

Solvent Effects on Flavin Electron Transfer Reactions[†]

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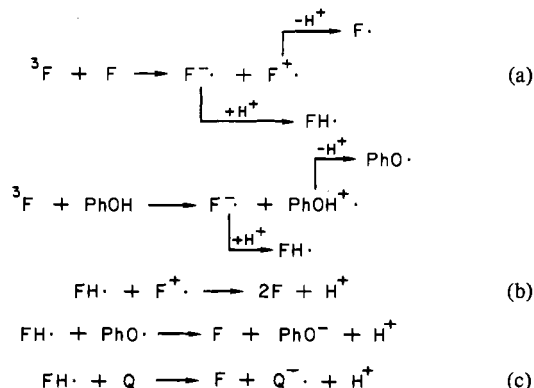
ABSTRACT: The effects of solvent environment on the rates of several flavin redox reactions have been studied by using laser flash photolysis. These include electron transfer to the flavin triplet state (a measure of oxidized flavin electrophilicity) and the oxidation of flavin semiquinone by oxidized flavin radical, oxidized phenol radical, and quinone. The rate constant for triplet quenching by 2,6-dimethylphenol was found to be proportional to the inverse of solvent viscosity, as would be expected for a diffusional process. The flavin semiquinone yield due to flavin reduction during the quenching reaction was linearly dependent on the solvent dielectric constant. This implies the existence of a polar or charged intermediate along the reaction pathway. A similar effect of solvent dielectric was found for the self-quenching reaction, which produces

semiquinone and an oxidized flavin radical. The rate constants for all three of the semiquinone oxidation reactions studied were found to exhibit a biphasic dependence on solvent dielectric, being virtually independent of dielectric at low values and sharply increasing at high values. This is interpreted in terms of a change in mechanism with solvent polarity. Specifically, we propose a neutral transition state and hydrogen atom transfer in low dielectric media and a dipolar transition state and electron transfer in high dielectric media. No specific effects of hydrogen-bonding interaction between flavin and solvent were observed for any of the processes studied. The mechanistic implications of these results for flavoenzyme catalysts are discussed.

The molecular environment of the flavin isoalloxazine ring in many flavoenzymes is generally thought to include both hydrogen-bonding and nonpolar interactions [see, for example, Edmonson & Tollin (1971), Mayhew & Ludwig (1975); and Yagi et al. (1980)]. This has been confirmed for at least one group of flavoproteins, the flavodoxins, by high-resolution X-ray crystal structure analysis (Mayhew & Ludwig, 1975; Smith et al., 1977; Watenpaugh et al., 1973). It is thus of great interest to inquire into the effects of such environmental factors on the chemical reactivity of the flavin molecule. Recent experimental and theoretical studies (Yagi et al., 1980; Nishimoto et al., 1978, 1980) have suggested that hydrogen bonding can have an important influence upon flavin electron transfer properties. Although Yagi et al. (1980) have reported a 50–60% increase in the rate of steady-state photoreduction of flavins by a substituted ethylenediamine in an aprotic solvent upon the addition of hydrogen-bonding substances, no systematic studies of environmental effects upon redox reactions have been reported. The present work was undertaken in an attempt to remedy this situation.

We have utilized laser flash photolysis techniques (Tollin et al., 1979) to investigate the solvent dependence of the following reactions (Scheme I). (a) The first reaction is electron transfer to the flavin triplet state (³F) from oxidized flavin (F) (self-quenching) and from 2,5-dimethylphenol (external quenching). These are measured by triplet-state quenching constants (*k_q*) and formation of flavin semiquinone (FH•). On the basis of theoretical considerations, Song and co-workers (Sun & Song, 1973; Song et al., 1976) have concluded that the reactivity of ³F is a measure of the electrophilicity of the ground state. (b) The second is electron transfer from FH• to either the oxidized flavin radical (Ballard et al., 1976) or the oxidized phenol radical which is produced by the above-mentioned electron transfer quenching of the triplet. Previous studies (Vaish & Tollin, 1971) have shown that these reverse electron transfer reactions compete favorably with radical disproportionation. (c) The third is electron

Scheme I: Triplet Quenching and Electron Transfer Reactions of Flavins^a

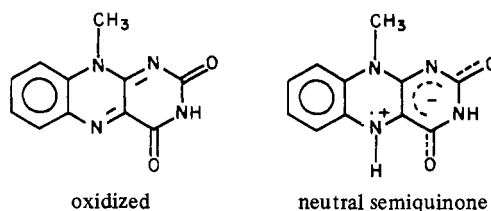


^a For convenience, reactions are written as e⁻ transfer, followed by protein equilibration. However, H• transfer may also occur, especially in low polarity, aprotic solvents (see text).

transfer from FH• to added 2,5-dichlorobenzoquinone. As will be documented below, although appreciable solvent effects were observed with these reactions, the correlations appear to involve viscosity and the dielectric constant rather than hydrogen-bonding capability. The solvent polarity effects suggest a possible mechanism for protein control of flavin catalysis.

Materials and Methods

10-Methylisoalloxazine was prepared as previously described



(Guzzo & Tollin, 1963). 2,6-Dimethylphenol and 2,5-dichloro-*p*-benzoquinone were obtained from Eastman Organic Chemicals. AR-grade solvents (Fisher Scientific Co. and Mallinckrodt Chemical Co.) were further purified by vacuum

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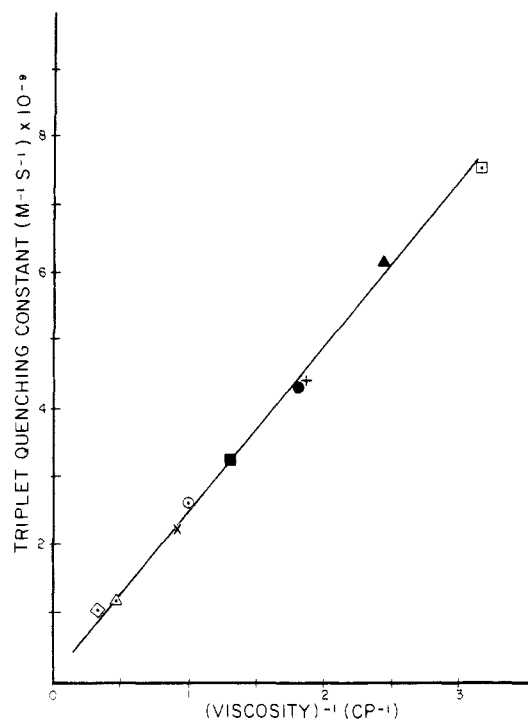


FIGURE 1: Dependence of flavin triplet state quenching by 2,6-dimethylphenol upon solvent viscosity. (○) Water; (●) methanol; (×) ethanol; (Δ) 1-propanol; (◇) 1-butanol; (□) acetone; (▲) dichloromethane; (■) dichloroethane; (+) chloroform.

distillation and stored over 3-Å molecular sieves to reduce water content. A 0.005 M sodium acetate-acetic acid buffer, pH 5.0, was used for reactions in aqueous media.

Samples containing 4×10^{-5} M 10-methylisoalloxazine and appropriate concentrations of the substrate (phenol or both phenol and quinone) dissolved in the solvent were placed in a 1 cm path-length cuvette, deoxygenated by bubbling with nitrogen gas for 15–20 min, and flash photolyzed. The flash photolysis experiments were conducted with a pulsed nitrogen laser pumped dye laser as previously described (Tollin et al., 1979). All samples were prepared in minimal light. Photolysis experiments were carried out at room temperature.

Concentrations of the flavin triplet and the neutral semiquinone radical were determined from their respective ab-

sorptions at 665 and 560 nm extrapolated to zero time after the flash by using the following values for the molar absorptivities: $\epsilon_{665}(^3F) = 4600 \text{ M}^{-1} \text{ cm}^{-1}$ (Knowles & Roe, 1968); $\epsilon_{560}(FH\cdot) = 4700 \text{ M}^{-1} \text{ cm}^{-1}$ (Land & Swallow, 1969). The values used for the solvent dielectric constants and viscosities are those reported in the *Handbook of Chemistry and Physics* (1980). Some dielectric constant values were obtained from the *Tables of Dielectric Constants of Pure Liquids* (1951).

Results and Discussion

Laser flash photolysis of 10-methylisoalloxazine in various solvents results in the formation of the flavin triplet state. At the flavin concentrations used (4×10^{-5} M), this species decays by pseudo-first-order kinetics due to self-quenching, i.e., 3F plus F to give flavin semiquinone and an oxidized flavin radical (Vaish & Tollin, 1970; Ballard et al., 1976). The structure of this oxidized radical has not been determined. The triplet yields, obtained by extrapolating the decay curves to zero time after the flash, were found to be essentially the same ($\pm 10\%$) in all of the solvents used. The triplet lifetimes varied as expected depending upon the solvent characteristics. The flavin triplet was further quenched in all solvents upon adding 2,6-dimethylphenol (Vaish & Tollin, 1971) which competes with ground-state flavin. The rate constants for triplet quenching by the phenol (k_q), determined at room temperature from Stern-Volmer plots (not shown), are reported in Table I. The values range from $1.0 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (1-butanol) to $7.6 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (acetone) and are dependent upon solvent viscosity (Figure 1). Thus, no specific solvent effects upon the second-order rate constants appear to be occurring beyond those related to the rates of solute diffusion.

The flavin semiquinone concentrations at zero time after the flash in the absence of quencher in various solvents were determined by extrapolating the second-order plots of the decay curves. These were found to be directly proportional to the solvent dielectric constant (Figure 2A). The same relationship (Figure 2B) was observed for the semiquinone concentrations obtained at 50% quenching of the triplet by 2,6-dimethylphenol. The latter values were determined from a plot of the concentration of $FH\cdot$ vs. $1 - \tau/\tau_0$ (the fraction of triplet quenched at various phenol concentrations). This value is used so as to correct for the solvent dependence of k_q . We interpret the results shown in Figure 2A,B as strong evidence that triplet

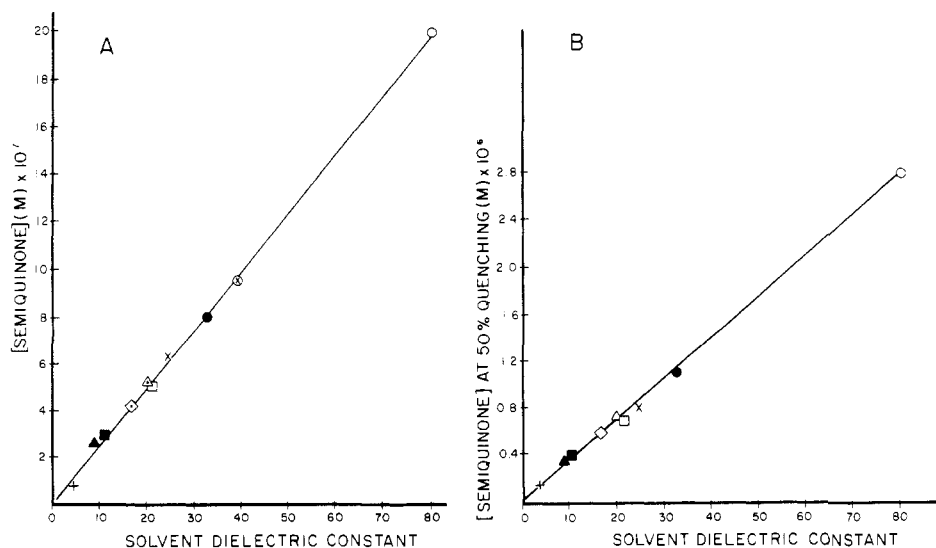


FIGURE 2: (A) Dependence of flavin semiquinone concentration at zero time after the flash upon the solvent dielectric constant. No added quencher. (⊙) Acetonitrile. All other symbols are as in Figure 1. (B) Dependence of flavin semiquinone concentration at zero time after the flash upon the solvent dielectric constant. Quencher was 2,6-dimethylphenol at a concentration which produced 50% quenching of the flavin triplet. Symbols are as in Figure 1.

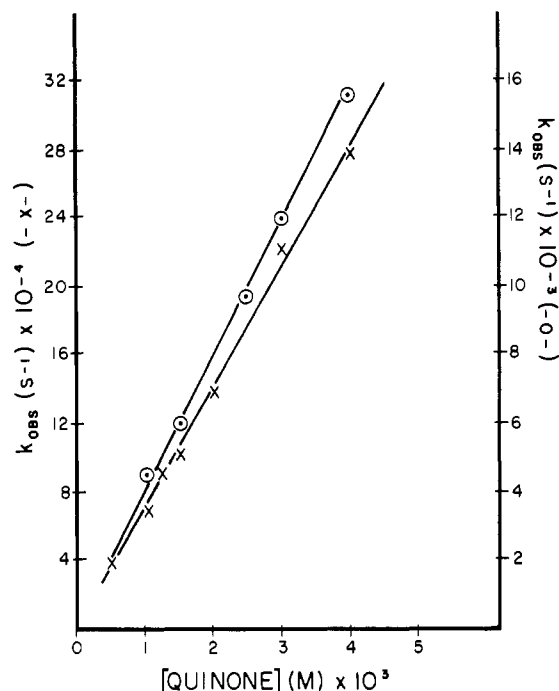


FIGURE 3: Plots of k_{obsd} for flavin semiquinone oxidation vs. 2,5-dichlorobenzoquinone concentration. (O) Methanol; (X) methanol-water (3:1).

reduction proceeds via a dipolar intermediate in all solvents [i.e., ${}^3F + AH \rightarrow (F^{\bullet} \cdots H \cdots A^{\bullet+})$]. The degree to which this collapses back to starting materials or proceeds to form separated radicals depends on the extent of solvent interaction. We, therefore, visualize the primary process as being basically electron transfer, with a variable degree of coupling to proton transfer. This is not unreasonable for an excited-state reaction. One would expect the electron affinity to be greatly increased over that of the ground electronic state because of the orbital vacancy created by the excitation process. Thus electron transfer should be the favored pathway compared with hydrogen atom transfer.

So that the influence of solvent properties on the reactivity of flavin semiquinone could be understood, second-order rate constants for the oxidation of this species by oxidized flavin radical, by oxidized phenol radical, and by 2,5-dichloro-*p*-benzoquinone have been determined in various solvents (Table

Table I: Second-Order Rate Constants^a for Triplet Quenching by 2,6-Dimethylphenol and for the Oxidation of Flavin Semiquinone by Oxidized Flavin Radical, Oxidized Phenol Radical, and 2,5-Dichlorobenzoquinone

solvent	k_q ($M^{-1} s^{-1}$)	k_2 ($M^{-1} s^{-1}$) (flavin alone)	k_2 ($M^{-1} s^{-1}$) (plus phenol)	k_2 ($M^{-1} s^{-1}$) (plus quinone)
water, pH 5.0	2.6×10^9	5.0×10^9	5.4×10^9	
acetonitrile		1.9×10^9	2.0×10^9	
methanol	4.3×10^9	1.6×10^9	1.7×10^9	4.0×10^6
ethanol	2.2×10^9	5.4×10^8	6.0×10^8	2.1×10^6
1-propanol	1.2×10^9	3.0×10^8	3.0×10^8	
1-butanol	1.0×10^9	2.2×10^8	2.2×10^8	9.9×10^5
acetone	7.6×10^9	7.1×10^8	7.1×10^8	
dichloroethane	3.2×10^9	3.3×10^8	3.2×10^8	
dichloromethane	6.3×10^9	3.2×10^8	3.1×10^8	8.2×10^5
chloroform	4.4×10^9	2.4×10^8	2.4×10^8	5.0×10^5
methanol-water (3:1)				3.5×10^7
methanol-water (1:1)				6.7×10^7

^a Estimated error is $\pm 10\%$.

I). The former two reactions account for the decay of FH· subsequent to laser photolysis in the absence and presence of phenol, respectively. The oxidation of FH· by quinone was accomplished by carrying out the photolysis with 2,6-dimethylphenol in the presence of large excesses of the quinone. This allows oxidation to compete effectively with the reverse electron transfer. It can be seen from Table I that the two radical reactions have similar rate constants ($\sim 10^9 M^{-1} s^{-1}$). However, the rate constants for the quinone reaction are considerably slower (10^6 – $10^7 M^{-1} s^{-1}$). The latter values were determined from the slopes of plots of k_{obsd} (the pseudo-first-order rate constant for radical decay in the presence of excess quinone) vs. quinone concentration. Two typical plots, obtained in methanol and methanol-water (3:1), are shown in Figure 3. No saturation effects were observed over the concentration ranges employed. This indicates that if an intermediate is present, its rate of decay must be greater than 10^5 – $10^6 s^{-1}$. The rate constants for all three reactions were found to correlate with the dielectric constants of the solvents used (Figure 4A,B,C). Again, no effects which could be attributed to the hydrogen-bonding capability of the solvent were observed. Note that a biphasic dependence of k_2 on ϵ

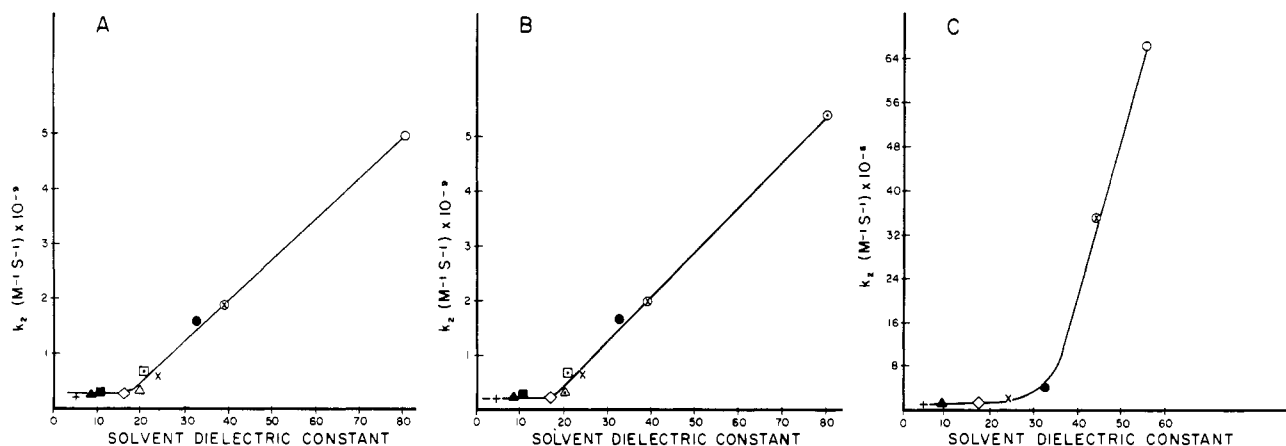


FIGURE 4: (A) Dependence of the second-order rate constant for oxidation of flavin semiquinone by oxidized flavin radical upon the solvent dielectric constant. Symbols are as in Figure 1. (B) Dependence of the second-order rate constant for oxidation of flavin semiquinone by the oxidized 2,6-dimethylphenol radical upon the solvent dielectric constant. Symbols are as in Figure 1. (C) Dependence of the second-order rate constant for oxidation of flavin semiquinone by 2,5-dichlorobenzoquinone upon the solvent dielectric constant. (O) Methanol-water (1:1); (●) methanol-water (3:1). Other symbols are as in Figure 1. It was assumed that the dielectric constants of the solvent mixtures were equal to the weighted average of the dielectric constants of the components [cf. Cheddar & Tollin (1980)].

was obtained. We interpret this as being due to a change in mechanism. Thus, in low polarity media, semiquinone oxidation proceeds via an electrically neutral transition state and a primarily hydrogen atom transfer mechanism which does not respond to solvent dielectric. At a certain minimum value of ϵ (which is in the range of 10–15 for the radical–radical reactions and approximately 30 for the much slower quinone reaction), solvent interaction is sufficient to stabilize a dipolar transition state and thus to favor a primarily electron transfer mechanism. Such minimum values for dielectric constant effects have been observed previously in other systems (Masuhara et al., 1975; Cheddar & Tollin, 1980). The rate constants are clearly much larger, and hence the activation energies are smaller, for the electron transfer pathway. This is presumably a consequence of the solvent participation in reducing the energy required to reach the transition state. For the less reactive quinone, one would anticipate a less polar transition state and hence a higher minimum value of the dielectric constant.

Conclusions

The results presented here indicate that, at least for the reactions studied, placing the flavin molecule in a hydrogen-bonding medium does not result in any significant effects upon electron transfer beyond those which can be ascribed to solvent polarity and viscosity. This does not support the results of theoretical treatments of the electronic structure of oxidized flavin (Nishimoto et al., 1978, 1980). It is possible, of course, that the semiquinone because of its different electron distribution behaves differently with respect to its response to hydrogen-bonding interactions. It should also be noted that theory predicts that the major enhancements of the electrophilic properties of the oxidized flavin ring will occur upon hydrogen bonding to N-1, N-3H, and the ring carbonyls. Hydrogen bonding to N-5 should produce the reverse effect. In fact, Yagi et al. (1980) observed that an excess of hydrogen-bonding reagent did decrease the magnitudes of the rate enhancements by 10–20%. They ascribed this to interaction occurring at the less basic N-5 position. In the present work, of course, because the molecules are totally surrounded by solvent, there is probably no selectivity with respect to which ring position participates in hydrogen bonding. Thus it is possible that some cancellation effects may be occurring, although these are likely to be small.

We have also found that the polarity of the solvent environment can exert a profound influence on the mechanism of the redox process. Thus, high polarity favors a dipolar transition state and an electron transfer mechanism. Low polarity favors a neutral transition state and hydrogen atom transfer. It is significant that reaction rates are considerably greater for the electron transfer pathway. Thus, an apoprotein to which a flavin prosthetic group is bound could exert control over the rates of the redox reaction catalyzed by the flavoenzyme by manipulating the dielectric microenvironment in such a way as to favor either electron transfer or hydrogen atom transfer from the semiquinone state. An example of this may be the flavodoxins. For these flavoproteins both the formation and oxidation rates of the semiquinone intermediate are quite slow compared with those of free flavin (Mayhew & Ludwig, 1975; Simonsen & Tollin, 1980). Since FH• is the predominant form of the semiquinone and the flavin is in

a largely nonpolar environment, it is not unreasonable to suppose that the protein constrains both reactions to proceed by the relatively sluggish hydrogen atom transfer pathway. Inasmuch as flavodoxin functions biochemically by shuttling between the fully reduced and semiquinone states, a kinetic stabilization of FH• toward oxidation would be advantageous. Similarly, according to this view flavoenzymes such as the oxidases, which react by a two-electron mechanism, would be expected to destabilize the semiquinone state by allowing electron transfer to proceed in a high dielectric environment. Consistent with this is the fact that oxidases invariably generate the flavin anion radical upon photoreduction (Massey et al., 1969). This is a consequence of a shift in the pK for the FH• to F• conversion to lower pH values, which implies a highly polar environment for the semiquinone. We believe that the control of the reaction mechanism by the dielectric environment represents a hitherto unrecognized principle operating in flavoenzyme catalysis.

References

- Ballard, S. G., Mauzerall, D. C., & Tollin, G. (1976) *J. Phys. Chem.* 80, 341.
- Cheddar, G., & Tollin, G. (1980) *Photobiochem. Photobiophys.* 1, 235.
- Edmondson, D. E., & Tollin, G. (1971) *Biochemistry* 10, 113.
- Guzzo, A. V., & Tollin, G. (1963) *Arch. Biochem. Biophys.* 103, 231.
- Handbook of Chemistry and Physics* (1980) (Weast, R. C., Ed.) 61st ed., CRC Press, Boca Raton, FL.
- Knowles, A., & Roe, E. M. F. (1968) *Photochem. Photobiol.* 7, 421.
- Land, E. J., & Swallow, A. J. (1969) *Biochemistry* 8, 2117.
- Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Mathews, R. G., & Foust, G. P. (1969) *J. Biol. Chem.* 244, 3999.
- Masuhara, T., Hino, T., & Mataga, N. (1975) *J. Phys. Chem.* 79, 994.
- Mayhew, S. G., & Ludwig, M. L. (1975) *Enzymes*, 3rd Ed. 12, Chapter 2.
- Nishimoto, K., Watanabe, Y., & Yagi, K. (1978) *Biochim. Biophys. Acta* 526, 34.
- Nishimoto, K., Watanabe, Y., & Yagi, K. (1980) in *Flavins and Flavoproteins* (Yagi, K., & Yamano, T., Eds.) p 493, University Park Press, Baltimore.
- Simonsen, R. P., & Tollin, G. (1980) *Mol. Cell. Biochem.* 33, 13.
- Smith, W. W., Burnett, R. M., Darling, G. D., & Ludwig, M. L. (1977) *J. Mol. Biol.* 117, 195.
- Song, P. S., Choi, J. D., Fugate, R. D., & Yagi, K. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) p 381, Elsevier, Amsterdam.
- Sun, M., & Song, P. S. (1973) *Biochemistry* 12, 4663.
- Tables of Dielectric Constants of Pure Liquids* (1951) *Natl. Bur. Stand. (U.S.), Circ. No. 514*.
- Tollin, G., Chan, R. L., Malefyt, T. R., & Bruice, T. C. (1979) *Photochem. Photobiol.* 29, 233.
- Vaish, S. P., & Tollin, G. (1971) *J. Bioenerg.* 2, 61.
- Watenpugh, K. D., Sieker, L. C., & Jensen, L. H. (1973) *Proc. Natl. Acad. Sci. U.S.A.* 70, 3857.
- Yagi, K., Ohishi, N., Nishimoto, K., Choi, J. D., & Song, P. S. (1980) *Biochemistry* 19, 1553.