Pulse Radiolysis Study of Optical Absorption and Kinetic Properties of Dithiothreitol Free Radical[1a]

Phillip C. Chan*[1b] and Benon H. J. Bielski[1c]

Contribution from the Department of Biochemistry, State University of New York Downstate Medical Center, Brooklyn, New York 11203, and the Department of Chemistry, Brookhaven National Laboratory, Upton, New York 10973.
Received June 23, 1973

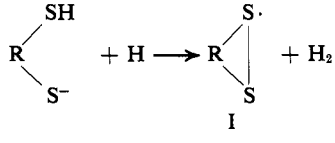
Abstract: The absorption spectrum of the dithiothreitol free radical was determined in N_2O-saturated aqueous solution (pH 2–10) by pulse radiolysis. Variation in molar extinction with pH suggests that the radical can exist in an ionized form I or as a neutral species IV with a pK of 5.5. At pH 8.3, variation of dithiothreitol from 0.1 to 10 mM did not affect the formation nor the disappearance of the radicals. The radicals decay by second-order kinetics throughout the entire pH range. Product analyses by thin-layer chromatography and spectrophotometry indicate that the radicals disproportionate to the oxidized and reduced forms of dithiothreitol. A mechanism is proposed which accounts for the observed acid-catalyzed decay. The radical is oxidized by molecular oxygen at a rate $k = (1.37 \pm 0.15) \times 10^9\ M^{-1}\ \text{sec}^{-1}$.

Since Cleland first introduced dithiothreitol as a protective reagent for protein sulfhydryl groups in 1964,[2] it has been used extensively in biochemistry laboratories. Besides its application for preserving or regenerating sulfhydryl groups which are essential to enzyme activities, it has been shown to be effective in protecting cells against ionizing radiations.[3] Moreover, Rhoads and Udenfriend have reported that dithiothreitol can substitute for ascorbic acid in the hydroxylation of proline in collagen.[4] The essential feature of dithiothreitol in serving these various functions is its two reactive sulfhydryl groups. Upon oxidation the compound forms a stable six-member, disulfide ring. Cleland determined that the redox potential of dithiothreitol at pH 7 is -0.33 V;[2] therefore, it can be used as a scavenger of free radicals or some other oxidants. Presumably the oxidation of dithiothreitol must go through a free radical intermediate. It is of interest to study the characteristics of the dithiothreitol free radical, not only because the compound is a sulfhydryl group protector, but also because the free radical mechanism of oxidation of dithiothreitol may serve as a model for biological sulfhydryl compounds.

The dithiothreitol free radical (here denoted as I) can be generated from the reduced parent compound ($R(SH)_2$) by oxidation with OH radicals during radiolysis of aqueous solutions containing nitrous oxide. The mechanism for formation (reactions 1–4) and disappearance (reaction 5) of the dithiothreitol free radical is based on experimental results obtained from ^{60}Co γ-ray and pulse radiolysis studies.

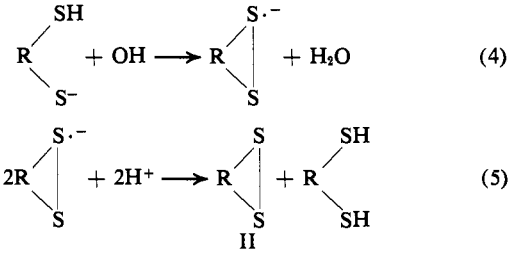
$$H_2O \xrightarrow{\sim\sim} H_3O^+,\ e_{aq}^-,\ H,\ OH,\ H_2,\ H_2O_2 \qquad (1)$$

$$N_2O + e_{aq}^- + H_3O^+ \longrightarrow N_2 + OH + H_2O \qquad (2)$$



(3)

I



II is the oxidized form of dithiothreitol.

Experimental Section

Apparatus. A pulsed 1.95-MeV Van de Graaff generator served as an electron source. The pulse length was kept constant at 5 μsec throughout this study, unless specified otherwise. All pulse radiations were carried out in a Suprasil quartz cell ($2 \times 2 \times 0.9$ cm) with one 2×2 cm window thinned to 0.4 mm to allow penetration of the electrons. Analyzing light from a G.E. 100-W quartz-iodine lamp passed through the cell three times with a total optical path length of 6.1 cm. The emerging light passed through a Bausch & Lomb $f/3.5$ monochromator, set for 2-nm resolution, to a 7200 RCA photomultiplier. The signal generated by the photomultiplier was subsequently fed into an oscilloscope where it was recorded. Interference from Cerenkov radiation was monitored over the spectral range investigated and it was found to be negligible under present experimental conditions. Yields were determined by ferrous sulfate dosimetry.[5]

A ^{60}Co γ-ray source of the Schwarz-Allen[6] design with an intensity of 570 krads/hr was used for routine γ-ray irradiations. All irradiations were made at $24 \pm 0.5°$ in 50-ml syringes. The energy absorption in solutions was determined with the Fricke ferrous sulfate dosimeter.[7,8] The dose rate for the source was calculated on the basis of $G(Fe^{3+}) = 15.50$. Gas chromatographic measurements were carried out with a Perkin-Elmer vapor fractometer, Model 154. The pH measurements were taken on an Orion Research ionalyzer, Model 801.

Chemicals. All solutions were prepared in triply distilled water. The pH of the solutions was adjusted by addition of either sulfuric acid or sodium hydroxide. Reduced and oxidized dithiothreitol were purchased from Calbiochem. Both chemicals were used without further purification.

Results

^{60}Co γ-Ray Studies. When a 5-mM aqueous solution of reduced dithiothreitol, saturated with nitrous oxide (pH 8.6, 0.02 M phosphate), was irradiated with

(1) (a) Research performed in part under the auspices of the U. S. Atomic Energy Commission. (b) State University of New York Downstate Medical Center. (c) Brookhaven National Laboratory.
(2) W. W. Cleland, *Biochemistry*, 3, 480 (1964).
(3) Y. A. E. Bick and W. D. Jackson, *Nature (London)*, 217, 479 (1968).
(4) R. E. Rhoads and S. Udenfriend, *Arch. Biochem. Biophys.*, 139 329 (1970).
(5) A. O. Allen, "The Radiation Chemistry of Water and Aqueous Solutions," Van Nostrand, Princeton, N. J., 1961.
(6) H. A. Schwarz and A. O. Allen, *Nucleonics*, 12, 58 (1954).
(7) H. Fricke and S. Morse, *Phil. Mag.*, 7, 129 (1929).
(8) A. O. Fregene, *Radiat. Res.*, 31, 256 (1967).

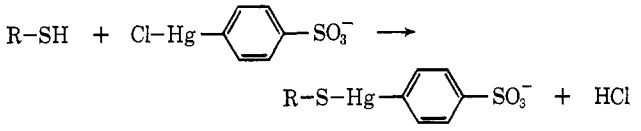
^{60}Co γ-rays, linear product formation was observed up to 50 krads. The product (cyclic monomer of oxidized dithiothreitol) was identified by thin layer chromatography and quantitatively determined spectrophotometrically.

For thin layer chromatography analysis a nitrous oxide saturated solution of 1×10^{-2} M dithiothreitol was irradiated in the ^{60}Co source for 1 hr. The change in absorption at 283 nm indicated about 40% of the compound was oxidized. Part of the solution was reacted with a slight excess of *p*-chloromercuriphenyl sulfonate (PCMPS) and then chromatographed on a thin layer silica gel plate. The solvent system was 1-butanol, methanol, and concentrated NH_4OH in the volume ratio of 7:2:1. The components were detected by exposing the plate to iodine vapor (Chart I).

Chart I

Samples	R_f of spots detected
PCMPS	0.08
PCMPS + DDT (ox)	0.08, 0.81
DTT (ox)	0.79
PCMPS + DTT (red)	0.09, 0.28, 0.80 (tr)
PCMPS + irradiated sample	0.06, 0.30, 0.78

Only reduced sulfhydryl groups are expected to react with *p*-chloromercuriphenyl sulfonate.



The irradiated sample showed spots of *p*-chloromercuriphenyl sulfonate, the mercuri complex of reduced dithiothreitol, and the product with similar R_f as the oxidized dithiothreitol.

Alternately the irradiated sample was chromatographed on a $CdSO_4$-treated silica gel plate according to the procedure of Gascoigne and Radda.[9] Two solvent systems were used (see Chart II): (a) 1-butanol–NH_4OH (10:1, v/v), (b) chloroform–methanol (3:2, v/v). The plates were developed in iodine vapor. Again the irradiated sample showed only the original compound and one product corresponding to the R_f of the oxidized dithiothreitol.

Chart II

Solvent system	Samples	R_f of detected spots
a	DTT (ox)	0.68
	DTT (red)	0.04
	Irradiated sample	0.05, 0.67
b	DDT (ox)	0.87
	DDT (red)	0.10
	Irradiated sample	0.13, 0.85

The absorption spectrum of the cyclic monomer of the oxidized dithiothreitol has a maximum at 283 nm, with a molar extinction coefficient $\epsilon = 273$ M^{-1} cm^{-1}. For quantitative evaluation of the product formation at this wavelength, a molar extinction coefficient $\epsilon = 283$ M^{-1} cm^{-1} was used, which takes into account absorbancy changes due to disappearance of reduced dithiothreitol. G(II) = 3.20 ± 0.10 molecules/100 eV, calculated from several experimental runs, implies a G value of 6.4 for dithiothreitol radical formation. This high G value for dithiothreitol radical (due to high solute concentration, *e.g.*, 2×10^{-2} M N_2O) is in close agreement with earlier reports.[10,11]

(9) I. M. Gascoigne and G. K. Radda, *Biochim. Biophys. Acta*, 131, 498 (1967).

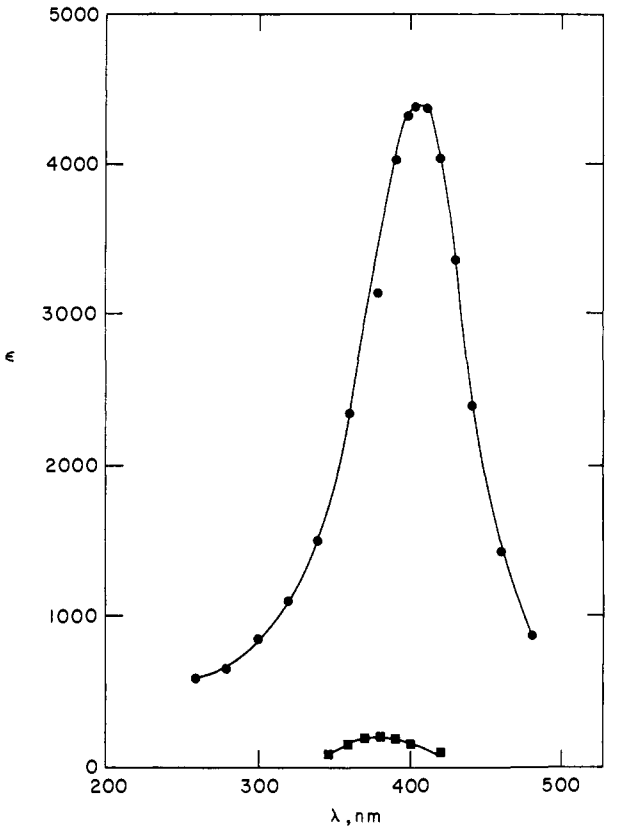


Figure 1. Absorption spectra of dithiothreitol free radical: (●) pH 7.9, (■) pH 2.1.

A nitrous oxide saturated solution of 1 mM reduced dithiothreitol was irradiated in the ^{60}Co source for different time intervals, and the N_2 and H_2 produced were determined by gas chromatography. The following values were found: $G(H_2) = 0.93 \pm 0.05$, and $G(N_2) = 3.10 \pm 0.10$. Although the true molecular yield for hydrogen $G(H_2)$ is not known for this system (it is a function of both N_2O and dithiothreitol concentration), the observed yield of total hydrogen production $G(H_2) = 0.93$ suggests direct reaction of H with dithiothreitol as shown in reaction 3. Hence taking $G_H = 0.55$[12] and $G_{OH} = 2.74$,[12] the G for dithiothreitol radical formation is

$$G(\mathrm{I}) = G(\mathrm{H}) + G(N_2) + G(\mathrm{OH}) = 0.55 + 3.10 + 2.74 = 6.39$$

a value in good agreement with the above value for product formation, $G(\mathrm{II}) = {}^1/_2 G(\mathrm{I})$.

Pulse Radiolysis Studies. The absorption spectra of the dithiothreitol radical at pH 7.9 and 2.1 are shown in Figure 1. They represent the molar absorbance as a function of wavelength at the end of a 5-μsec pulse. The optical absorbance per unit of absorbed energy (OD/rad) at the end of the pulse in 5 mM dithiothreitol saturated with nitrous oxide was constant up to 2.7 krads/pulse. The individual experimental points

(10) E. J. Land and A. J. Swallow, *Arch. Biochem. Biophys.*, 145, 365 (1971).
(11) B. H. J. Bielski, D. Comstock, and R. A. Bowen, *J. Amer. Chem. Soc.*, 93, 5624 (1971).
(12) B. H. J. Bielski and A. O. Allen, *Int. J. Radiat. Phys. Chem.*, 1, 153 (1969).

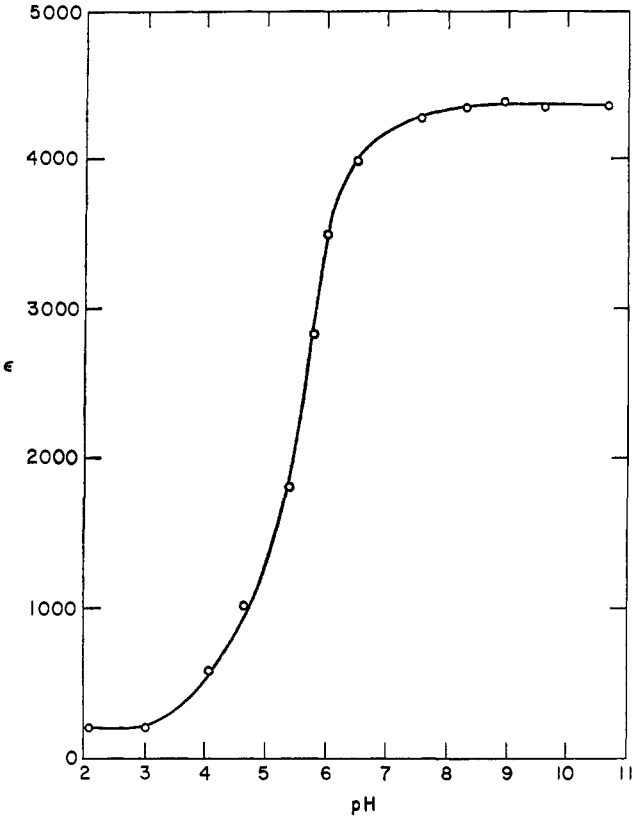


Figure 2. Molar extinction coefficients of dithiothreitol free radical at 405 nm as a function of pH at 23.5°.

were obtained by extrapolation of second-order decay plots to the end of the pulse. A study of the effect of dithiothreitol concentration (0.1 to 10 mM) upon the optical absorbance at the end of the pulse was carried out at pH 8.3. The observed optical density per unit absorbed energy at the end of the pulse was constant within experimental error over the entire concentration range studied. Consequently only one concentration of dithiothreitol, 5 mM, was used for determination of both spectra in Figure 1.

The energy input per pulse was kept constant during the determination of a particular spectrum (430 rads/pulse at pH 7.9 and 2.5 krads/pulse at pH 2.1). As can be seen in Figure 1, change in pH causes a blue shift of the absorption maximum from 405 to 380 nm. The associated change in molar extinction with pH at 405 nm (Figure 2) gives a pK of 5.5 for the dithiothreitol free radical.

A second-order decay of the dithiothreitol free radical was followed spectrophotometrically at 405 nm. As shown in Figure 3, strict second-order kinetics were obtained over many half-lives. The observed second-order decay constants, $k_{obsd} = (1/C - 1/C_0)/2t$, as a function of pH are given in Figure 4. The effect of dithiothreitol concentration upon the rate of decay of its free radical was studied at pH 8.3 over a concentration range from 0.1 to 10 mM at a constant energy input of 430 rads/pulse. The experiments showed no change (within experimental error) in the decay rate over the concentration range studied.

In order to determine if the radical is a neutral or a charged species, the rate of disappearance of the radical was studied in the presence of varying concentration of $NaClO_4$ up to 0.1 M at pH 9. Figure 5 shows that the

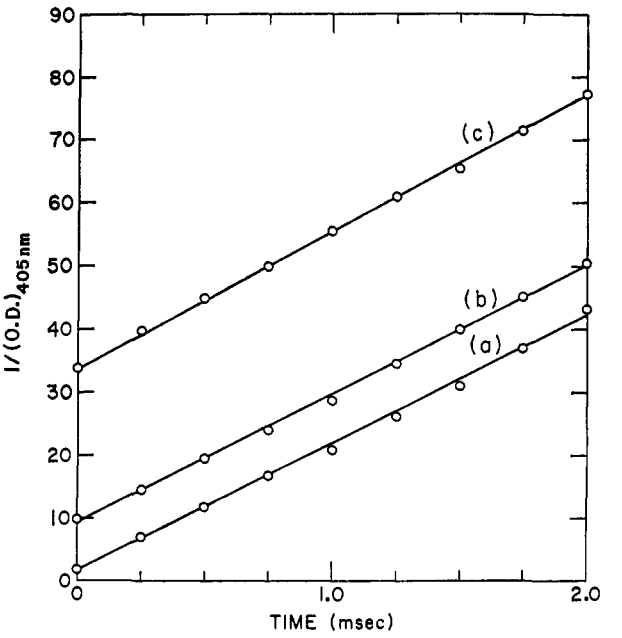
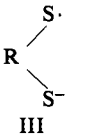


Figure 3. The second-order decay of dithiothreitol free radical at 23.5°, pH 8.3. Concentration of the radical at the end of a 10 μsec pulse of varying intensity: (a) 23.30 μM/3.90 krads; (b) 3.74 μM/0.56 krad; (c) 1.11 μM/0.166 krad.

change in ionic strength has an effect on k_{obsd}, which indicates that the free radical is monovalent. Consequently, I may be designated as the reacting species under these conditions (the data do not exclude an alternate structure III).



III

The rate of disappearance of dithiothreitol free radical in an air-saturated solution follows pseudo-first-order kinetics. The absorption spectrum of the product was identical with the spectrum observed in N_2O-saturated solutions. A systematic study of the rate of reaction of dithiothreitol free radical with molecular oxygen was carried out at pH 8.3. For a given experiment an air-saturated dithiothreitol solution (5 mM) was divided into two portions of which one was bubbled for 20 min with nitrous oxide. Then the air-saturated solution was diluted with the nitrous oxide saturated solution to the desired O_2 concentration. Pseudo-first-order rate constants for the disappearance of dithiothreitol free radical at 405 nm were obtained in solutions containing from 1.25×10^{-4} to 2.08×10^{-5} M oxygen (Table I).

Discussion

Earlier irradiation studies[13,14] on monothiol compounds have encountered complex mechanisms in radical formation and disappearance. The radical RS· tends to interact with the ionized species of the thiol compound

$$RS\cdot + RS^- \longrightarrow RSSR^-$$

(13) G. E. Adams, G. S. McNaughton, and B. D. Michael, "Energetics and Mechanisms in Radiation Biology," G. O. Phillips, Ed., Academic Press, New York, N. Y., 1968, pp 342–347.

(14) M. Simic and M. Z. Hoffman, *J. Amer. Chem. Soc.*, **92**, 6096 (1970).

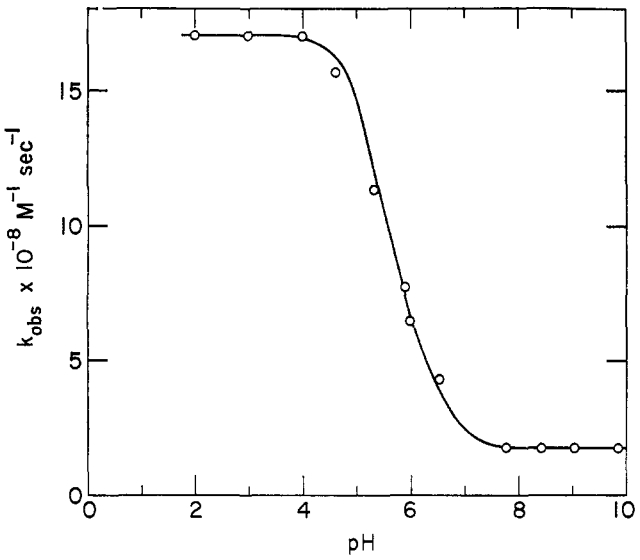


Figure 4. Second-order decay constants, k_{obsd}, for dithiothreitol radicals as a function of pH: (O) experimental rate constants; (——) curve computed from eq I.

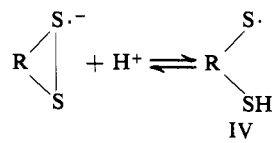
Table I. Rates of Disappearance of I in the Presence of Molecular Oxygen at pH 8.3 and 23.5°

O_2, M	k, sec^{-1}	k, $M^{-1}\ sec^{-1}$
2.08×10^{-5}	3.22×10^{4}	1.55×10^{9}
6.25×10^{-5}	7.92×10^{4}	1.27×10^{9}
1.25×10^{-4}	1.63×10^{5}	1.30×10^{9}

therefore, the rate of formation becomes dependent on both the solute concentration and the pH.

Cleland[2] has indicated that the structure of dithiothreitol is sterically favorable for a cyclic disulfide formation. Consequently, during the oxidation process it tends to form an intramolecular disulfide ring rather than to react intermolecularly to form dimers or polymers. This steric consideration is verified by our experimental results. First, both the formation and the rate of disappearance of the radical were found to be independent of dithiothreitol concentration. Second, the irradiation product was identified as the oxidized form of dithiothreitol by thin layer chromatography and by its absorption spectrum.

Changes in molar extinction at 405 nm with pH (Figure 2) suggest that the dithiothreitol free radical exists in two forms, as a negatively charged species I in the alkaline range and a neutral form IV in the acid range. Hence the determined pK value of 5.5 represents the equilibrium



(6)

IV

This pK value is similar to that of the lipoyl radical recently reported.[15]

The pH dependence of the dithiothreitol free radical

(15) M. Z. Hoffman and E. Hayon, *J. Amer. Chem. Soc.*, **94**, 7951 (1972).

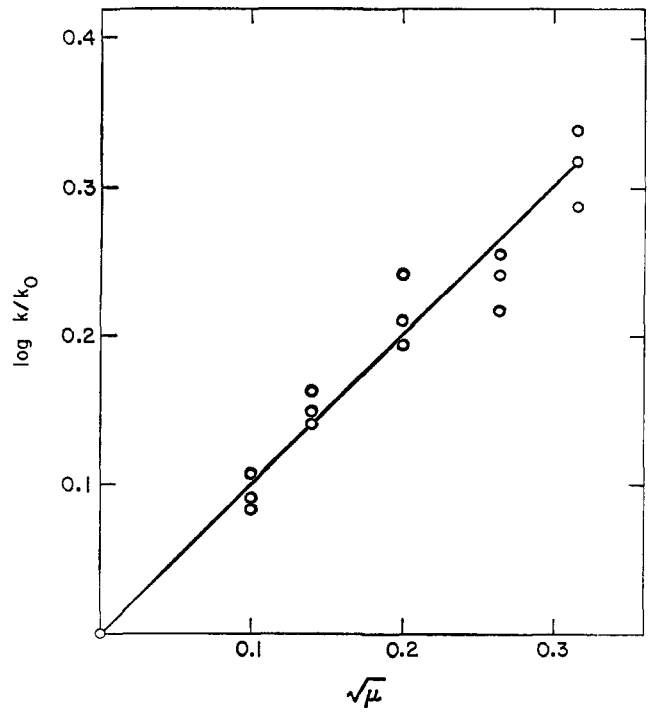


Figure 5. The effect of ionic strength on the rate of disappearance of dithiothreitol radicals. The solutions contained 5 mM dithiothreitol and varying amounts of sodium perchlorate, nitrous oxide saturated, adjusted to pH 9 with NaOH. The solid line indicates a slope of 1.0.

decay by second order can be explained by the following mechanism

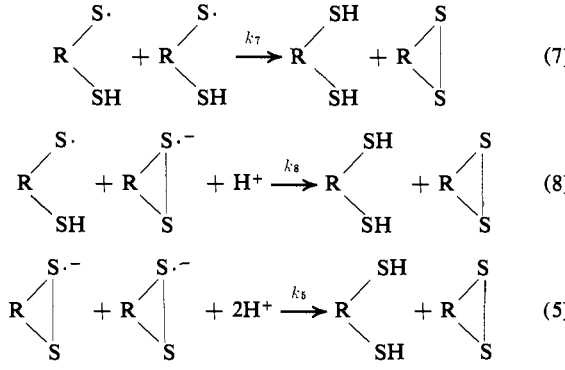


Table II. Rate and Equilibrium Constants for the Dithiothreitol Free Radical at 23.5°

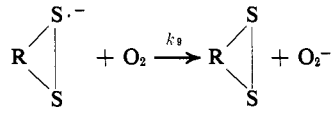
$k_7 = (1.70 \pm 0.28) \times 10^{9}\ M^{-1}\ sec^{-1}$
$k_8 = (2.50 \pm 0.26) \times 10^{9}\ M^{-1}\ sec^{-1}$
$k_5 = (1.70 \pm 0.21) \times 10^{8}\ M^{-1}\ sec^{-1}$
$K = 3.17 \times 10^{-6}$

The overall rate of decay in the pH range studied (2.0 to 10.5) is given by the equation

$$k_{obsd} = \frac{k_7 + k_8(K/[H^+]) + k_5(K/[H^+])^2}{(1 + (K/[H^+]))^2} \qquad (I)$$

which was used to compute the solid line in Figure 4. The best values of the rate and equilibrium constants are given in Table II.

The faster rate of disappearance of dithiothreitol free radical in the presence of molecular oxygen can be represented by eq 9 and 10, where $k_9 = (1.37 \pm 0.15) \times 10^9\ M^{-1}\ \text{sec}^{-1}$ and $k_{10} < 10^2\ M^{-1}\ \text{sec}^{-1}$.[16,17]



(9)

$$O_2^- + O_2^- + 2H^+ \xrightarrow{k_{10}} O_2 + H_2O_2 \qquad (10)$$

Acknowledgment. We wish to thank Dr. A. O. Allen for his constructive criticism and for having read the manuscript. Thanks are also due to Mr. D. Comstock for his excellent technical assistance.

(16) J. Rabani and S. O. Nielsen, *J. Phys. Chem.*, 73, 3736 (1969).
(17) D. Behar, G. Czapski, L. M. Dorfman, J. Rabani, and H. A. Schwarz, *J. Phys. Chem.*, 74, 3209 (1970).

A Novel Fluorescent Probe. Use of Time-Correlated Fluorescence to Explore the Properties of Micelle-Forming Detergent[1]

Richard R. Hautala, Neil E. Schore, and Nicholas J. Turro*

Contribution from the Department of Chemistry, Columbia University, New York, New York 10027. *Received December 20, 1972*

Abstract: A technique is described which utilizes the fluorescence lifetime of naphthalene as a probe for solutions containing micelle-forming detergents. The processes of micellar catalysis and inhibition have been directly observed using the probe. A direct measure of the partitioning of naphthalene between the micelle and aqueous phases has been made and the results are compared with a simple model treating the micellar system as a simple two-phase distribution. The naphthalene probe provides important information concerning the solvent properties and the effective local oxygen concentration of the micelle phase for several detergents. A comparison is made with other types of fluorescence probes commonly used in systems of biological interest.

One of the most fascinating properties of biological systems is their ability to achieve chemical efficiency and specificity by means of organization of reactants.[2] Recently, attempts to map out the distinct microenvironments of biological macromolecules, such as proteins and polynucleotides, have been the subject of intensive research. In particular, investigations of organized multimolecular aggregates such as membranes have received special attention.[3] Fluorescent probes[4] have been elegantly and effectively employed to study the structure of such organized systems. The general idea behind a fluorescent probe is that a molecule, whose fluorescent emission can serve as a sensor of microenvironments, will display a specific affinity for a given site (*e.g.*, the active site of an enzyme relative to bulk solution) *and* will also display *distinct* fluorescent properties which uniquely characterize each environment. In practice a common technique is to employ a probe which is highly fluorescent in apolar environments and is virtually nonfluorescent in water.[4]

Thus, the fluorescent probe technique usually depends on the assumption and hope that any observed fluorescence arises predominately from probe molecules that are bound or associated with the apolar regions of the macromolecules. A decided disadvantage of the fluorecence probe technique is the empirical finding that relatively large and highly polar (or ionic) molecules are required in order to observe a sensitivity of fluorescent properties to medium polarity. Clearly, a probe which has considerable molecular size and is inherently polar is very likely to cause significant perturbations of the very apolar regions which are being explored. Furthermore, one can imagine complications in an analysis due to solvent reorientation at the local environment of the probe upon electronic excitation (indeed, large spectral shifts of emission relative to absorption are a common feature of the commonly employed fluorescence probes). It is quite possible, therefore, that time-dependent fluorescent properties can severely complicate and even vitiate interpretations of results on both a quantitative and qualitative level.[5]

We report here the use of a fluorescent probe that is based on precise measurements of fluorescence decay constants which allows a distinction between two or more different environments available to the probe. The specific method described here is the time-correlated fluorescence of naphthalene dissolved in aque-

(1) The authors thank the Air Force Office of Scientific Research (Grant AFOSR-70-1848) and the National Science Foundation (Grant NSF-GP-26602X) for their generous support of this research.
(2) M. Dixon and E. C. Webb, "Enzymes," Longmans, London, 1964.
(3) (a) D. Chapman, Ed., "Biological Membranes," Academic Press, New York, N. Y., 1968; F. Snell, J. Wolken, G. Iverson, and J. Lam, Ed., "Physical Principles of Biological Membranes," Gordon and Breach, New York, N. Y., 1970; (b) E. Sackmann and H. Trauble, *J. Amer. Chem. Soc.*, **94**, 4482, 4492, 4499 (1972), and references therein.
(4) Review: G. M. Edelman and W. O. McClure, *Accounts Chem. Res.*, 1, 65 (1968); L. Brand and J. R. Gohlke, *Annu. Rev. Biochem.*, 41, 843 (1972).
(5) For typical examples concerning a variety of systems employing fluorescent probes see: (a) I. Tasaki, A. Watanabe, and M. Hallett, *Proc. Nat. Acad. Sci. U. S.*, **68**, 938 (1971); (b) B. Rubalcava, D. Martinez de Munoz, and C. Gitler, *Biochemistry*, **8**, 2742 (1969); (c) A. S. Waggoner and L. Stryer, *Proc. Nat. Acad. Sci. U. S.*, **67**, 579 (1970); (d) C.-G. Rosen and G. Weber, *Biochemistry*, **8**, 3915 (1969); (e) L. Stryer, *Science*, **162**, 526 (1968); (f) J. Schlessinger and I. Z. Steinberg, *Proc. Nat. Acad. Sci. U. S.*, **69**, 769 (1972).