PICOSECOND SPECTROSCOPY OF Cu(II) CYTOCHROME *C*

A. H. REYNOLDS
Bell Laboratories, Murray Hill, New Jersey

K. D. STRAUB
Veterans Administration Medical Center and University of Arkansas for Medical Sciences, Little Rock, Arkansas

P. M. RENTZEPIS
Bell Laboratories, Murray Hill, New Jersey

ABSTRACT We have observed a strong pH dependence in the relaxation rate of Cu(II) cytochrome *c* following excitation at 532 nm. At pH 8.0 the excited state relaxes with a lifetime of 10 ± 5 ps while at pH extremes of 2.5 and 13.0 we find that the lifetime becomes longer than 1 ns. This change of more than two orders of magnitude in the lifetime may be due to the Cu coordination number, which is six at neutral pH but five at pH extremes.

INTRODUCTION

The transfer of energy from the porphyrin ring system to other reaction centers is a central process in the sequence of events that allows useful chemical work to be done in many biological systems, including photosynthesis and oxidative phosphorylation. The role the protein plays in the transfer and dissipation of energy in hemoproteins is not yet understood. However, the relaxation of electronically excited metalloporphyrins has been widely studied by emission (1–6) and absorption (7,8) spectroscopy. In addition, theoretical studies have contributed considerably to the understanding of the mechanisms of radiative and nonradiative transitions (3,6,9,10).

The utilization of picosecond spectroscopy (11–13) has made it possible to monitor directly ultrafast relaxation of excited states and record the absorption spectra of the intermediates. These techniques have been able to verify and extend the studies on metalloporphyrin electronic relaxation (5,7,8,13). Thus, the iron porphyrin complexes in organic solvents that are known to be nonfluorescent and nonphosphorescent have been shown to relax to the ground state within 6 ps after excitation (7). When the iron porphyrin is the prosthetic group of a hemoprotein such as myoglobin or cytochrome *c*, the relaxation time was found to be faster than 6 ps. Therefore, to measure and assess the effect of the protein on the relaxation process of the hemoproteins, it is necessary to choose a metalloporphyrin for the prosthetic group that has a relatively slow relaxation to be time resolvable. We have shown that Cu(II) protoporphyrin IX dimethyl ester in benzene and glacial acetic acid relaxes to the ground state through the triplet state by the following mechanism: $^2S_1 \rightarrow {}^2T_1$ (ps); $^2T_1 \rightarrow {}^4T_1$ (450 ps); $^2T_1 \rightarrow {}^2S_0$, $^4T_1 \rightarrow {}^2S_0$ (1 ns) (14). Because the lifetime of the $^2T_1 \rightarrow {}^4T_1$ transition is within our experimental time range, we have used the copper derivative of cytochrome *c* (15) for studying the effect of protein on the electronic relaxation of hemoproteins.

MATERIALS AND METHODS

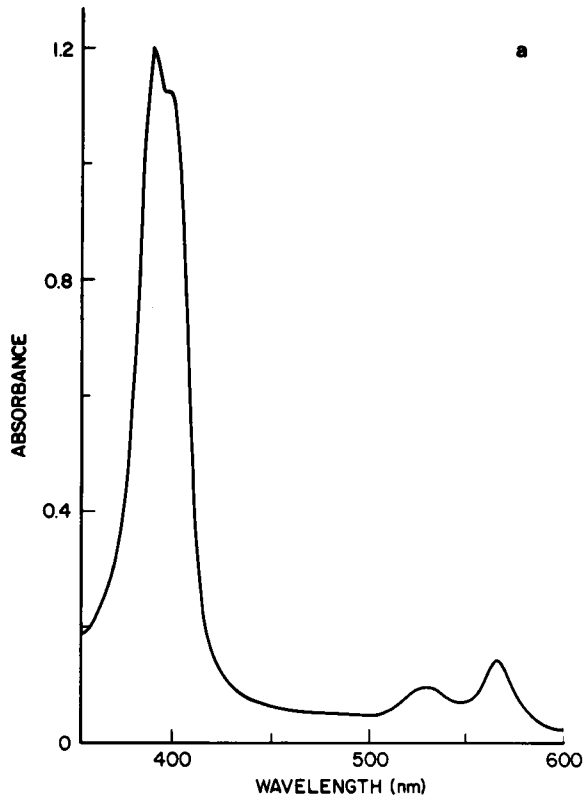
Cytochrome *c* and hemin were purchased from Sigma Chemical Co.,St. Louis, MO. All other chemicals were reagent grade. Cu(II) cytochrome *c* was prepared by the method of Vanderkooi et al. (16). All experiments discussed in this paper were performed in 0.01 M potassium phosphate at the indicated pH.

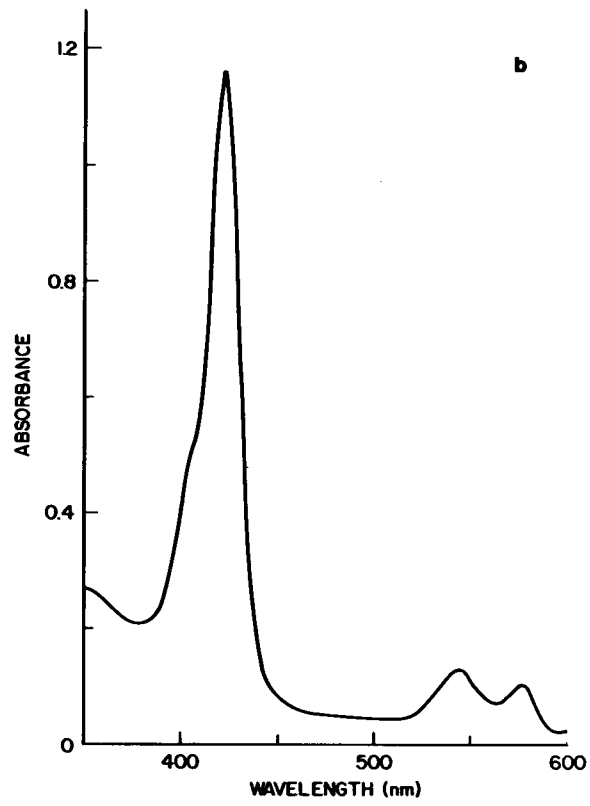
The final purification was done by chromatography on a CM Sephadex column 2 × 10 cc equilibrated with 0.05 M phosphate buffer and the Cu cytochrome *c* eluted with a 0–0.4 M NaCl linear gradient. The first peak at ~0.15 M NaCl was saved. This peak is the same as the Cu cytochrome *c* "A" peak described by Findlay et al. (15), which was eluted from Amberlite CG-50 ion exchange resin, Rohm and Haas Co., Philadelphia, PA and has characteristics of the native of Fe cytochrome *c*. From the persistent 405 shoulder on the Soret band at pH 8.0, we know that somewhat less than 10% of this preparation is in the Cu cytochrome *c* "B" form. All experiments were performed in 0.05 M potassium phosphate at indicated pH. Cu(II) protoporphyrin IX was prepared by removing the iron (Fe^{++}) from hemin and inserting Cupric ion after the method of Dorough (17). One preparation of metal-free protoporphyrin IX was made with HF for comparisons with the Cu cytochrome *c*.

Optical density at 500 mm was adjusted to 0.2–0.3 for excitation at 355, nm and for excitation at 530, the optical density at 570 nm was adjusted to ~1.6. Absorption spectra were recorded on an Aminco DW-2 (American Instrument Co., Silver Spring, MD) spectrophotometer.

The weighted-average molecular weight for the Cu(II) cytochrome *c* was determined at pH 2.5, 12.5, and 8.0 in 0.05 M PO_4 buffer by ultracentrifugation on a Model E Beckman analytical ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). For both velocity and sedimentation equilibrium, no evidence for dimerization was found at these pH values. A small amount of 6,000 MW component was determined on sedimentation equilibrium for all pH values.

The picosecond transient spectra were generated by a picosecond pulse emitted by a Nd^{3+}/YAG laser system (Quantel International, Santa Clara, CA), previously described by Reynolds et al. (22). In brief it consists of an Nd^{3+}/YAG oscillator emitting a train of 25 ps 1,064 nm pulses. A single pulse is extracted from the train, amplified, and converted





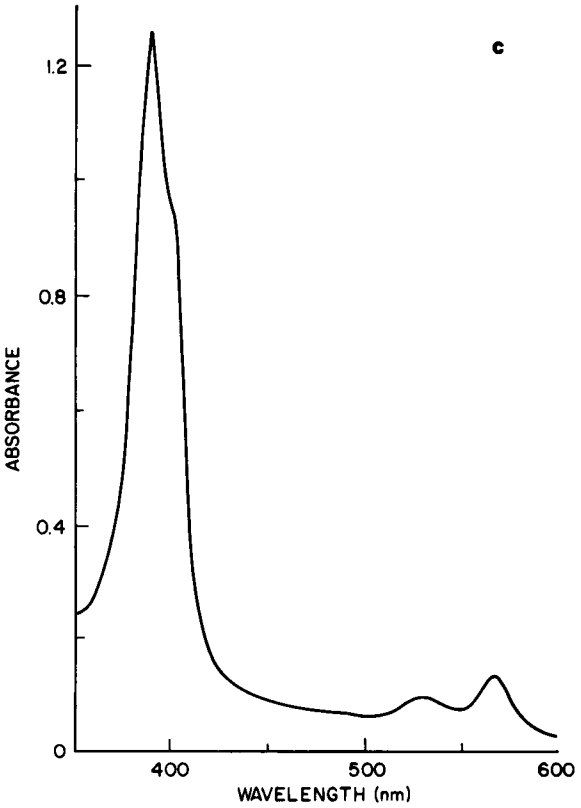


FIGURE 1 UV-visible absorption spectra for Cu(II) cytochrome *c* in 10 mM phosphate buffer, 290 K, at (*a*) pH 2.5, (*b*) pH 8.0, and (*c*) pH 13.0.

to 530 nm which is used for excitation. The remaining 1,064 nm light is amplified again and impinges on a cell of water where it generates a broad-band picosecond continuum that completely covers the 400–650 nm region under investigation. The excitation and interrogating beams are delayed relative to each other by changing the travel distance of the interrogating beam to the sample cell, relative to the excitation beam. The continuum is focused, after passing through the reaction cell, onto the slit of a 1/4 m monochromator and then onto the face of an intensified (Princeton Applied Research, Princeton, NJ) ISIT vidicon. An OMA2 is coupled to a Data General Eclipse S/130 mini computer (Data General Corp., Westboro, MA) for analysis and spectra display.

RESULTS

The visible absorption spectra of the Cu(II) cytochrome *c* at various pH values are shown in Fig. 1. As can be seen, the neutral pH range yields a Cu(II) porphyrin spectrum with inverted α—β band intensities. The α band is larger than the β band in Cu(II) protoporphyrin IX dimethyl ester in benzene and in Cu(II) protoporphyrin IX in 0.05 M phosphate buffer or pyridine. However, at pH 2.5 and 12.5 the Cu(II) cytochrome *c* spectra have unusual α:β ratios, and the Soret band is blue-shifted to a position closer to Cu protoporphyrin IX dimethyl ester in benzene. These spectra are identical with those reported for the "A" fraction of Cu(II) cytochrome *c* by Findlay et al. This fraction has been shown to be indistinguishable by electrophoresis from the native protein (15).

Picosecond spectra recorded at 30 and 80 ps after excitation with 530-nm 30-ps pulse for Cu(II) cytochrome *c* at several pH values are shown in Figs. 2, 3, 4, and 6. The difference spectra displayed in these figures result from

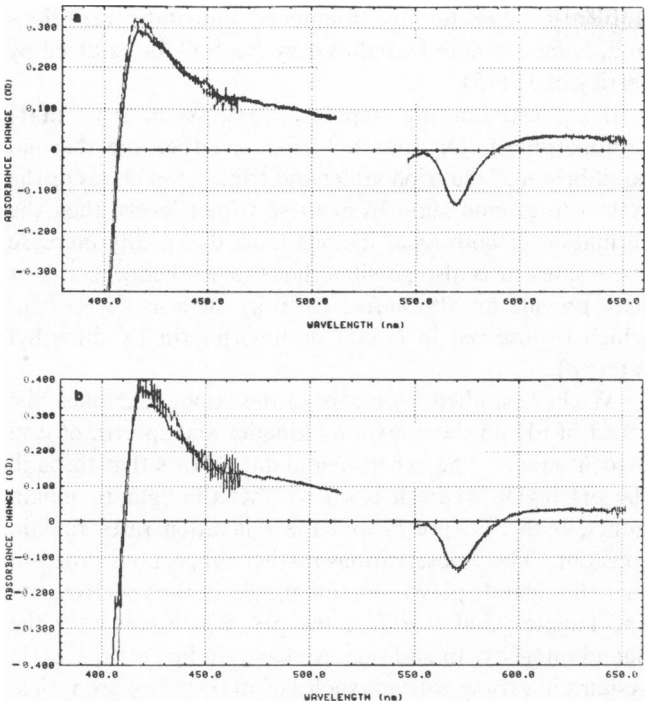


FIGURE 2 Transient absorption spectra at 30 ps (——). and 80 ps (· · ·) ps for Cu(II) cytochrome *c* at 290 K in 10 mm phosphate buffer at (*a*) pH 2.5 and (*b*) pH 13.0.

ground-state bleaching and excited-state absorption. Examination of Fig. 2 indicates that the spectra recorded at 30 and 80 ps after excitation at pH 2.5 and 13.0, show no measurable change over this time scale. However, at pH 8.0 (Figs. 3 and 4) we observe that the excited state band disappears between 30 and 80 ps. The kinetics at pH 8 show the presence of two components; a slow one, which may represent a 10% B species impurity, with a lifetime of at least a few hundred picoseconds, and the other, corresponding to the A fraction, which is responsible for the faster decay lifetime of 10±5 ps. The kinetics for the fast process are shown in Fig. 5. In the spectral range of 450–650 nm the spectra of two species are mixed, resulting in the complex difference spectra presented in Fig. 4*a*. To resolve the spectra of each component the long-lived component as represented by the 100 ps difference spectrum was subtracted allowing the presence of a homogeneous short-lived species to be evident as shown in Fig. 4*b*. The spectra at pH 3.5 and 11.0 qualitatively appear to be combinations of the pH 8.0 and either 2.5 or 13.0 spectra respectively (Fig. 5).

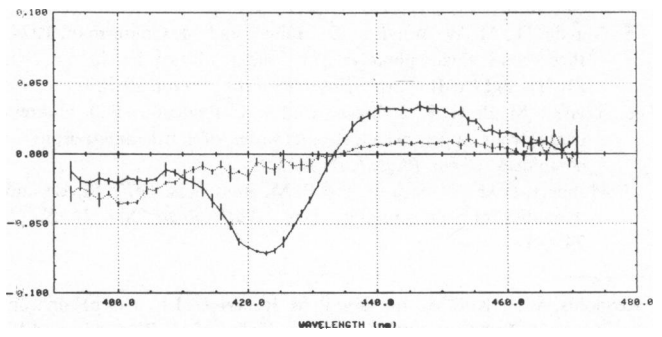


FIGURE 3 Transient absorption spectra in the soret region at 15 ps (——) and 65 ps (· · ·) for Cu(II) cytochrome *c* at 290 K and pH 8.0.

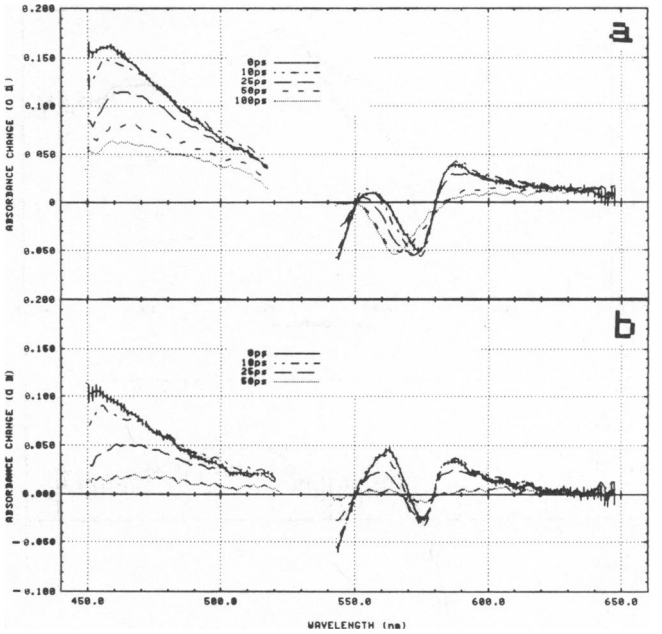


FIGURE 4 (*a*) Transient absorption spectra in the α—β band region for Cu(II) cytochrome *c* at pH 8.0. (*b*) Spectra of (*a*) with the 100 ps spectrum subtracted to reveal the transient spectra of short-lived component.

DISCUSSION

The dependence of Cu(II) cytochrome *c* spectrum on pH is very similar to the behavior of Fe(III) cytochrome *c* previously observed (19,20). It is expected that the acidic form would correspond to a five ligand complex of Cu(II)

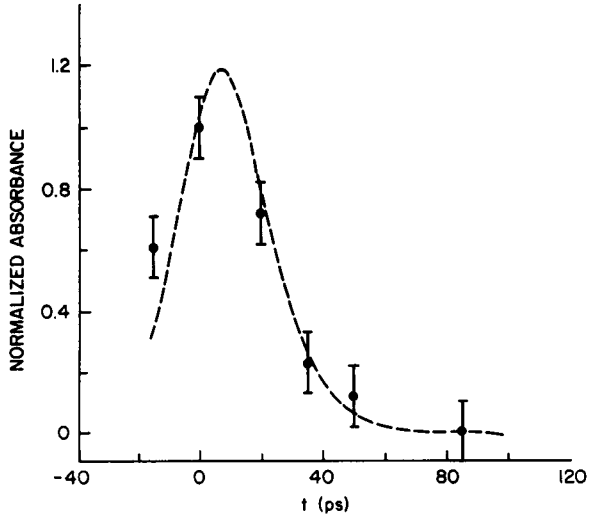


FIGURE 5 Kinetics of the fast component of Cu(II) cytochrome *c* at pH 8.0., 290 K, $\tau \sim 10 \pm 5$ ps.

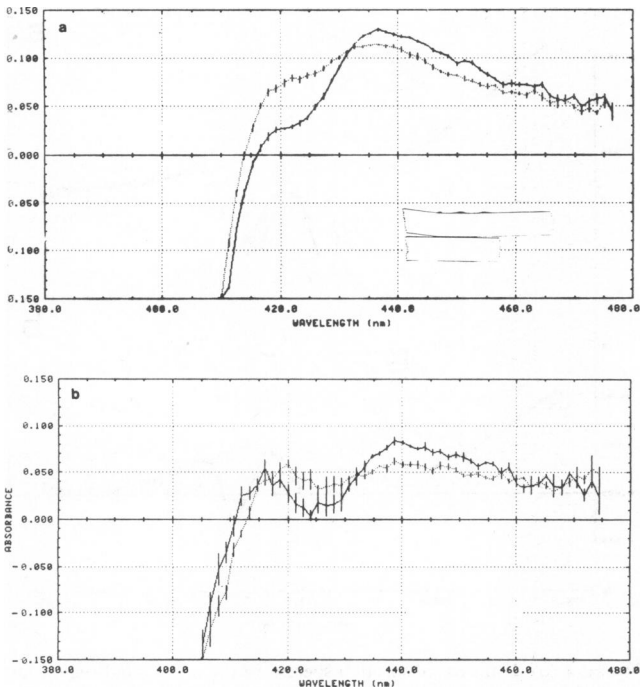


FIGURE 6 Transient absorption spectra for Cu(II) cytochrome *c* at 30 ps (——) and 60 ps (· · ·) for (*a*) pH 3.5 and (*b*) pH 11.8.

porphyrin with methionine sulfur (20) in one axial position. As the pH increases, an imidazole nitrogen from histidine takes the sixth ligand position and the Cu(II) cytochrome *c* has an octahedral metalloporphyrin configuration (15). At pH 13.0 the methionine ligand is removed, again leaving a five-ligand Cu(II) porphyrin complex. The five-coordinated form of Cu(II) cytochrome *c* has a spectrum similar to the square-planar Cu(II) protoporphyrin dimethyl ester in organic solvents, with similar Soret band maxima and similar α and β band maxima and intensities. The octahedral complex indicates significant electronic rearrangement compared with the five-coordinated forms, with shifts in absorption maxima and reversal of the α:β ratio.

The fact that the relaxation of the excited state takes place within picoseconds indicates that the pathways for electronic relaxations are different for the octahedral complex and five-coordinated form. Glatz et al. (21) have obtained luminescent spectra of Cu(II) cytochrome *c* at pH 7.4 50% glycerol at low temperatures and found that the phosphorescent lifetimes differed from free Cu(II) porphyrins by one to two orders of magnitude. The picosecond relaxation data shows that Cu(II) cytochrome *c* at pH 8.0 has an excited state lifetime of 10 ps while Cu(II) protoporphyrin IX dimethyl ester in benzene has a lifetime of at least 450 ps. It appears, therefore, that the octahedral Cu(II) protoporphyrin IX configuration with imidazole nitrogen and methionine sulfur in cytochrome *c* provides a very fast relaxation pathway for electronic relaxation. This enhancement of the relaxation rate cannot be due to increased intersystem crossing secondary to changes in spin state because Cu(II) does not have low enough unfilled orbitals for ligand-induced spin-state transitions and, in fact, cannot be reduced by $Na_2S_2O_4$ or oxidized by ferricyanide (15).

If the rate-limiting step for relaxation of the Cu(II) protoporphyrin IX dimethyl ester is either the thermal equilibrium of the tripdoublet and tripquartet states or the return to ground state from these triplet levels, then the formation of both axial ligands must drastically increase these rates. It is also possible that the octahedral complex may provide an alternative pathway such as $^2S_1 \rightarrow {}^2S_{dz^2}$, which is observed in Ni(II) protoporphyrin IX dimethyl ester (8).

We have studied, by means of the same techniques, the effect of pH on the relaxation kinetics and spectra of iron cytochrome *c*.[1] The experimental data shows that through the pH region where it is known that the ligands remain intact, both the spectra and the relaxation rates remain constant.[1] These observations further support our proposal that the ligands play a dominant role in the spectroscopy and kinetics, and therefore the pH, which can alter the ligand integrity. In addition, we have studied other Cu(II) systems in various solvents such as Cu(II) protoporphyrins[1] and the data further support the results presented for Cu(II) cytochrome *c*. Yet, we do not suggest that this proposal should find a general application to biological systems. However, we expect that these results and the application of this method would provide a means for extending our understanding of energy (electron) transfer.

Received for publication 14 January 1982 and in revised form 25 March 1982.

REFERENCES

1. Allison, J. B., and R. S. Becker.1960. Effect of metal atom perturbations on the luminescent spectra of porphyrins. *J. Chem. Phys.* 32:1410.
2. Becker, R. S., and M. Kasha. 1955. Luminescence spectroscopy of porphyrin-like molecules including the chlorophylls. *J. Am. Chem. Soc.* 77:3669.
3. Eastwood, D., and M. Gouterman. 1969. Porphyrins XII. Luminescence of copper complexes at liquid nitrogen temperature. *J. Mol. Spectrosc.* 30:437.
4. Hopf, F. R., and D. G. Whitten. 1972. Photochemistry of porphyrins and metalloporphyrins. *In* Porphyrins and Metalloporphyrins. K. M. Smith, editor. Elsevier, New York, 667.
5. Magde, D., M. W. Windsor, D. Holten, and M. Gouterman. 1974. Picosecond flash photolysis: transient absorption in Sn(IV), Pb(II), and Cu(II) porphyrins. *Chem. Phys. Lett.* 29:183.
6. Tsvirko, M. P., G. F. Stelmakh, and V. E. Pyatosin. 1980. Fluorescence from upper psi electronic states of lanthanide-porphyrin complexes. *Chem. Phys. Lett.* 273:80.
7. Huppert, D., K. D. Straub, and P. M. Rentzepis. 1977. Picosecond dynamics of iron proteins. *Proc. Natl. Acad. Sci. U. S. A.* 74:4139.

[1]Reynolds, A. H., K. D. Straub, and P. M. Rentzepis. Effect of pH on iron cytochrome *c*. To be submitted. Straub, K. D., A. H. Reynolds, and P. M. Rentzepis. Solvent effects on Cu(II) protoporphyrin. To be submitted.

8. Kobayashi, T., K. D. Straub, and P. M. Rentzepis. 1979. Energy relaxation of mechanism in NI(II), Pd(II), Pt(II) and Zn(II). *Photochem. Photobiol.* 229:925.

9. Ake, R. L., and M. Gouterman.1969. Theory for the luminescent state in VO, Co, Cu complexes. *Theor. Chim. Acta.* 15:20.

10. Gouterman, M., F. P. Schwarz, P. D. Smith, and D. Dolphin. 1973. Porphyrins XXVII. Spin-orbit coupling and luminescence in group (IV) complexes. *J. Chem. Phys.* 59:679.

11. Rentzepis, P. M. 1968. Direct measurements of radiationless transitions in liquids. *Chem. Lett.* 22:117.

12. Huppert, D., W. S. Struve, and P. M. Rentzepis.1975. Subpicosecond relaxation of localized electrons in liquid ammonia. *J. Chem. Phys.* 63:1205.

13. Busch, G. E., D. Huppert, and P. M. Rentzepis. 1976. Picosecond time resolved spectroscopy. *Proc. Soc. Photo-Optical Instr. Eng.* 82:80.

14. Kobayashi, T., D. Huppert, K. D. Straub, and P. M. Rentzepis. 1979. Picosecond kinetics of copper and silver protoporphyrins. *J. Chem. Phys.* 70:1720.

15. Findlay, M. C., L. C. Dickinson, and J. C. W. Chien. 1977. Copper cytochrome *c*. *J. Am. Chem. Soc.* 99:5168.

16. Vanderkooi, J. M., B. Chance, and A. Waring. 1978. Luminescence of Cu-cytochrome *c*. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 88:273.

17. Dorough, G. D., J. R. Miller, and F. M. Huennekens.1951. Spectra of the metallo-derivatives of α, β, γ, δ-tetraphenylporphine. *J. Am. Chem. Soc.* 73:4315.

18. Reynolds, A. H., and P. M. Rentzepis. 1982. Kinetics and temperature dependence of carboxymyoglobin-ligand photodissociation. *Biophys. J.* 38:15–18.

19. Theorell, H., and A. Akesson. 1941. Studies on cytochrome *c*. II. The optical properties of pure cytochrome *c* and some of its derivatives. *J. Am. Chem. Soc.* 63:1812.

20. Boeri, E., A. Ehrenberg, K. G. Paul, and H. Theorell. 1953. On the compounds of ferricytochrome *c* appearing in acid solution. *Biochem. Biophys. Acta.* 12:273.

21. Glatz, P., B. Chance, and J. M. Vanderkooi.1979. Microseconds luminescence emission from copper cytochrome *c*. *Biochemistry.* 18:3466.