by pumping on a vacuum line. The lipids were swollen with 1.0 ml of deionized water containing Tempol (1×10^{-3} *M*) and the mixture was shaken on a vortex shaker for 30 sec. When commercially available, solid protein was used; 11.7 mg of the protein constituent was dissolved in 1.0 ml of the Tempol solution. The swollen lipid solution was added dropwise to the protein solution with vigorous stirring. The resulting red, flocculent precipitate was centrifuged at 5950*g* for 8 min. The pellet was washed with deionized water and recentrifuged at 5950*g* for 4 min. The supernatant was removed, and the pellet was taken up in a small capillary tube which was placed in a quartz esr tube. Usually 15 to 30 min were required before the initial spectrum could be recorded. When ferrocytochrome *c* prepared by dialysis was used, the procedure above was the same except that the protein, being in solution already, was not diluted further by radical solution.

The model membranes were analyzed for composition by dissolving the membrane in a 1 *M* NaCl solution (see Results and Discussion) and recording the uv-visible spectrum of the resulting clear solution on a Cary 15 spectrophotometer. Protein extinction coefficients were those of Margoliash and Frohwirt[4] while experimentally determined values were used for the lipids.

B. Lipid–H_2O Systems. The binary lipid-water systems were prepared by suspending a known amount of lipid by a known vol-

ume of water containing Tempol to give solutions of various per cent lipid by weight. Often the lipids were first dissolved in chloroform which was then evaporated (see above). These various lipid-water solutions were then put in a capillary tube, which was in turn put in a standard esr tube.

C. Protein-H_2O Systems. To study the radical decay kinetics in these systems, various amounts of cytochrome *c* were mixed with an aqueous solution of Tempol of known concentration, the resulting solution having a pH of 8.0 ± 0.1. In order to ascertain the protein concentration two methods were employed. The more usual one involved recording the absorption spectrum of the protein.[4] The second method utilized involved the calculation of the concentration of protein from a knowledge of the weight of protein, the resulting weight of the solution, and its density. The last quantity was calculated by reference to a curve which was determined by measuring the density of solutions of different per cent protein by weight. The two methods used gave protein concentrations which were identical.

D. $Fe(phen)_3(ClO_4)_2$-H_2O System. A kinetic experiment similar to those involving the protein-H_2O systems was carried out with $Fe(phen)_3(ClO_4)_2$. The concentration of a 4.2 m*M* aqueous solution of $Fe(phen)_3(ClO_4)_2$ was determined from absorbance measurements at 510 nm.[5] Equal volumes of this solution and 1 m*M* aqueous solutions of Tempol were mixed and the esr spectra were recorded as a function of time at 23°.

E. Esr Measurements. The electron spin resonance (esr) measurements were carried out on a standard Varian V-4502-15 system equipped with frequency and power monitoring devices. Care was taken to avoid both saturation and modulation broadening by working at low microwave power (below 10 mW incident on the microwave cavity) and small modulation amplitudes. (Tempol in the current studies is saturated at powers greater than 25 mW and is over modulated at modulation amplitudes greater than 0.2 G.) A Varian V-4540 temperature control unit was employed to maintain the desired temperature to within ±1° as measured by a copper-constantan thermocouple in thermal contact with the sample. Since the observed line widths in each experiment were constant in time, radical concentrations were taken to be proportional to the amplitude of a given line of the first derivative spectrum.

Results and Discussion

Composition of the Model Membranes. The composition of the model membranes was determined spectrophotometrically using the following extinction coefficients of cytochrome *c*[4] and the 2:1 PC-PS lipid mixture (Chart I). A

Chart I.

λ, nm	ϵ_P, M^{-1} cm^{-1}	ϵ_L, M^{-1} cm^{-1}
409	105,300	525
300	13,000	1527

previously weighed model membrane was dissolved in 1 *M* NaCl and the amount of protein and phospholipid in the sample was determined. The amount of water was determined by the difference in weight of the sample and that of the protein and phospholipids. The water that is associated with the model membrane is held quite loosely; desiccation over P_2O_5 or $CuSO_4$ removed all the water from the membrane as determined by the above difference method. The preparative technique which gave the best reproducible results involved placing the model membrane on filter paper so that excess surface water would be absorbed. In this way the intrinsic water of the model membrane was not lost. Table I shows the weight per cent of each component as determined experimentally and the molecular weight (mol wt) of each component. The result of the analysis of the lipid to protein ratio in nine different membranes is also indicated along with model calculated ratios to be discussed later. Shipley, *et al.*,[6] using X-ray diffraction as a tool, have shown that a weight per cent of water of 22.5% is necessary to maintain the structural integrity of model membranes composed of cytochrome *c*, phosphatidylcholine-phosphatidylserine (2:1), and water. The model membranes used in this present work were prepared in a similar manner and, as can be seen, were composed of the same chemicals. Two different model membranes were analyzed for water by the difference method indicated above. A third sample was analyzed for water content by the Karl Fischer method. The results of these three determinations indicated a water content in our model membranes of 24.7 ± 4.6 wt %, a value that agrees favorably with that reported by Shipley, *et al.*[6]

Table I. Composition of Model Membranes

	Wt % of each constituent	Mol wt	No. of molecules/protein[a] Exptl	Calcd
Protein	20.6 ± 1.6	12,384	1	1
Lipid	54.7 ± 1.6	760	43 ± 4	36–47
Water	24.7 ± 4.6	18	825 ± 154	806

[a] See text for description of model involved.

Further indication that the experimentally determined composition of our model membranes is compatible with that of others comes from the work of Ivanetich, *et al.*[7] They determined the phopholipid to protein ratio in model membranes composed of cytochrome *c*, water, and the total lipid extract of the mitochondrial membrane were cytochrome *c* is found *in vivo*. Their results indicated a phospholipid to cytochrome *c* ratio of 44, to be compared to the value of 43 ± 4 obtained in the present study.

Cytochrome *c*, which possesses a +8 charge at pH 7,[8] forms artificial membranes with acidic phospholipids[9,10] or with mixtures of acidic and neutral phospholipids[7,9-12] but not with zwitterionic phospholipids alone.[12,13] These artificial membranes have been studied by a number of different techniques but principally X-ray diffraction has been used. These studies show that a hydrated cytochrome *c* layer is held between lipid bilayers; such is also the case for reconstituted mitochondrial lipids and cytochrome *c* model membranes.[12] When the ionic strength of the aqueous phase is increased, the structural integrity of these model membranes sharply diminishes[7,12] indicating that the principal forces stabilizing the membrane are electrostatic in nature. Such is the case for cytochrome *c* in mitochondrial membranes as well, since it is readily removed by washing these membranes with a 1*M* NaCl solution.[8] In both mitochondrial and artificial membranes when cytochrome *c* is so removed, no lipid is associated with it. This enzyme is then a peripheral protein according to Singer.[14]

To account for the molecular ratios observed the simplest model "unit cell" consistent with these facts consists of a nearly spherical cytochrome *c* circumscribed by a cube, the free volume of which contains all the water molecules, and a section of the phospholipid bilayer, the polar head group of one side in contact with one face of the cube. Since Tempol has an isotropic coupling constant of 16.9 G in these membranes and 16.9 G in water,[2] it is reasonable to assume that Tempol molecules are also to be found in the free volume water portion of the cube. Cytochrome *c* is an oblate spheroid of axial lengths 30 × 34 × 34 Å.[15] Simplifying the situation by taking the protein to be spherical with a diameter of ⅓ (30 + 34 + 34) = 32.7 Å, the free volume accessible to water is 1.66×10^4 Å^3, from which a water to protein ratio of 806 is obtained, assuming a molecular volume for water of 20.6 Å^3.[16] The calculated value of 806 compares well with the experimental value of 825 ± 154. The number of phospholipids available per cytochrome *c* molecule can be obtained from twice the product of the quotient of the surface area of one face of the cube (edge length 32.7 Å) and the surface area of a head group of a phospholipid, a quantity ranging from 45 to 60 Å^2.[12,17-19] Using these values a phospholipid to protein ratio of 36 to 47 is calculated, to be

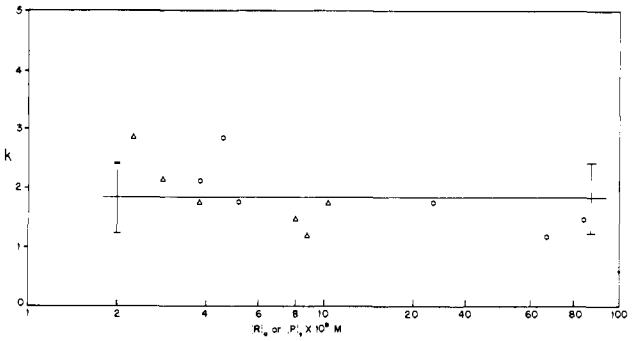


Figure 1. A plot of $-(d[R]/dt)/[R][P]$ evaluated at $t = 0$ as a function of both initial Tempol concentration (Δ) and initial protein concentration (O). The 12 points represent the results of six separate experiments. The data were collected at 23° and pH 8.

compared with a value of 43 ± 4 obtained experimentally. This simplistic model accounts remarkably well for the experimentally observed phospholipid-protein and water-protein ratios.

From the above discussion, it is seen that the model membranes have structural and chemical properties that resemble those of mitochondria and thus should be suited as a model to study the mode of action of the electron transport enzyme cytochrome *c* in a membrane.

Kinetic Results. Our primary concern is with a kinetic study of the spin label reduction (ferrocytochrome *c* oxidation) in a model membrane. It will, however, be easier to discuss the model membrane results after having first discussed the kinetic results obtained with binary aqueous protein-Tempol systems.

A. Protein-H_2O Systems. When ferrocytochrome *c* is mixed with an aqueous solution of Tempol, the radical is reduced. The amplitudes of the central line of each experiment were fitted to equations quadratic in time in order to determine initial rates by an extrapolation procedure. If the reduction of Tempol is assumed to be simple second-order reaction, then the rate is given by

$$-d[R]/dt = k[R][P] \quad (1)$$

where [R] and [P] are the Tempol and ferrocytochrome *c* concentrations, respectively, and *k* is the rate constant. Using data derived from the initial rate method, the quantity $(-d[R]/dt)/[R][P]$ evaluated at time zero is shown plotted against both initial radical and protein concentration in Figure 1. The data are scattered about the expected straight line of zero slope. The mean value of the rate constant so derived is $(1.8 \pm 0.6) \times 10^{-2}\ M^{-1}\ \text{sec}^{-1}$ at 23°. Finally, the full data of each run were plotted using the second-order rate function

$$Z \equiv \frac{1}{[R]_0 - [P]_0} \ln \left[\frac{[P]_0[R]}{[R]_0([P]_0 - [R]_0 + [R])} \right] = kt \quad (2)$$

where $[P]_0$ and $[R]_0$ are the initial protein and radical concentrations and [R] is the concentration of radical at any time *t*. This latter quantity can be obtained by knowing the amplitude of the esr signal at time zero, the amplitude at the time in question, and the initial free radical concentration. Each individual plot shows good linearity over at least 75% of the reaction indicating that the overall process is indeed second order and first order with respect to each reactant. The mean results of six runs are shown in Figure 2. The mean second-order radical decay rate constant using second-order plots is $(1.7 \pm 0.5) \times 10^{-2}\ M^{-1}\ \text{sec}^{-1}$ at 23°, shown as the straight line in Figure 2.

Deviations from linearity in individual second-order rate

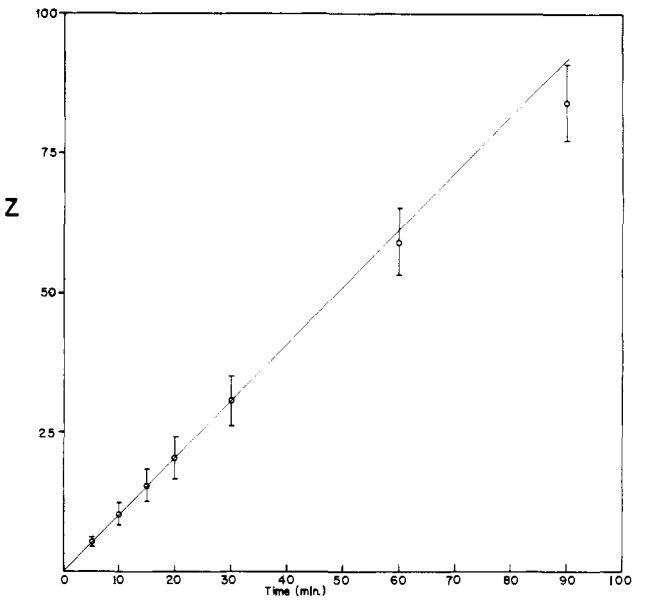


Figure 2. Averaged data for six runs at 23° (pH 8) plotting the second-order rate function *Z* (see eq 2 of text) *vs.* time. The range of initial radical and protein concentrations corresponds to the data of Figure 1. The exhibited experimental uncertainties represent the error averaged over the various experiments while the straight line exhibited is the mean slope observed, $1.7 \times 10^{-2}\ M^{-1}\ \text{sec}^{-1}$.

plots were observed over long reaction times (>75% completion). This may be due to a regeneration of the Tempol radical caused by an oxidation of the reduced form by O_2. Rozanstev[20] reports that sterically protected nitroxides which are reduced to the corresponding hydroxylamines are gradually reoxidized by O_2. Deviations from linearity are not the result of an appreciable amount of reverse reaction occurring since the data do not fit a straight line equation for a reversible second-order reaction.[21] Air oxidation of ferrocytochrome *c* can also be discounted as a competing reaction since the protein is nonautoxidizable.[8]

The reduction of Tempol by ferrocytochrome *c* was also monitored spectrophotometrically at 550 nm to ensure that no rapid reaction was occurring that could not be observed in the esr experiments. Spectra were obtained for ferrocytochrome *c* solutions before and immediately after the addition of a tenfold excess of Tempol. No dramatic spectral changes were observed indicating the radical decay reaction measured by esr techniques was the only one occurring.

It was found that ferricytochrome *c* and porphyrin cytochrome *c* cause no decay of the free radical. Thus the reduction of Tempol is not caused by its interaction with any amino acid residue in the protein but is caused by an electron from the iron atom in ferrocytochrome *c*.

B. Model Membrane Systems. When model membranes composed of ferrocytochrome *c*, synthetic L-α-lecithin-bovine phosphatidylserine (2:1) and water (containing Tempol) are made as described previously, a decrease in the amplitude of the Tempol esr signal is observed. Since the membrane contains an excess of ferrocytochrome *c* (see Table I), the kinetics of this radical decay process were monitored under pseudo-first-order conditions. The results of a typical experiment are shown in Figure 3. As can be seen, the radical decay obeys pseudo-first-order kinetics over more than 75% of the reaction. Due to the nature of the experiment involving the preparation of the model membrane and mixing with Tempol, it is not possible to obtain data during the initial stages of the reaction. However, extrapolation of the log amplitude data to zero time is in agreement with the predicted value. Also, it should be recalled that in the binary

system described above no rapid reaction other than the radical decay was observed.

The second-order rate constant for the model membrane system was calculated as the quotient of the experimentally observed pseudo-first-order rate constant, k_{obsd}, and the initial effective ferrocytochrome *c* concentration, $[Fe^{2+}cc]_0$:$k = k_{obsd}/[Fe^{2+}cc]_0$. The initial effective ferrocytochrome *c* concentration was calculated according to eq 3, where mol

$$[Fe^{2+}cc]_0 = \frac{(\text{wt \% P})\rho \times 10^3(\text{fraction } (Fe^{2+}cc))}{\text{mol wt}_P} \quad (3)$$

wt_P is taken as 12,384.[8] The weight per cent of protein is obtained by reference to the composition of the model membrane in Table I and is assumed to involve only the protein and water components. The density, ρ, is obtained by reference to the previously mentioned calibration curve. The fraction of the total protein used to make the model membrane which was in the reduced form is necessary in order to calculate the initial ferrocytochrome *c* concentration. When ferrocytochrome *c* was prepared from pure ferricytochrome *c* by the dithionite-dialysis method (see Material and Methods), approximately 70% of the total protein was in the reduced form but varied from preparation to preparation. The actual per cent ferrocytochrome *c* was determined for each preparation spectrophotometrically. Commercially available ferrocytochrome *c* also contained a substantial fraction of the oxidized form but this fraction was constant in each preparation made from this commercially available product. The ferrocytochrome *c* in this product was 34.9%, also determined spectrophotometrically. The mean value for the second-order rate constant obtained from five independent experiments as described above is $1.9 \pm 1.2 \times 10^{-2}$ M^{-1} sec^{-1} at 23°. It can be seen that the radical decay rate constants for the model membrane system and the binary ferrocytochrome *c*-water system are the same, within the limits of experimental error. The application of Student's *t*-test indicates no statistically significant difference.

To ensure that the reducing agent in the model membrane system was in fact ferrocytochrome *c*, model membranes composed of synthetic L-α-lecithin and phosphatidylserine or bovine phosphatidylserine and either ferricytochrome *c* or porphyrin cytochrome *c* and water (containing Tempol) were monitored for radical decay. No radical decay was observed in these systems or of Tempol in the presence of the binary lipid-H_2O systems using a 2:1 PC-PS mixture.

The equality of the rate constants suggests that the radical reduction mechanism, and hence the oxidation mechanism of ferrocytochrome *c* by Tempol, is the same in both cases. To further test this suggestion a limited temperature study of the radical decay process was carried out on the binary protein-water and model membrane systems. In addition to the rate constants obtained at room temperature (23°), data were also collected near 30° and 40°. The energies of activation for the oxidation of ferrocytochrome *c* by Tempol in the binary protein-water and model membrane systems are found to be 7.9 ± 2.2 and 7.2 ± 1.5 kcal/mol, respectively. These values are seen to be nearly equal and are so within experimental error; the Student *t*-test analysis of these data again shows that there is statistically no significant difference in ΔE_{Act} for the oxidation of ferrocytochrome *c* by Tempol in the two systems. This result coupled with the previous result that there is no significant difference in the rate constants for the oxidation of ferrocytochrome *c* by the free radical strongly suggests that the mechanism of oxidation of ferrocytochrome *c* by Tempol is the same in the protein-water binary solution and in the model membrane.

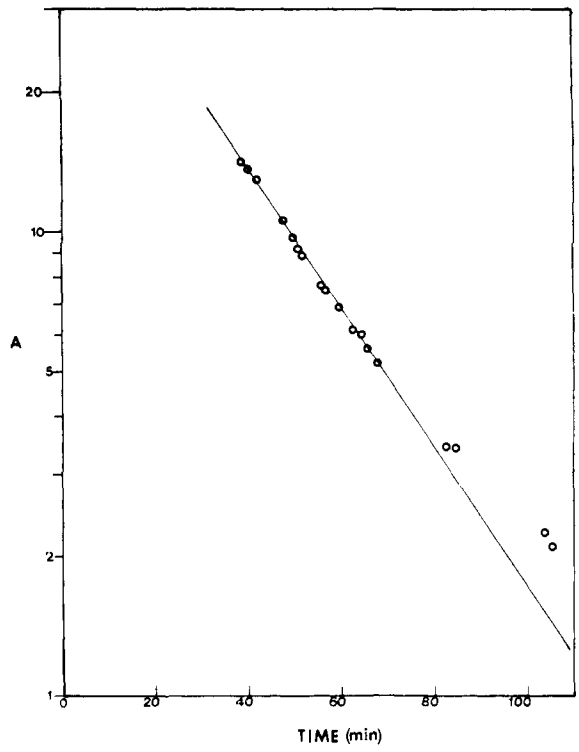


Figure 3. A semilogarithmic plot of a typical experiment at 23° involving the reduction of Tempol in a model membrane composed of $Fe^{2+}cc$, PC-PS (2:1), and water. The peak first derivative amplitude (arbitrary units) of the $m_I = 0$ line was used as a measure of the radical concentration.

1,10-Phenanthroline is a strong field aromatic nitrogen donor ligand, and, in that respect, is similar to the heme portion of cytochrome *c*. The second-order radical decay rate constant for the reaction of $Fe(phen)_3^{2+}$ and Tempol was determined in aqueous solution at 23° and found to be $(4 \pm 2) \times 10^{-2}$ M^{-1} sec^{-1}. The rate constant for phenanthroline dissociation from $Fe(phen)_3^{2+}$ is *ca.* 10^5 smaller than the rate constant for the oxidation of $Fe(phen)_3^{2+}$ by Tempol. This suggests that direct iron-Tempol bond formation in the octahedral complex does not occur prior to electron transfer and that an outer-sphere mechanism is operative in the $Fe(phen)_3^{2+}$-Tempol system.[22]

The rate constant for the $Fe(phen)_3^{2+}$-Tempol system is essentially the same as that obtained for both the cytochrome *c* model membrane and the aqueous solution systems. This suggests that ferrocytochrome *c* reacts as a typical low spin d^6 iron complex (such as $Fe(phen)_3^{2+}$) in reducing Tempol and that all three systems react *via* similar mechanisms. That is, a direct iron-Tempol linkage is not formed prior to radical decay in the protein system and hence the reaction can be classified as outer sphere. Further evidence for an outer-sphere mechanism being operative in the ferrocytochrome *c* systems comes from a consideration of the Tempol nitrogen isotropic coupling constant. If a direct iron-nitroxide bond were formed, one might expect a decrease in the isotropic coupling constant since the heme crevice area contains many hydrophobic residues. More importantly, a redistribution of spin density might well be expected to occur giving rise to a greatly altered spectrum. In addition, one might expect the rotational correlation time of the spin label to increase considerably. The absence of all of these effects plus the similarity of rate constants for the protein and $Fe(phen)_3^{2+}$ systems supports the idea that an outer-sphere electron-transfer process is operative.

Kinetic Results and the Oxidative Pathway of Cytochrome *c*. In order to properly assess the potential implica-

tions of the results of our current work to the behavior of cytochrome *c in vivo,* it is necessary to briefly review some of the structural and chemical properties of this very important enzyme. Cytochrome *c* is an electron transport enzyme located on the cytoplasmic side of the inner of the two membranes of mitochondria present in all aerobic organisms and is an essential enzyme in the terminal electron transport chain. It is a basic, small heme protein of molecular weight 12,384, containing one polypeptide chain of 104 amino acids covalently attached to the heme group. The crystal structures of both the oxidized and reduced form of the protein are known.[15,23]

There are a number of surface features of the protein which are probably important in the function of the molecule; these include the heme crevice, the left and right channels, and the patches of both positively and negatively charged groups on the molecule. The heme crevice is open in the oxidized form and is therefore readily accessible to solvent molecules. In the reduced form of the protein the crevice is blocked by the repositioning of the Phe 82 residue. The right and left channels are surrounded on the surface by positively charged lysines and arginine residues. The two hydrophobic channels as well as the heme crevice are of vital concern in proposed mechanisms for the action of the enzyme.

In the electron transport chain cytochrome *c* reductase reduces ferricytochrome *c* which is then oxidized by the complex cytochrome a + a_3, cytochrome *c* oxidase. Margoliash[24] has shown that the reductase and oxidase bind to different sites in cytochrome *c;* in particular, chemical modification of residues near the left channel results in an inability of the enzyme to react with the reductase while at the same time reacting with the oxidase with the rate essentially that of the native enzyme.[25] Modification of Lys 13, which is near both the heme crevice and the right channel, results in loss of activity with respect to the oxidase without modification of the ability to react with the reductase. Dickerson[15,23,26,27] has proposed two possible pathways for the oxidation of ferrocytochrome *c* involving either a direct electron transfer through the heme crevice or transfer through the right channel. Margoliash[24] has made a similar proposal with regard to the direct interaction of the heme group.

These proposed pathways have been based on X-ray evidence of the crystalline compound while, of course, the actual redox reaction takes place in a membranous system. Since our model membranes possess some characteristics of mitochondrial membranes, it should be possible to discuss the potential mechanism *in vivo* in terms of the kinetic results of our present study. Cytochrome *c* is held in the mitochondrial inner membrane primarily by electrostatic forces. Recall here that both the left and right channels are surrounded by the positively charged Lys and Arg residues and that in order to form model membranes it is necessary that negatively charged phospholipids be present. It seems clear then that the sites of binding of the negatively charged lipids needed to form the model membranes involve probably both the right and left channels of the protein. Consequently, the right channel, potentially involved in the oxidation of the enzyme, should be considerably more blocked in the model membrane than in aqueous solutions where no lipids are present. Because the rate constants and activation energies for the oxidation of ferrocytochrome *c* by the nitroxide free radical Tempol in both of these systems are not significantly different, the mechanism for the oxidation of the reduced protein is probably the same. Since the right channel is presumably blocked by the lipids in the model membrane and is clearly free of lipids in the binary system, the most obvious pathway for oxidation of ferrocytochrome *c* in our systems would seem to involve the exposed edge of the heme group.

A major problem of extending this mechanism to the *in vivo* oxidation of cytochrome *c* has to do with the fact that in the present system we are looking at the reaction of a small organic free radical while in the biological membrane large and complex proteins are involved. The data of Smith, *et al.*,[25] can be used to calculate[28] a rate constant for the oxidation of ferrocytochrome *c* by cytochrome oxidase in a suspension of beef heart muscle particles of approximately $1 \times 10^3\ M^{-1}\ sec^{-1}$. Performing the same type of calculation on the data of Minnaert[29] involving similar systems yields a second-order rate constant no greater than $30\ M^{-1}\ sec^{-1}$; other workers[30,31] have attained much larger values in *in vitro* systems where no membrane fragments are present. The fact that these rate constants are orders of magnitude greater than that observed in our systems may well reflect the relatively large stability of nitroxide free radicals in general.[20,32]

Such a discussion as this must clearly be of a speculative nature because of the complicated systems involved. The equality of the rate constants and activation energies for the oxidation of ferrocytochrome *c* by Tempol in the binary ferrocytochrome *c*–water system and the model membrane system clearly suggests that the protein oxidation mechanism is the same in both cases. The similarity of the rate constants in these systems and that of the $Fe(phen)_3^{2+}$–Tempol system suggest that the electron transfer occurs *via* an outer-sphere mechanism involving the exposed edge of the heme group. It is clear that one can interpret our results as supportive of the proposal that the exposed edge of the heme group is involved in electron removal from cytochrome *c.* To the extent that these considerations and results apply to cytochrome *c* in the mitochondrial membrane we have gained some additional knowledge about the mechanism of this important electron transport enzyme.

References and Notes

(1) (a) Supported in part by the National Science Foundation (Grant GP-22546, D.A.B. and D.B.C.), the Duke University NIH Biomedical Support Grant (D.A.B. and D.B.C.), a Research Award from the Graduate School of Duke University (D.A.B.), and a Frederick Gardner Cottrell Grant from the Research Corporation (A.L.C.). (b) Abbreviations: esr = electron spin resonance; Tempol = 2,2,6,6-tetramethylpiperidin-1-oxyl-4-ol; Fe^{2+}cc = ferrocytochrome *c;* PC = synthetic L-α-lecithin; PS = bovine phosphatidylserine; $Fe(phen)_3^{2+}$ = ferrous phenanthroline.
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The Hypervalent Molecules Sulfurane (SH_4) and Persulfurane (SH_6)

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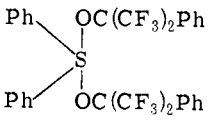
Abstract: The electronic structures of SH_2, SH_4, and SH_6 have been investigated by ab initio theoretical methods. The geometry of each species has been predicted using self-consistent-field wave functions employing a S(12s 9p/7s 5p), H(5s/3s) basis set of contracted Gaussian functions. Using these geometries, the effects of hydrogen scale factor, d functions on sulfur, and p functions on hydrogen have been explored. It is concluded that SH_4 lies energetically above $SH_2 + H_2$, and that SH_6 lies at least 36 kcal/mol above $SH_2 + 2H_2$. Thus SH_4 and SH_6 at best represent local minima on their respective potential energy surfaces. The structure predicted for SH_4 is quite reminiscent of the known geometry of SF_4. The bonding in these two model systems is discussed making use of population analyses. A number of molecular properties are predicted.

The well characterized SF_4 and SF_6 molecules are the simplest known hypervalent[1] sulfur compounds, i.e., the simplest S(IV) and S(VI) compounds. SF_6 is of course octahedral, with S-F bond distance[2] 1.564 Å. The more interesting structure of the SF_4 molecule[3] is seen in Figure 1 and might be considered "nearly octahedral." That is, its geometry is roughly approximated by the removal of two adjacent F atoms from the SF_6 structure. However, there are significant deviations from the octahedral model. In particular the axial S-F bond lengths are 0.101 Å longer than the equatorial ones. In addition the F-S-F bond angles are somewhat distorted from their idealized values of 180 and 90°. Finally, we note in Figure 1 that all four fluorine atoms lie in the right hemisphere of the molecule.

Polyfluoro compounds of the types RSF_3 and RSF_5 have been known for some time. Perhaps the earliest research in this area was the preparation of CF_3SF_3 and CF_3SF_5 by Tyczkowski and Bigelow[4] via the fluorination of carbon disulfide. One of the most important *recent* developments in organosulfur research has been the preparation, particularly by Denney[5] and by Martin,[6] of a series of more general hypervalent sulfuranes and persulfuranes. Among the most interesting compounds discovered to date is



(1)

which Martin and coworkers have found to be very useful as a reagent in the dyhydration of alcohols, and to react with amides and amines in a unique manner.[6] These recent developments certainly suggest that hypervalent sulfur compounds have a rich chemistry, only the surface of which has been touched to date.

Concurrent with these experimental developments, Musher[1,7,8] has developed a qualitative theory of the electronic structure of hypervalent sulfur molecules. In addition to providing a framework for the understanding of the known properties of sulfuranes and persulfuranes, Musher has made several intriguing predictions concerning their chemistry.[1,7] In his most recent paper Musher (with Koutecky)[9] has reported semiempirical CNDO/2 calculations of the electronic structures of SF_2, SF_4, SF_6, SH_2, and the two hypothetical molecules SH_4 and SH_6. Since SH_4 and SH_6 are the very simplest hypervalent sulfur molecules, these species serve as models for the many more complicated S(IV) and S(VI) compounds. For this reason, Musher has given the simple name sulfurane to SH_4 and called SH_6 persulfurane.

The present ab initio theoretical study may be viewed as the logical extension of Koutecky and Musher's semiempirical studies of SH_2, SH_4, and SH_6. However, this paper is also a sequel to two semiempirical[10,11] studies and one ab initio treatment[12] of the model hypervalent phosphorous compound PH_5. We note that Rauk, Allen, and Mislow predicted PH_5 to lie ~50 kcal/mol above $PH_3 + H_2$. In this regard it should be noted that the bonds in, e.g., SH_6 must be much stronger than those in SF_6 in order for SH_6 to exist. This is because the bond in the F_2 molecule is rather weak (~39 kcal/mol[13]), so that stability with respect to $S + 3F_2$ requires that each S-F bond energy be only ~20 kcal/mol. On the other hand, the stronger H_2 bond (109 kcal/mol[14]) requires an S-H bond strength of 55 kcal/mol in order that SH_6 lie energetically below $S + 3H_2$.

The goal of the present study, then, is first to predict the geometries of SH_4 and SH_6 and whether either molecule is a thermodynamically stable entity. Second, we seek to elucidate the electronic structures of these two prototype molecules, in particular the nature of the occupied molecular orbitals. In addition we will attempt to determine the importance of d functions on sulfur and p functions on hydrogen.

Initial Basis Sets. Although the H_2S molecule has been