Hydrogen Atom Exchange between Nitroxides and Hydroxylamines[1]

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Abstract: Nitroxides and their hydroxylamines exchange hydrogen atoms. The exchange of hydrogen atoms between tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) and the reduced form of the quaternary ammonium nitroxide (2,5,5-trimethyl-2-[2-(*N,N,N*-trimethyl)amino]ethylpyrrolidine-1-oxyl iodide) follows the second-order rate law, rate = k_0 [nitroxide]·[hydroxylamine] where $k_0 = 9.5 \pm 0.4$ M^{-1} s^{-1}. The reaction rate is catalyzed by copper ions. At low copper ion concentrations the catalyzed reaction obeys the rate law, rate = $k_1[Cu^+]$[nitroxide] where $k_1 = 2.2 \pm 0.1 \times 10^4$ M^{-1} s^{-1}. These reactions can be used for studies of the kinetics of immunochemical reactions at membrane surfaces.

Introduction

Several biophysical problems have been solved by taking advantage of the free-radical reactivity of nitroxide spin labels. Kornberg and McConnell[3] used ascorbate, a membrane-impermeable reducing agent, to determine the rate at which head-group-labeled phospholipids diffuse from the protected inner monolayer of bilayer vesicles to the exposed outer monolayer of these vesicles. Sheats and McConnell[4] developed alkylpentacyanocobaltates that when photolyzed produce alkyl radicals which can react with nitroxides to destroy their paramagnetism. This reaction has been employed for determinations of the rates of lateral diffusion of spin-labeled phospholipids.[5] Schwartz and McConnell[6] used a cobalt complex which produced a membrane-impermeable radical to measure the proportion of spin-labeled phospholipid on the outer surface of multilamellar liposomes. The radical reduced the spin labels exposed on the outer surface of the liposomes but did not destroy the protected nitroxides inside. Certain problems involving proteins, however, require gentler, more sensitive methods.

We are currently investigating the binding of antibodies to membrane antigens. Since the binding of antinitroxide antibodies to head-group spin-labeled phospholipids in model membranes provides a convenient model system,[7] we needed a superior chemical method to distinguish antibody-bound spin labels from free spin labels. This paper developes an unexplored area of nitroxide chemistry which is useful for problems of this type.

Experimental Section

Synthesis of 2,5,5-Trimethyl-2-[2-(*N,N,N*-trimethyl)amino]ethylpyrrolidine-1-oxyl Iodide. 2-Methyl-2-nitro-5-pentanone was prepared by condensing ethyl vinyl ketone (Aldrich) with 2-nitropropane (Aldrich, redistilled) according to Shechter et al.,[8] and was purified by vacuum distillation (bp 69–74 °C, 0.1 Torr). This compound was stirred in aqueous ammonium chloride with zinc dust, according to Bonnett et al.,[9] to yield 2,2,5-trimethylpyrrolidine-1-oxyl, which was vacuum distilled (bp 75–80 °C, 0.1 Torr) and characterized by NMR. Nitrile nitroxide was prepared by adding the nitrone to a solution of $LiCH_2CN$ in THF–hexane (−78 → 0 °C) by the procedure of Kaiser and Hauser[10] and then oxidizing the resultant hydroxylamine with cupric acetate and air by the procedure of Keana et al.[11] The crude product (10.8 g) was taken up in THF and added to 2.8 g of $LiAlH_4$ in THF, at such a rate that it refluxed gently. The reaction mixture was refluxed for 2 h and then was let stand overnight. It was quenched with 10 mL of 1 M NaOH and filtered, and the solvent was evaporated. The dark oil was taken up in 1 M NaH_2PO_4 and extracted with ether to give 3 g of a mixture of nitrone and starting material (characterized by IR). The aqueous layer was then brought to pH 12 and extracted with ether to yield 3.04 g of amino nitroxide. The amine was quaternized with methyl iodide and 1,2,2,5,5-pentamethylpiperidinol as described by Sommer et al.,[12] and purified on a column of neutral alumina packed in ethyl acetate and eluted with a methanol–ethyl acetate gradient. The product moved as one spot on alumina TLC developed with 10:1 ether–methanol, and was characterized by its paramagnetic resonance spectrum (purity by EPR assay, 100%; $A = 15.8$ G).

Stopped-Flow Apparatus. In order to mix the hydroxylamine solution with the nitroxide anaerobically in the EPR cavity, a stopped-flow apparatus was constructed from a piece of 10-mm diameter quartz tubing, a 50-μL glass capillary tube, two 100-μL gas-tight syringes, and a Teflon mixing chamber (Figure 1). The mixing chamber was built from a 2-in. length of 0.7-in. diameter Teflon rod as follows. A large hole was machined 0.400 in. deep into the end of the Teflon rod so that the quartz tube fit snugly. A concentric hole (0.055 in. diameter, 0.1 in. deep) was drilled into which the 50-μL pipet fit. A third concentric hole (0.040 in. diameter, 0.020 in. deep) was then drilled to form the actual mixing chamber. Two delivery holes (0.018 in. diameter) were then drilled from opposite sides of the Teflon rod, 0.010 in. off-center, as shown in cross section (Figure 1). The apparatus, assembled as shown in Figure 1, is held in place with conventional EPR cavity hardware.

To keep out oxygen, the entire region between the magnet poles was enclosed with polyethylene sheet and this region was then filled with nitrogen. Thin latex was used as material for diaphragms to form air-tight seals around the wave guide and the cables to the cavity. This material was also used for a septum through which the microliter syringe needles were passed before inserting them into the mixing chamber delivery holes. The nitrogen flow was adjusted to maintain a slight positive pressure inside the enclosure. For each experiment, the syringes were loaded with the appropriate solutions and the needles inserted into the mixing chamber. The plungers were pushed by hand, so that the solutions were mixed and injected into the EPR cavity in about 1 s. Reproducibility was about 5%.

Preparation of Samples. Hydroxylamines were prepared by reducing an aqueous solution of nitroxide with hydrogen over platinum.[13] Large (~0.07 g) pieces of platinum were used for the reduction, which had the advantage that the hydroxylamine solution could be removed directly from the reaction flask without taking an additional step to remove fine pieces of platinum. Reduction with 6–12 pieces takes about 2 h. After each reduction, platinum was regenerated by washing with concentrated nitric acid.

Solutions of nitroxide were deaerated by stirring under nitrogen in a 25-mL two-necked pear-shaped flask, and the syringes were loaded under a stream of nitrogen. The syringes were then taken to the spectrometer, the needles pushed through the diaphragms into the nitrogen atmosphere, a few microliters of solution squirted out to get rid of oxygen at the tip of the needle, and the needles inserted into the delivery holes of the mixing chamber.

Miscellaneous. Solutions were buffered to pH 6 since the copper-catalyzed reaction rate is maximal there. 2-(*N*-Morpholino)ethanesulfonic acid (MES) buffer (Calbiochem) at 50 mM was chosen because it has a p*K* of 6.2 and does not complex copper.

Since bacteria interfere with the exchange reaction, stock solutions of all reagents were stored in the freezer, and the reagent solutions were made up each day.

Chemical Kinetics. Spectra were taken on a Varian E-12 EPR spectrometer. The concentrations of tempol and the ammonium ni-

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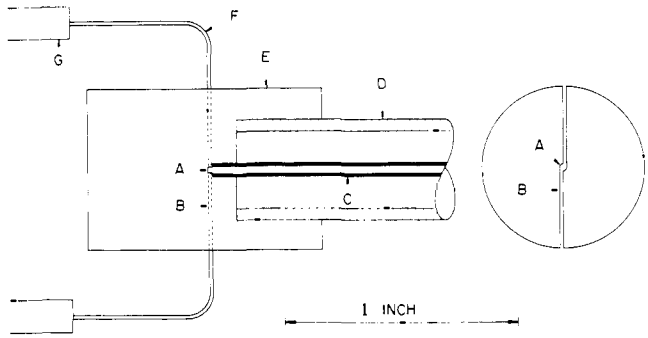
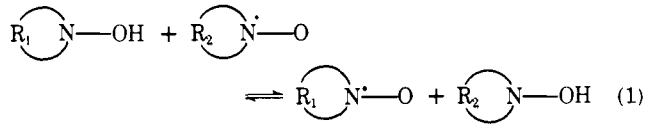


Figure 1. Stopped-flow device: A, mixing chamber; B, delivery holes; C, 50-μL glass capillary; D, quartz tube; E, Teflon block; F, needle; G, 100-μL syringe.

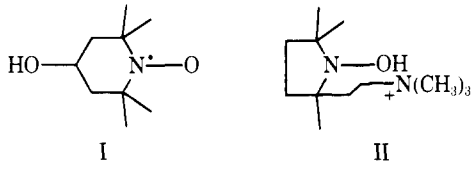
troxide were monitored by locking onto their respective low-field lines with a Varian E-272B field frequency lock; reaction rates were measured by locking onto either nitroxide signal, mixing the sample solutions in the stopped-flow device, and measuring the change in signal intensity with time. The initial rate of reaction was calculated directly from the change in signal intensity, and the rate law was discovered by measuring the initial rate for various reactant concentrations.

Results and Discussion

We have found that nitroxides and their hydroxylamines exchange hydrogen atoms according to reaction 1. Typical reaction kinetics are shown in Figure 2.



This reaction occurs between all the nitroxides we have examined, including nitronyl nitroxides[14] and doxyl nitroxides, but we have chosen for detailed study the exchange between tempol (I) and the hydroxylamine of the quaternary ammonium nitroxide (II).



I II

Compound II was chosen for four reasons. It is completely stable to repeated reduction and oxidation, as indicated by complete recovery of EPR signal intensity upon air oxidation. (Nitronyl nitroxides and doxyl nitroxides are not sufficiently stable under these conditions.) It binds only weakly to antinitroxide antibodies. It has a very low reduction potential so that the exchange reaction goes to completion. (Measurement of EPR signal intensities showed the equilibrium constant corresponding to the reaction of I and II according to eq 1 to be greater than 1×10^4.) And it has a very large hyperfine splitting (15.8 G) so that the low-field lines of tempol and the ammonium nitroxide are resolved; thus their concentration in mixtures can be determined independently from their spectral intensities.

As shown in Figures 3 and 4 the initial reaction rate varies linearly with both nitroxide and hydroxylamine. This suggested the second-order rate law $-d[I]/dt = k_0[I][II]$ where $k_0 = 9.5 \pm 0.4$. This was verified by plotting the concentrations of nitroxide and hydroxylamine from Figure 2 according to the integrated form of the rate law. As shown in Figure 5, this gives a straight line of slope $9.5 \pm 0.4\ M^{-1}\ s^{-1}$. The reaction is independent of pH between 5 and 8, and presumably occurs by a simple bimolecular mechanism.

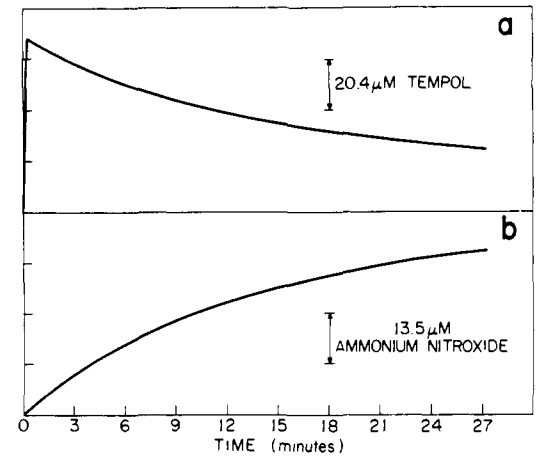


Figure 2. Reaction between 68 μM tempol (I) and 102 μM hydroxylamine (II). Figure 2a shows the EPR signal intensity of tempol during the course of the reaction. The rapid initial increase of the signal upon injection of reactants into the cavity is followed by a slow decrease as tempol is reduced to hydroxylamine. Figure 2b shows the increase in signal intensity of the ammonium nitroxide as it forms from the oxidation of its hydroxylamine (II).

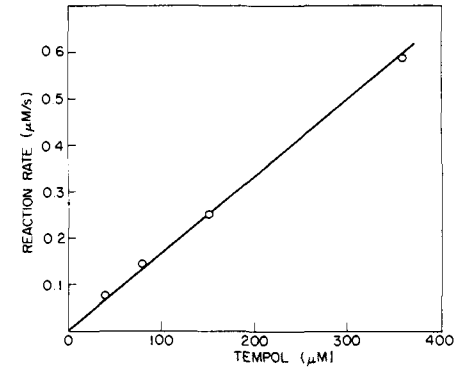


Figure 3. Dependence of the exchange reaction rate upon nitroxide (I) when the hydroxylamine concentration is held constant at 100 μM.

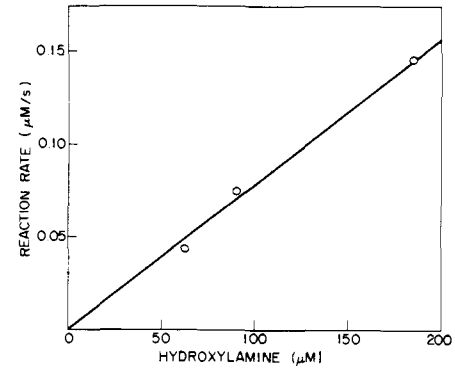


Figure 4. Dependence of the exchange reaction rate upon hydroxylamine (II) when the nitroxide concentration is held constant at 100 μM.

Effect of Copper. This reaction is catalyzed by cupric ion. As shown in Figure 6, cupric sulfate at low concentrations increases the reaction rate dramatically. The catalysis probably occurs by a two-step mechanism in which copper cycles between Cu^+ and Cu^{2+}.[13] We have observed both of these reactions independently: cupric ion oxidizes hydroxylamines to their nitroxides rapidly and completely, and cuprous ion in excess reduces nitroxides. Reaction 3 is rate limiting, as indi-

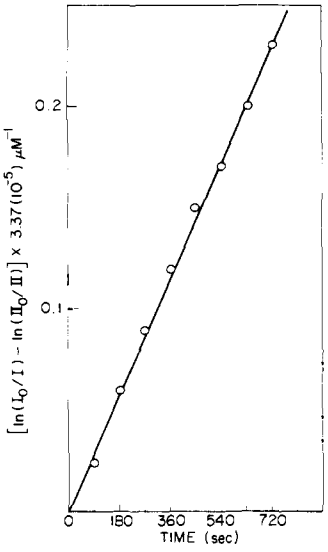


Figure 5. The concentrations of I and II from Figure 2 are plotted according to the integrated form of the rate law, $-d(I)/dt = k_0[I][II]$. The result is a straight line of slope k_0.

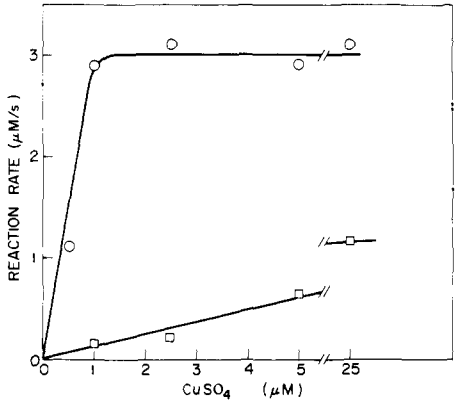
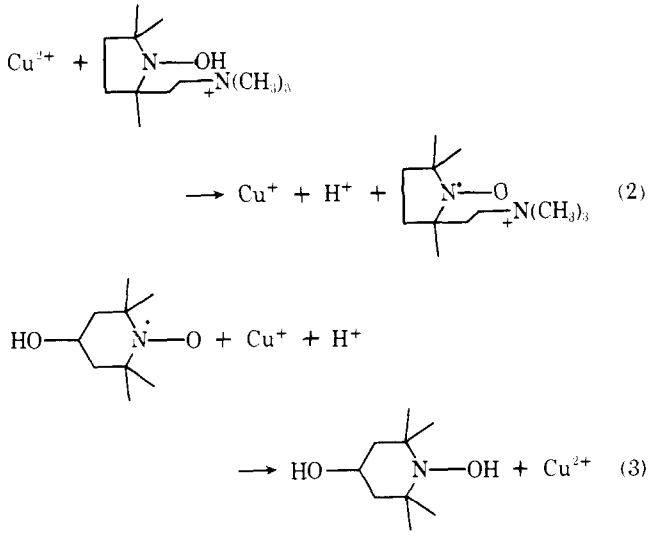


Figure 6. Dependence of the exchange reaction rate on copper concentration. The circles show the dependence of the exchange rate of $CuSO_4$ concentration when tempol and the hydroxylamine concentrations are held constant at 200 μM each. The squares show the reaction rates when tempol and hydroxylamine concentrations are 50 μM each.

cated by the very rapid oxidation of hydroxylamines by Cu^{2+}, and by the kinetic results presented below.



A peculiar feature of the catalysis is that the reaction rate levels off when the concentration of copper is as large as 1/200th of the reactant concentrations. We decided to investigate this behavior further. We found that the saturation effect is independent of pH (pH 7-3) and ionic strength (0-0.5), but depends on the reactant concentrations. At lower reactant concentrations, higher copper concentrations are necessary to observe saturation. Furthermore, there is a striking difference in the kinetics above, below, and in the vicinity of the saturation point. At high $CuSO_4$ concentrations the reaction obeys the rate law $-d[I]/dt = k_2[I]/[II]$ where $k_2 = 4.1 \times 10^{-4}$. Well below the saturated region, $-d[I]/dt = k_1[Cu^+][I]$, where $k_1 = 2.2 \pm 0.1 \times 10^4\ M^{-1}\ s^{-1}$. In the intermediate region, at least qualitatively, $-d[I]/dt \propto [Cu^+]^x[I]/[II]^y$, where x varies between 1 and 0 and y varies between 0 and 1 as the saturation region is approached.

The unexpected inverse dependence of the reaction rate on hydroxylamine concentration, and the inverse dependence of the saturation point on reactant concentrations, suggested that hydroxylamine binds to cuprous ion. Thus, as more copper is added, it is complexed and cannot catalyze the reaction. It is clear that a simple 1:1 soluble complex is not consistent with the shape of the curve in Figure 6. However, we have shown in theoretical calculations not given here that a soluble polymeric complex, $Cu_n(II)_n$, with n between 10 and 100, can account for the curve in Figure 6 to within the experimental accuracy. We have no data directly supporting the existence of this type of complex, but polymeric cuprous complexes are well-known.[15]

In work not reported here we studied the effect of Ag^+ on the copper-catalyzed reaction between I and II, since this ion should complex hydroxylamine[16] like Cu^+, but should not catalyze the exchange reaction because of the high oxidation potential to Ag^{2+}. The effect of Ag^+ was studied at copper concentrations corresponding to the higher copper concentrations. All the observed effects of Ag^+ on the exchange rate as a function of silver concentration support the view that Ag^+ can compete with Cu^+ in the hydroxylamine complex.

Conclusions

The exchange reaction between nitroxides and hydroxylamines has several advantages over other methods for destroying the paramagnetism of nitroxides. First, the course of reaction may be monitored either by watching the disappearance of the nitroxide whose state of protection is being studied or by the appearance of the nitroxide derived from the hydroxylamine. When the nitroxide under study has a broad, immobilized EPR spectrum and the hydroxylamine is free in solution, this may give a large increase in the signal-to-noise ratio. Second, by varying copper and hydroxylamine concentrations, the half-life of the exchange reaction may be varied from a few seconds to hours. Thus this chemistry could be used to investigate processes which occur on time scales from seconds to days. Third, the hydroxylamine may be altered to fit particular needs; it may be made more or less bulky, more or less hydrophobic, or with a higher or lower reduction potential. Unfortunately, the utility of copper as a catalytic agent in biochemical and biophysical studies is severely limited owing to the binding of copper ions to proteins.

We are presently studying the kinetics of specific antinitroxide antibody binding to phospholipid spin-label haptens in model membranes, by measuring the rate of reaction 1 in the presence and absence of specific antibody. When antibody is bound to the nitroxide lipid hapten, the nitroxide group is protected by the combining site region of the antibody, and reaction 1 is prevented. These preliminary studies demonstrate the utility of this method, but detailed quantitative kinetic studies await the production of monoclonal antinitroxide antibodies (in progress in this laboratory).

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References and Notes

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Syntheses and Structure Studies of Stable Difluoro- and Dichlorosulfuranes. Apicophilicity Orders in Sulfuranes[1]

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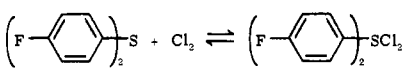
Abstract: Difluoroalkoxysulfurane **7** in solution is found to be in a conformation with an apical alkoxy ligand and an equatorial fluorine ligand. A similar conformation is postulated for dichloroalkoxysulfurane **8** in solution, but in the crystalline form **8** exists as a tetramer with bridging chlorine ligands and an octahedral arrangement of ligands around sulfur. Ring strain and electronegativity as factors affecting relative apicophilicities in sulfuranes and phosphoranes are discussed. Evidence that alkoxy groups may be more apicophilic than fluoro and chloro ligands in sulfuranes is also discussed.

Introduction

Tetracoordinate, tetravalent sulfur species, sulfuranes,[2] have been known for a long time. Sulfur tetrachloride was first reported in 1873[3] and sulfur tetrafluoride was reported in 1911.[4] Sulfur tetrachloride is unstable at temperatures above −20 °C; however, it has been employed in the synthesis of sulfur tetrafluoride.[5] Sulfur tetrafluoride is[6] a "trigonal bipyramid" (TBP) with the sulfur lone pair occupying an equatorial position. An approximate bonding scheme for this type of structure, the hypervalent bond model, has been described by Musher.[2]

Trichloro-[7] and dichlorosulfuranes,[8] like sulfur tetrachloride, are unstable with respect to the loss of chlorine at room temperature in solution, whereas trifluoro-[9] and difluorosulfuranes,[10] like sulfur tetrafluoride, do not show the analogous loss of fluorine. Wilson and Chang[8b] have reported the equilibrium constant for the formation of dichlorosulfurane **1** from the corresponding sulfide and chlorine to be $4.04 \times 10^5\ M^{-1}$ (eq 1). The first report, of which we are aware, of a dichlorosulfurane (**2**) was that by Price and Smiles;[11] however, controversy has surrounded this report.[12] Some workers[12a,b] have proposed that the true structure is **3** on the basis of products studies and IR data; however, others[12c,d] have supported the dichlorosulfurane structure on the basis of products and the IR spectrum of freshly made material. Livant and Martin[12d] have argued from the IR spectrum that the two compounds are



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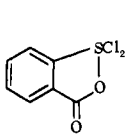

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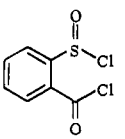

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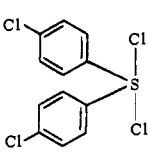
in equilibrium. Some dichlorosulfuranes have been found to be useful synthetic intermediates[13] or reagents.[14]

Dichlorosulfurane **4**[8a] was found to be a TBP, like sulfur tetrafluoride, with the chlorine ligands occupying the apical



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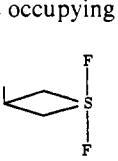

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positions, as is expected from the relative apicophilicities of chlorine and carbon ligands that have been established[15] for phosphoranes. On the basis of ^{19}F NMR spectroscopic data, Denney et al.[10c] have determined that sulfurane **5**, like **4**, has its halogen ligands in the apical positions.

Monochloroalkoxysulfuranes have been implicated by spectroscopic methods as unisolated intermediates[16] or prod-

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