Determination of Radioactivity. In the experiments with nonstereospecifically tritiated mevalonic acids the determination of tritium and ^{14}C was carried out by a dry combustion method, which involves physical separation of the two isotopes, followed by gas phase proportional counting.[49] All other isotope determinations were carried out directly in a Packard Tricarb or Beckman LS 100 liquid scintillation counter, using Bray's solution[50] or PPO and POPOP in toluene as scintillators. Counting efficiencies were determined by adding internal standard to every sample. It was noted that ergot alkaloids gave a temperature-dependent chemoluminescence reaction with the peroxide present even in reagent grade, stabilized dioxane, which contributed considerably to the tritium counts, when these samples were counted in Bray's solution in the ambient-temperature Beckman instrument, but not in the refrigerated Tricarb. Thus, a nonlabeled sample of elymoclavine dissolved in dioxane without scintillator gave several thousand counts in the tritium channel. The dioxane-containing scintillation mixture was therefore not used with the Beckman counter. Radioactivity on paper and thin-layer chromatograms was detected using a Packard chromatogram scanner.

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Acknowledgments. We are greatly indebted to Drs. A. Brack, A. Hofmann, and D. Stauffacher of Sandoz Research Laboratories, Basel, Switzerland, who generously provided us with reference alkaloids and a strain of ergot. Our thanks are also due to Drs. D. Arigoni and W. Acklin, Eidgen. Technische Hochschüle, Zürich, Switzerland, for communicating to us experimental details (*e.g.*, some of the tlc systems for the separation of chanoclavine isomers) and results of their work prior to publication. Financial support of this work by Deutsche Forschungsgemeinschaft, Eli Lilly and Co., and the National Institutes of Health (Grant No. AM 11662-01) is gratefully acknowledged.

Mechanism of the Photodephosphorylation of Menadiol Diphosphate. A Model for Bioquantum Conversion[1]

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Abstract: Photooxidation and photodephosphorylation of menadiol diphosphate by riboflavin and oxygen have been investigated as a model for biological quantum conversion. The details of the mechanism for this process in water have been studied experimentally. The results obtained suggest that the singlet oxygen generated by the triplet energy transfer from riboflavin is the photochemically reactive species which oxidizes and dephosphorylates menadiol diphosphate. The implication of this photoprocess as a bioquantum conversion model has been discussed.

Recently, we made a preliminary attempt at understanding the biological quantum conversion process in terms of a model system.[2] The model consists of a flavin and menadiol diphosphate. These compounds occur in chloroplasts in the form of FMN and menadiones, respectively. In our preliminary investigation, it was found that oxygen is required to oxidize and dephosphorylate menadiol diphosphate in the flavin-sensitized photoreaction. It was, however, not possible to elucidate the mechanism of the photoprocess due to a lack of understanding of the nature of the flavin triplets and insufficient kinetic data. We now report a detailed mechanistic elucidation of the photodephosphorylation of menadiol diphosphate in the light of additional kinetic data as well as theoretical and spectroscopic results on flavin triplets.[3-5]

Experimental Section

Materials. Riboflavin (6,7-dimethyl-9-ribitylisoalloxazine), Sigma Grade (recrystallized from acetic acid), was obtained from Sigma Chemical Co. Menadione (2-methyl-1,4-naphthoquinone, vitamin K, M=O), of spf grade, was obtained from Sigma Chemical Co. and was used after purification by vacuum sublimation. Menadiol sodium diphosphate (tetrasodium 2-methyl-1,4-naphthalenediol bis(dihydrogen phosphate), synkayvite, M) was a gift from Dr. W. E. Scott of Hoffmann-La Roche Ind. *p*-Benzoquinone was obtained from Eastman Organic Chemicals and was used after purification by vacuum sublimation. Potassium ferrioxalate for the chemical actinometry was purchased from City Chemical Co. (New York) and was recrystallized from warm water before use. A reagent grade potassium iodide (J. T. Baker Co.) was used without further purification. Water was first deionized, followed by distillation. Acetic acid was purified and dehydrated for use as solvent. Matheson Research Grade N_2 and O_2 were used without further purification.

Photolysis and Actinometry. Photolysis of the reaction mixture was done with a Bausch and Lomb xenon light source with a high-intensity monochromator with grating (catalog no. 33-86-07). This unit was fitted with a water-cooled aluminum cell holder (23 ± 1°) for Beckman 46005 10-mm path cells or equivalent. The entrance slit width was 3.56 mm and exit slit width 2.00 mm for all photolysis experiments. This gives a bandpass of 14.8 nm. The absorbance measurements were made on a Beckman DB spectrophotometer. The quantum yields were determined on the basis of orthophosphate produced and the chemical actinometry using potassium ferrioxalate as described by Parker and Hatchard.[6] The analytical method for determination of orthophosphate was that of Murakami and Martell.[7]

(1) (a) Part VI of Electronic Structure and Photochemistry of Flavins. (b) To whom reprint requests should be sent.
(2) P. S. Song and T. A. Moore, *Photochem. Photobiol.*, **7**, 113 (1968).
(3) P. S. Song and W. E. Kurtin, *J. Am. Chem. Soc.*, **89**, 4248 (1967).
(4) P. S. Song, *J. Phys. Chem.*, **72**, 536 (1968).
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A typical quantum yield measurement was as follows. A 3-ml solution (pH 7.5) of riboflavin (6.2×10^{-5} *M*) and menadiol diphosphate (*e.g.*, 1×10^{-3} *M*) was photolyzed for 2 min, flushing with oxygen during photolysis, and the amount of orthophosphate was determined. A 3-ml solution of potassium ferrioxalate with the absorbance identical with that of the riboflavin–menadiol diphosphate mixture at the same wavelength (450 nm) was photolyzed for 2 min, and the amount of Fe^{2+} produced was determined (see ref 6 for detailed procedure). The quantum yield of orthophosphate is given by

$$\Phi = \frac{(\Phi_{Fe^{2+}})(\text{no. of } PO_4^{3-} \text{ ions}/2)}{(\text{no. of } Fe^{2+} \text{ ions})}$$

The number of phosphate ions must be divided by 2, as two orthophosphates are produced per quantum of light. An alternative method was also employed in which the potassium ferrioxalate solution was concentrated such that all the light at 450 nm was absorbed, and then the total quanta per second per volume was calculated. The quantum yield in this case is given by

$$\Phi = \frac{(\text{no. of } PO_4^{3-} \text{ ions}/2)}{(\text{no. of quanta absorbed by riboflavin})}$$

This procedure gave identical results with the first method. The actinometry was carried out along with each sample photolyzed. All of the quantum yields reported in this paper have been obtained after averaging several repeated measurements (five to ten measurements). The least-square analysis of the data was also carried out using a FORTRAN program on an IBM 7040, and the straight lines drawn in the Results and Discussion section are from these data treatments.

To determine the oxygen requirement a solution was degassed either by flushing with N_2 or by the freeze–thaw method. No significant difference in photolysis was noticeable, *i.e.*, the photolysis of the degassed solution for approximately 1 hr yielded no detectable amount of menadione or orthophosphate. The extent of the photoreaction was also followed by measuring the difference spectrum at 350 nm due to the $^1(\pi \rightarrow \pi^*)$ transition of the organic product, menadione. This method correlated nicely with the orthophosphate produced and was used to determine the extent of the reaction on several occasions (*e.g.*, pH-dependence experiments).

Product Identification. Orthophosphate was identified chromatographically, as previously described,[2,8] and was quantitatively determined as mentioned above. The organic photoproduct was extracted from the aqueous reaction mixture with cyclohexane, and the absorption spectrum was compared with an authentic sample of menadione and with the spectrum published by Tomasi and Dallam.[8] The photoproduct absorbs at 262 (shoulder), 250 (λ_{max}), and 244 nm (shoulder). The authentic menadione has a shoulder at 263, λ_{max} at 250, and a shoulder at 244 nm. Both the authentic and photoproduced menadiones gave the same weak intensity band at the 350-nm region in water. We found no detectable amount of other photoproducts on the basis of the absorption spectra, fluorescence spectra, and chromatography of the photolyzed solution. Therefore, it appears that the reaction is clean.

Fluorescence and Phosphorescence Spectra. Uncorrected fluorescence and phosphorescence spectra of samples were measured in water at room temperature and in an ethylene glycol–water (50:50) matrix at 77°K, respectively, on an Aminco-Bowman spectrophotofluorometer. The emission intensity was detected by a PM tube (1P 28) and the spectra were recorded on a time-base recorder. The decay of the phosphorescence of menadiol diphosphate (10^{-4} *M*) was followed on this recorder.

Molecular Orbital Computations. Both HMO and ω-SCF procedures have been employed to calculate the reactivity indices of the reactant as well as inhibitors. The set of Coulomb and resonance integrals extensively tested by Pullman and Pullman[9] was chosen. Convergence in the ω technique was reached within five iterations. The methyl group in menadiol diphosphate and menadione was treated by the inductive model[10,11] using the Coulomb parameter of -0.5[12] for the substituted carbon. Computations were carried out on an IBM 7040 computer at the Texas Technological College Computer Center.

Results and Discussion

Figure 1 shows a typical rate curve obtained by following the orthophosphate produced in the photolysis (450-nm light). The rate of the reaction was alternatively followed as described in the Experimental Section and as shown in Figure 2. From Figure 2, it can be seen that a small amount of *p*-benzoquinone effectively quenched the photoreaction. Figure 3 shows the Stern–Volmer-type plot of the quenching of the photodephosphorylation by two inhibitors. Both *p*-benzoquinone and KI are seen to be effective as quenchers of the photoreaction. However, *p*-benzoquinone is approximately 100 times more inhibitory than KI. The slope of the plot in Figure 3 is found to be 1.1×10^6 M^{-1} for the former and 1.9×10^4 M^{-1} for KI. Since the range of the concentrations of quenchers used did not significantly affect the fluorescence intensity of riboflavin in the reaction mixture, it can be concluded that the riboflavin singlet $^1(\pi,\pi^*)$ is not photochemically reactive in the photodephosphorylation of menadiol diphosphate. However, the riboflavin singlet is reactive with respect to intramolecular photodecomposition.[13] For this reason, we used lumiflavin in our preliminary work[2] in order to avoid the intramolecular photodecomposition. Subsequently, we found that the flavin sensitization of the photodephosphorylation of menadiol diphosphate is much more efficient ($\Phi = 0.133$ at menadiol diphosphate concentration 1×10^{-3} *M*) than the intramolecular photodecomposition *via* the singlet excited state and the intramolecular photoreduction *via* the triplet state[13–16] ($\Phi = 0.006$).[17] Thus, the quantum yields from lumiflavin and riboflavin sensitizations were found to be about the same. In the present work, riboflavin was used exclusively.

KI is a well-know triplet quencher of the flavin system.[13,15–18] The data in Figure 3 suggest that the quencher (KI) inhibits the photoreaction by depopulating the triplet riboflavin as a reactive species, since the singlet population is not sufficiently affected by the low concentrations of KI to quench the photoreaction. The same interpretation may be applicable to the quenching by *p*-benzoquinone. However, in view of the fact that KI is an effective spin decoupler and electron donor, while *p*-benzoquinone is an electron acceptor, and 100 times more effective as a quencher than the former, it seems likely that an alternative quenching mechanism is operating in the case of *p*-benzoquinone. A more detailed discussion of this aspect will be given later.

Our recent theoretical[3,4] and spectroscopic[5] studies of the triplet states of flavins indicate that the lowest triplet is of $^3(\pi,\pi^*)$ type with an energy of 2.05 eV above the ground state. In order to examine possible

(8) G. E. Tomasi and R. D. Dallam, *J. Biol. Chem.*, **239**, 1605 (1964).
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(12) A. Streitwieser, Jr., "Molecular Orbital Theory for Organic Chemists," John Wiley & Sons, Inc., New York, N. Y., 1961, pp 131–135.
(13) P. S. Song and D. E. Metzler, *Photochem. Photobiol.*, **6**, 691 (1967).
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(17) B. Holmström and G. Oster, *J. Am. Chem. Soc.*, **83**, 1867 (1961).
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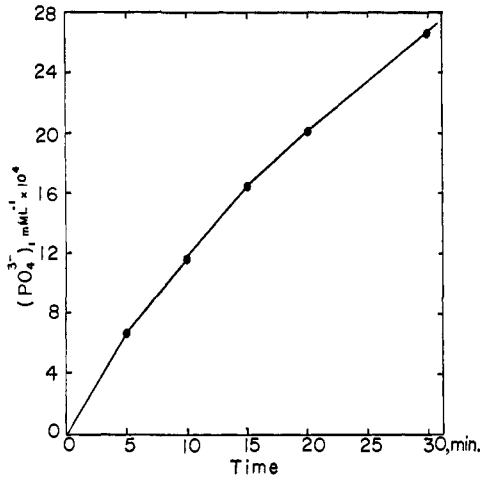

Figure 1. The rate curve for the photodephosphorylation of menadiol diphosphate (1×10^{-3} *M*) sensitized by riboflavin (*ca.* 6.2×10^{-5} *M*) and O_2 (saturated) in water (pH 7.9).

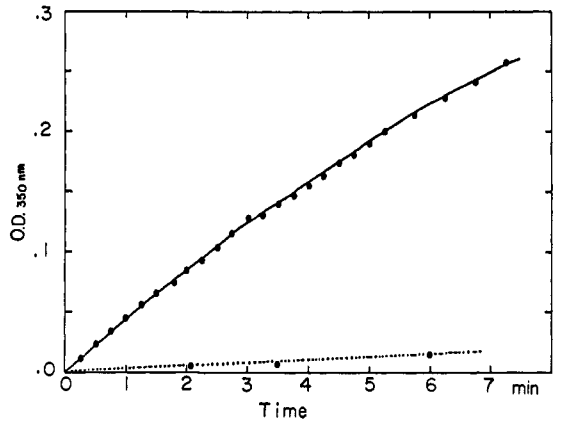

Figure 2. The rate (—●—) of formation (absorbance at 350 nm) of menadione from the photodephosphorylation of menadiol diphosphate (1×10^{-3} *M*) sensitized by riboflavin and O_2 in water (pH 7.9). The lower curve (· · · ●· · · ·) was obtained in the presence of *p*-benzoquinone (1×10^{-4} *M*).

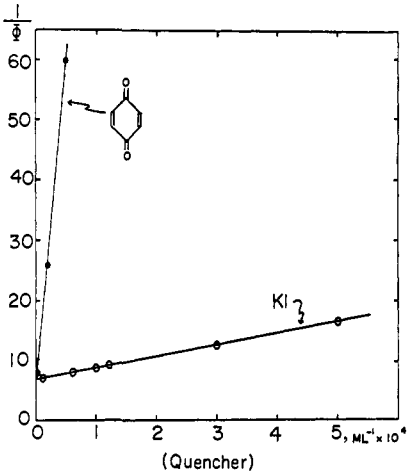

Figure 3. The quenching of the photodephosphorylation of menadiol diphosphate (1×10^{-3} *M*) sensitized by riboflavin (*ca.* 6.2×10^{-5} *M*).

energy-transfer mechanisms between the excited states of the sensitizer and menadione or oxygen, information concerning the energy levels of these molecules is essential. One possible energy transfer in the sensitized photodephosphorylation process in question is the singlet energy transfer from riboflavin to menadiol diphosphate, as shown in Figure 4. The Stern–Volmer K_q (quenching constant) was found to be 55.6 M^{-1} and k_3 (rate constant for the energy transfer from the singlet riboflavin to menadiol diphosphate) was estimated to be 1.07×10^{10} l. M^{-1} sec^{-1}, using a lifetime of 5.2×10^{-9} sec for the excited singlet flavin.[19] This value is approximately of the same magnitude as the Debye diffusion rate constant, $k_D = 8RT/3000\eta \cong 7.4 \times 10^9$ l. M^{-1} sec^{-1} at 24°. On the basis of the efficiency of the singlet excitation energy transfer, the transfer mechanism is probably of an exchange type. In addition, we have also noticed a static quenching of the riboflavin fluorescence by menadiol diphosphate at high concentrations ($>1 \times 10^{-2}$ *M*). However, it will be shown that any direct energy transfer from riboflavin to menadiol diphosphate is not important in the sensitized dephosphorylation itself. In order to examine this point (*i.e.*, possible energy transfer modes from riboflavin to menadiol diphosphate leading to the photodephosphorylation), emission spectra of menadiol diphosphate were obtained, as shown in Figure 5.

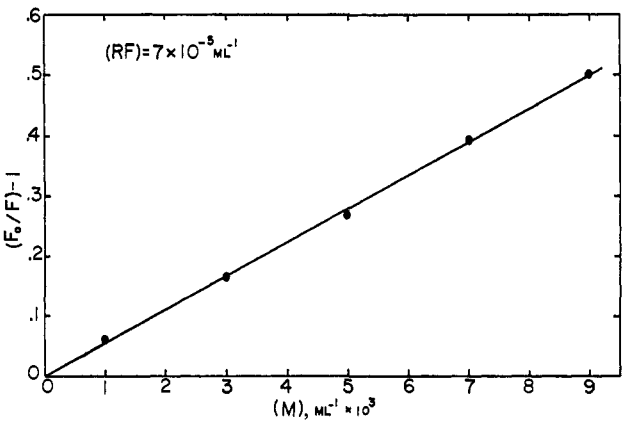

Figure 4. The fluorescence quenching of riboflavin by menadiol diphosphate in 0.1 *M* phosphate baffer (pH 6.5). F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively.

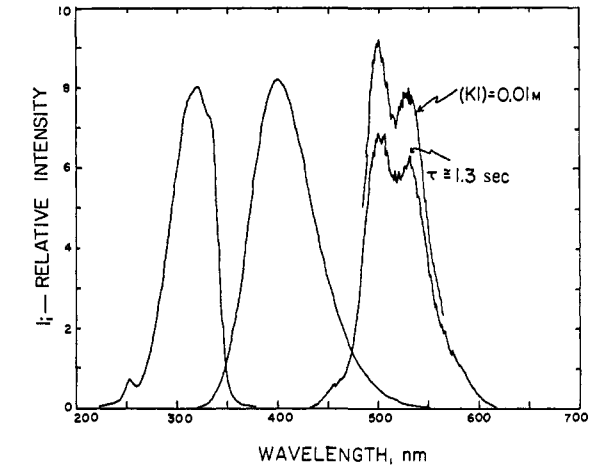

Figure 5. Spectra of menadiol diphosphate; from left to right: luminescence excitation spectrum, fluorescence emission spectrum, and phosphorescence emission spectrum.

(19) B. Holmström and L. Tegner, *Photochem. Photobiol.*, **5**, 207 (1966).

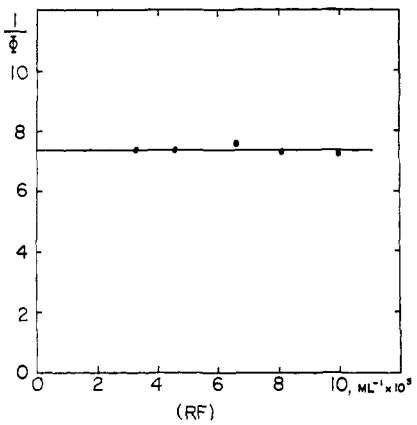

Figure 6. The reciprocal quantum yield of the photodephosphorylation of menadiol diphosphate (1×10^{-3} *M*) as a function of the flavin concentration.

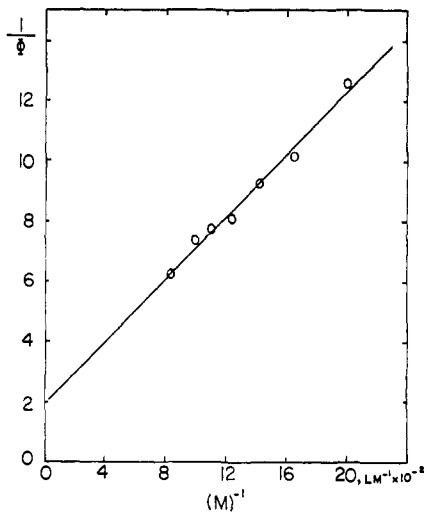

Figure 7. The reciprocal quantum yield of the photodephosphorylation as a function of the substrate concentrations (*M*; menadiol diphosphate) at pH 7.9.

Since the fluorescence and phosphorescence emission of riboflavin occur at 520- and 600-nm regions, respectively, possible combinations of energy transfer modes from riboflavin (RF) to the substrate are

$$^1RF + M_0 \longrightarrow RF_0 + {}^3M \quad (a)$$

$$^1RF + M_0 \longrightarrow RF_0 + {}^1M \quad (b)$$

Reaction a is energetically possible although it is a spin-forbidden process. Reaction b is unlikely since there is no spectral overlap between the absorption spectrum of menadiol diphosphate and the fluorescence spectrum of riboflavin. The spin-allowed energy transfer from riboflavin triplet (E_T = 2.05 eV) to menadiol diphosphate to yield the triplet state (E_T = 2.32 eV) of the latter is energetically unlikely, and the overlap between phosphorescence spectra of riboflavin and menadiol diphosphate is not sufficient to account for the relatively high quantum yields of the sensitized photodephosphorylation reaction. In view of the effect of KI on the quantum yield of the photoreaction and the above considerations, the energy transfer from riboflavin to menadiol diphosphate can be ruled out as an important mechanism for the reaction in question. This view will be further supported later in this paper. As to the nature of the triplet state of menadiol diphosphate, the relatively weak intensity of the phosphorescence and the long lifetime (1.3 sec) suggest that the lowest triplet is of π,π^* character. It can also be seen, from Figure 5, that KI has some emission-enhancing effect *via* the external spin–orbit perturbation.

At this point, we can rule out the singlet or triplet energy transfer from riboflavin to menadiol diphosphate as the directly responsible step for the sensitized photodephosphorylation of the latter. In an attempt at elucidating the mechanism of the photoprocess, additional data are presented below. Figure 6 shows the apparent independence of the quantum yield upon concentrations of the sensitizer (RF). This means that the quantum yield does not depend on the amount of light absorbed initially by the reaction mixture, as expected. Thus, the mechanism to be proposed should be consistent with the data in Figure 6. Figure 7 shows a typical Stern–Volmer type plot for the photodephosphorylation of menadiol diphosphate sensitized by riboflavin under constant oxygen pressure. The range of the substrate concentrations was chosen such that both ambiguity in the product determination (due to dilute concentration) and significant fluorescence quenching can be avoided. The least-square-treated slope and intercept of the plot were 5.12×10^{-3} M^{-1} and 1.997, respectively. It was found that a concentration of menadiol diphosphate above 1.2×10^{-3} *M* caused fluorescence quenching of riboflavin (see also Figure 4). The quantum yield is near zero in the absence of oxygen.

It is clear that the sensitization occurs *via* energy transfer from the riboflavin triplet to oxygen ($^3\Sigma_g^-$), on the basis of the results described so far.

$$^3RF + O_2(^3\Sigma_g^-) \xrightarrow{k_6} RF_0 + O_2(^1\Delta_g \text{ or } {}^1\Sigma_g^+) \quad (c)$$

The singlet oxygen can then react with menadiol diphosphate to yield menadione and orthophosphate in aqueous solutions. Since both $^1\Delta_g$ and $^1\Sigma_g^+$ states of the excited oxygen are 0.98 and 1.64 eV in energy, respectively,[20,21] reaction c is energetically possible and spin allowed. Furthermore, such a mechanism of energy transfer from dyes to oxygen has been well established and has recently been discussed extensively.[21] In order to verify mechanism c as the responsible step in the dephosphorylation of menadiol diphosphate, we generated the singlet oxygen chemically,[22] yielding both menadione and orthophosphate.

Before we propose a detailed reaction scheme and mechanism, we will first consider the mechanisms of

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(21) C. S. Foote, *Accounts Chem. Res.*, **1**, 104 (1968).

(22) Singlet oxygen can be generated by mixing hydrogen peroxide with sodium hypochlorite.[23-25] In our case, sodium hypochlorite itself oxidized menadiol diphosphate. It was, therefore, necessary to bubble the generated oxygen into a separate reaction vessel *via* glass tube (*ca.* 10 cm in total length) as quickly as possible using an inert gas pressure. Although one can observe the reddish emission from the excited oxygen gases in the dark, the population of the singlet oxygen by the time it reaches the reaction vessel decreases enough to lower the efficiency of the chemical dephosphorylation.

(23) (a) C. S. Foote and S. Wexler, *J. Am. Chem. Soc.*, **86**, 3879, 3880 (1964); (b) C. S. Foote, S. Wexler, W. Ando, and R. Higgins, *ibid.*, **90**, 975 (1968).

(24) J. S. Arnold, R. J. Browne, and E. A. Ogryzlo, *Photochem. Photobiol.*, **4**, 963 (1965).

(25) A. U. Khan and M. Kasha, *J. Am. Chem. Soc.*, **88**, 1574 (1966), and references therein.

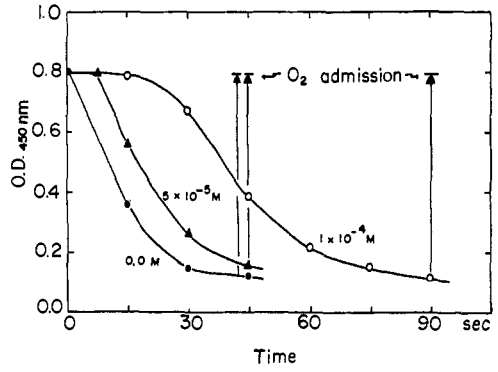

Figure 8. The effect of the concentration of *p*-benzoquinone on the anaerobic photoreduction of riboflavin (6.7×10^{-5} *M*) by EDTA (excess) in water. Light source: PCQ-011 photochemical grid lamp described previously.[14] OD_{450nm} is due to the absorbance of riboflavin. Note the recovery of the OD at the end of photolysis upon air admission.

the quenching by KI and *p*-benzoquinone, as described earlier. In connection with the data shown in Figures 2 and 3, we suggest that the mechanisms of the quenching by KI and *p*-benzoquinone are different. Namely, KI is an effective flavin triplet quencher, as established in our previous work and results of others on the photochemical reactions of flavins.[13,15-18] In view of the well-known ability of singlet oxygen to form peroxides with olefinic molecules,[21] it is very likely that the inhibition of the photoreaction by *p*-benzoquinone is due to the depopulation of the singlet oxygen in the reaction mixture. In order to demonstrate that *p*-benzoquinone does not inhibit the reaction *via* quenching of the riboflavin triplet, we carried out the photoreduction of riboflavin by EDTA. This reaction and the analogous photoreduction of riboflavin by N-benzyl-N,N′-dimethylethylenediamine and N,N′-dibenzylethylenediamine are known to proceed *via* the flavin triplet state.[14,16,18] Figure 8 shows the anaerobic photoreduction of riboflavin by EDTA as a function of concentration of *p*-benzoquinone. It can be seen that 5×10^{-5} *M* *p*-benzoquinone does not affect the rate of the photoreduction, except for the induction period due to the fast reoxidation (dark reaction) of leucoriboflavin by *p*-benzoquinone.[26] It should be recalled from Figure 3 that *p*-benzoquinone quenches the photodephosphorylation drastically at concentrations of 5×10^{-5} *M* or less, while the rate of the photoreduction in Figure 8 is not affected to any significant degree. At a slightly higher concentration of *p*-benzoquinone, the induction period, of course, is lengthened due to more oxidation of leucoriboflavin. A somewhat slower rate of the photoreduction at a *p*-benzoquinone concentration of 1×10^{-4} *M* is to be expected from the fact that hydroquinone produced after the induction period begins to quench the reaction by depopulating the flavin triplet state.[13] Therefore, it can be suggested that *p*-benzoquinone inhibits the photodephosphorylation of menadiol diphosphate by quenching the oxygen singlet state(s), rather than the

(26) (a) M. J. Gibian and J. A. Rynd, Abstracts of Papers, 155th National Meeting of the American Chemical Society, San Francisco, Calif., April 1968, Paper No. P-12. (b) This induction period is equivalent to the reoxidation of reduced flavins with *p*-benzoquinone. The addition of *p*-benzoquinone to the photoreduced solution of leucoriboflavin instantly restores the absorbance at 450 nm. The riboflavin phosphorescence was not quenched by *p*-benzoquinone (*ca.* 1×10^{-3} *M*).

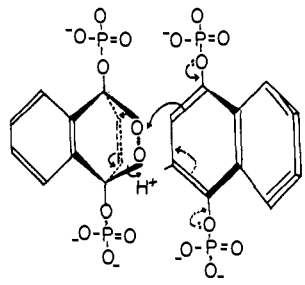

Figure 9.

riboflavin triplet. It is to be noted, in Figure 8, that admission of oxygen restores almost 100% of the absorbance of riboflavin at 450 nm, showing the absence of the intramolecular photoreactions.

We now propose the following scheme as the most likely mechanism for the photodephosphorylation of menadiol diphosphate

$$RF_0 + h\nu \xrightarrow{I_a} {}^1RF$$

$${}^1RF \xrightarrow{k_1} RF_0 + h\nu' \text{ (fluorescence)}$$

$${}^1RF \xrightarrow{k_2} RF_0 + \text{heat}$$

$${}^1RF + M \xrightarrow{k_3} RF_0 + M$$

$${}^1RF \xrightarrow{k_4} {}^3RF \text{ (intersystem crossover)}$$

$${}^3RF \xrightarrow{k_5} RF_0 + \text{heat}$$

$${}^3RF + O_2({}^3\Sigma_g^-) \xrightarrow{k_6} RF_0 + {}^1O_2({}^1\Delta_g \text{ or } {}^1\Sigma_g^+)$$

$${}^1O_2 + M \xrightarrow{k_7} MO_2$$

$$MO_2 \xrightarrow[2H_2O]{k_8} M{=}O + 2P_i + H_2O_2$$

$${}^1O_2 \xrightarrow{k_9} O_2 + \text{heat and } h\nu''$$

$${}^1O_2 + M{=}O \xrightarrow{k_{10}} O_2 \cdot M{=}O \text{ or } (O_2 + M{=}O)$$

where P_i represents orthophosphate. The above scheme is consistent with all the observed data described so far, as can be seen from the following steady-state expression derived for the above mechanism

$$\frac{1}{\Phi} = \frac{k_{1,2,4,3}}{k_4}\left[1 + \frac{k_5}{k_6(O_2)}\right]\left[1 + \frac{k_9 + k_{10}(M{=}O)}{k_7}\left(\frac{1}{(M)}\right)\right] \quad (1)$$

where $k_{1,2,4,3} = k_1 + k_2 + k_4 + k_3(M)$. The above equation accounts for the data shown in Figures 3, 4, 6, and 7, and for the oxygen requirement as well as the inhibition either by menadione produced or *p*-benzoquinone added (step k_{10}). The inhibition by menadione apparently is much less efficient than by *p*-benzoquinone. Therefore, the last step does not affect the quantum yield data significantly in the case of (M=O) = menadione, although it may affect the kinetics to some extent in the system of higher initial concentrations of menadiol diphosphate. This aspect will be discussed further. It was, however, not possible to quantitatively study the inhibition by the product due to the limited range of the solubility in water. A possible reason for the apparently lower quenching

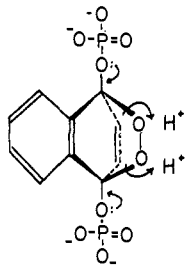

Figure 10.

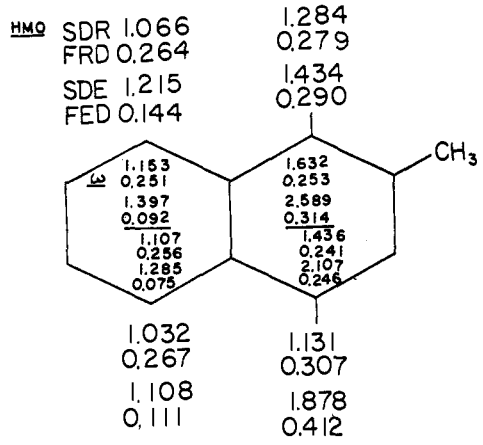

Figure 11. SDR (superdelocalizability for radical attack), FRD (frontier radical density), SDE (superdelocalizability for electrophilic attack), and FED (frontier electron density) in menadiol diphosphate calculated by HMO (values outside the ring structure) and ω-SCF methods. The phosphate groups (not shown) in menadiol diphosphate were explicitly included in the calculations. In general, the larger the values, the more reactive the sites. See K. Fukui, T. Yonezawa, and H. Shingu, *J. Chem. Phys*,. **20**, 722 (1952), for definitions and applications of the above indices.

efficiency of menadione than *p*-benzoquinone will be given later.

The following scheme (see also Figure 9) has been readily ruled out as the possible pathway of the sensitized reaction, as the reciprocal quantum yield depends on the inverse of $(M)^2$.

$$MO_2 + M \xrightarrow[2H_2O]{k_6'} M{=}O + M + 2P_i + H_2O_2 \qquad (d)$$

$$MO_2 \xrightarrow{k_9'} M + O_2 + \text{heat} \qquad (e)$$

$$M{=}O + {}^3RF \xrightarrow{k_{10}'} M{=}O + RF_0 \qquad (f)$$

or

$$M{=}O + {}^1O_2 \xrightarrow{k_{10}} O_2 \cdot M{=}O \text{ or } (M{=}O + O_2)$$

$$^1O_2 \xrightarrow{k_9} O_2 + \text{heat or } h\nu''$$

On the other hand, the mechanism involving direct oxidation of menadiol diphosphate by the singlet oxygen without the formation of the MO_2 peroxide complex of Kautsky's type[21,27] is kinetically permissible.

$$M + {}^1O_2 \xrightarrow{k_7'} M{=}O + 2P_i + H_2O_2 \qquad (g)$$

$$^1O_2 \xrightarrow{k_9} O_2 + \text{heat or } h\nu''$$

$$M{=}O + {}^1O_2 \xrightarrow{k_{10}} O_2 \cdot M{=}O \text{ or } (M{=}O + O_2)$$

(27) H. Kautsky and H. de Bruijn, *Naturwissenschaften*, **19**, 1043 (1931); (b) H. Kautsky, *Biochem. Z.*, **291**, 271 (1937).

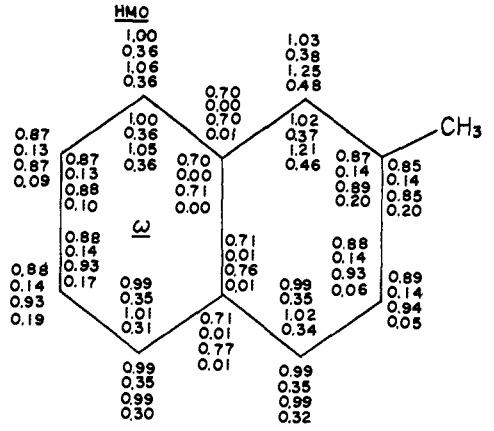

Figure 12. The reactivity indices in 2-methylnaphthalene; from top to bottom at each position: SDR, FRD, SDE, and FED.

The steady-state treatment for the above scheme leads to the following expression.

$$\frac{1}{\Phi} = \left[1 + \frac{k_9 + k_{10}(M{=}O)}{k_7'}\left(\frac{1}{(M)}\right)\right] \times \frac{k_{1,2,4,3}[k_5 + k_5(O_2)]}{k_4 k_6(O_2)} \qquad (2)$$

On the basis of the well-documented examples of the peroxide formations and of the theoretical considerations discussed below, it appears to us that the mechanism involving MO_2 (steps k_7 and k_8) is preferred to the direct oxidation mechanism (step k_7' above). We propose that Figure 10 represents a possible structure for MO_2. From Figure 10, it can be seen that two orthophosphates and menadione are readily produced in aqueous solution. The theoretical calculations shown in Figure 11 are consistent with the sites of peroxidation shown in Figure 10.

$O_2(^1\Delta_g)$ and $O_2(^1\Sigma_g^+)$ are probably different in their respective reactivity toward menadiol diphosphate. Due to the same irreducible representations of the wave functions for the ground and second excited singlet states ($^1\Sigma_g^+$), the latter may act as a radical reagent, while the lowest excited singlet oxygen may act as an electrophile.[21] Recently, theoretical studies of the model consisting of cyclobutadiene and singlet oxygen indicated that $O_2(^1\Delta_g)$ is the reactive species and $O_2(^1\Sigma_g^+)$ is unreactive.[28] Since our kinetic data are insufficient to choose either $O_2(^1\Delta_g)$ or $O_2(^1\Sigma_g^+)$ as the reactive species, both electrophilic and radical reactivity indices were considered in Figure 11. In either case, it can be seen that the carbons next to the phosphate groups (positions 1 and 4) are definitely more reactive than positions 5 and 8. The same prediction is made by both HMO and ω procedures. The calculated indices are consistent with the peroxide intermediate shown in Figure 10. It is interesting to compare the reactivity indices of menadiol diphosphate with those of its analog, 2-methylnaphthalene, shown in Figure 12. It can be predicted, from Figure 12, that 2-methylnaphthalene will be less reactive toward the singlet oxygen than menadiol diphosphate. We can also account for the reactivity (inhibitory) difference between

(28) A. U. Khan and D. R. Kearns, Abstracts of Papers, 155th National Meeting of the American Chemical Society, San Francisco, Calif., April, 1968, Paper No. S-161.

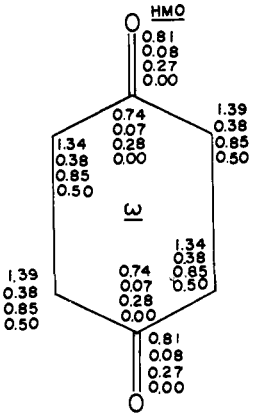

Figure 13. The reactivity indices in *p*-benzoquinone; from top to bottom at each position: SDR, FRD, SDE, and FED.

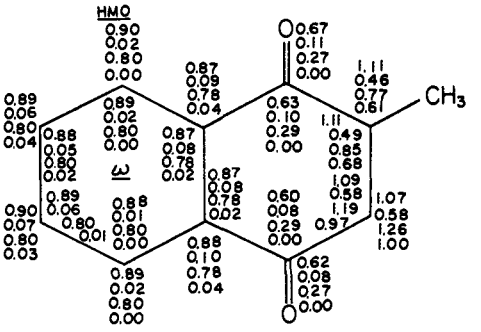

Figure 14. The reactivity indices in menadione; from top to bottom: SDR, FDR, SDE, and FED.

p-benzoquinone and menadione. From Figures 13 and 14, *p*-benzoquinone (combination of positions 2 and 5, or 3 and 6) is seen to be more reactive than menadione, consistent with our qualitative observation that the former is a much more efficient quencher for the photodephosphorylation of menadiol diphosphate.

It is instructive to compare the reactivity of the substrate (menadiol diphosphate) and inhibitor (*p*-benzoquinone) with the known reactivities of olefinic compounds toward singlet oxygen.[21] The comparison can be made in terms of β ($=k_9/k_7$), which is characteristic of the reactivity of a particular $^1O_2$ acceptor. If we assume that step k_8 is much faster than step k_7, a reasonable assumption, eq 1 can be expressed in the following simplified form

$$\frac{1}{\Phi} = \frac{k_9}{\Phi_{^1O_2} k_7(\mathrm{M})} + \frac{1}{\Phi_{^1O_2}} \qquad (3)$$

where $\Phi_{^1O}$ is the quantum yield for the singlet oxygen. Thus, from Figure 7 it is possible to evaluate β. The value of β is the concentration of M such that one-half the singlet oxygen decays and the other half is trapped to yield product, *i.e.*, $k_9 = k_7(\mathrm{M})$. In order for the above to be meaningful, it is necessary that the quantum yield be determined initially so that very little M has reacted. These conditions were met in this experiment, and $\beta = 2.5 \times 10^{-3}\ M^{-1}$ and $\Phi_{^1O_2} = 0.5$ were found. The β value indicates that menadiol diphosphate is indeed reactive and compares favorably with the olefinic acceptors studied by Foote.[21] This parameter was also determined for *p*-benzoquinone. In this case, eq 4 can be readily written

$$\frac{1}{\Phi} = \frac{k_9}{\Phi_{^1O_2} k_7(\mathrm{M})} + \frac{1}{\Phi_{^1O_2}} + \frac{k_{10}(\mathrm{M{=}O})}{k_7(\mathrm{M})\Phi_{^1O_2}} \qquad (4)$$

Assuming that the concentration ($1 \times 10^{-3}\ M$) of menadiol diphosphate is constant during the initial period (product less than 5%) and using the β value for menadiol diphosphate, eq 4 becomes

$$\frac{1}{\Phi} = \frac{3.5}{\Phi_{^1O_2}} + \frac{k_{10}(\mathrm{M{=}O})}{k_7(\mathrm{M})\Phi_{^1O_2}} \qquad (5)$$

Thus, from Figure 3 we obtain $k_{10}/k_7 = 1.61 \times 10^2$. In other words, *p*-benzoquinone is much more reactive (160 times) than the substrate, thus accounting for the efficient quenching of the photodephosphorylation by *p*-benzoquinone. The β value (k_9/k_{10}) was estimated to be 1.5×10^{-5}. It also turned out that $^1O_2$ from eq 5 and Figure 3 was found to be 0.46, in satisfactory agreement with 0.5 obtained from eq 3 and Figure 7.

The fluorescence quantum yield in water (pH 7) was found to be 0.26,[29] which implies that the quantum yield for the triplet must be 0.74 or less. Considering the approximations used and experimental errors, our value of the quantum yield for the singlet oxygen ($0.46 \sim 0.5$) is qualitatively reasonable. Estimation of the triplet flavin quantum yield is somewhat difficult, since an additional term containing k_6 would appear in eq 3 and 5. Efforts are under way in our laboratory to identify the products of the singlet oxygen addition to *p*-benzoquinone. A possible initial addition product may be a paramagnetic *p*-benzoquinone peroxide (peroxide linkage between positions 2 and 5), if the reaction proceeds as predicted by the data shown in Figure 13. Further decompositions or dimerization may follow such an initial formation of the inhibition product.

In the case of the quenching of the reaction by KI, the following expression can be derived, and, again, is consistent with all the data presented in the present study.

$$^3\mathrm{RF} + \mathrm{KI} \xrightarrow{k_{11}} \mathrm{RF_0} + \mathrm{KI}$$

$$\frac{1}{\Phi} = \frac{k_{1,2,4,3}}{k_4}\left[1 + \frac{k_5 + k_{11}(\mathrm{KI})}{k_6(\mathrm{O_2})}\right]\left[1 + \frac{k_9}{k_7}\left(\frac{1}{(\mathrm{M})}\right)\right] \qquad (6)$$

The pH dependence of the photodephosphorylation was also studied. From Figure 15, a sharp dependence of the reaction upon pH can be seen. Since pH affects so many variables such as the fluorescence intensity, the singlet oxygen lifetime, ionization of the phosphate groups in the substrate molecule, population of the flavin triplet,[30] and various rate constants, the interpretation of Figure 15 is extremely difficult at the present time.

In addition to the sensitized reaction, we also found that orthophosphate and menadione can be produced aerobically from uv irradiations of the aqueous solutions of menadiol diphosphate without riboflavin (no reaction with visible light, of course). Although details of the data on the nonsensitized reaction are lacking, it was found that the quantum yield is inde-

(29) G. Weber and F. W. J. Teale, *Trans. Faraday Soc.*, **53**, 646 (1957).

(30) T. Shiga and L. H. Piette, *Photochem. Photobiol.*, **3**, 213 (1964).

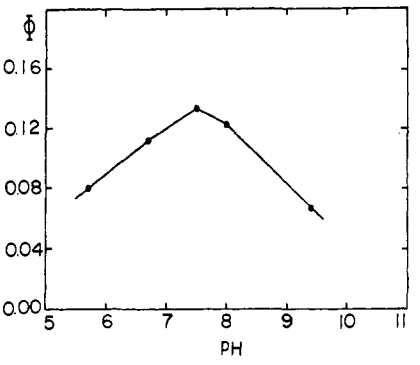

Figure 15. The pH dependence of the quantum yield of the photodephosphorylation of menadiol diphosphate (1×10^{-3} *M*).

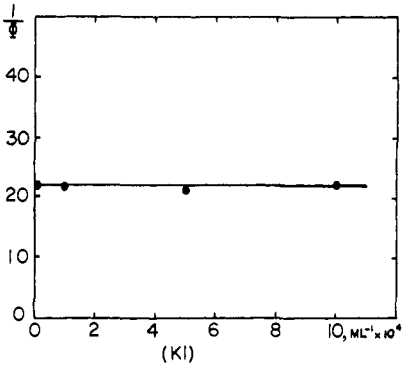

Figure 16. The effect of KI on the nonsensitized uv photolysis of menadiol diphosphate (1×10^{-3} *M*) in water (pH 7.9). Light source: PCQ-011 low-pressure mercury lamp.

pendent of KI, as shown in Figure 16. Two mechanisms may be feasible.

Mechanism A

$$M + h\nu \longrightarrow M^* \text{ (singlet or triplet)} \qquad (h)$$

$$M^* + O_2(^3\Sigma_g^-) \xrightarrow[2H_2O]{} M{=}O + 2P_i + H_2O_2 \qquad (i)$$

Mechanism B

$$M^* \text{ (triplet)} + O_2(^3\Sigma_g^-) \longrightarrow M + {}^1O_2(^1\Delta_g) \qquad (j)$$

$$M + {}^1O_2 \xrightarrow{k_7} MO_2$$

$$MO_2 \xrightarrow[2H_2O]{k_8} M{=}O + 2P_i + H_2O_2$$

It was found that (KI) = 1×10^{-3} *M* does not quench the fluorescence of menadiol diphosphate. Thus, it is not possible to rule out mechanism A. On the other hand, if mechamism B is correct, the absence of the quenching of the uv photolysis by KI (Figure 16) is consistent with our proposal that KI inhibits the sensitized reaction by quenching ^{3}RF, not $^1O_2$.[31] Further studies are needed to establish the mechanism of the nonsensitized reaction.

(31) However, the experiments do not differentiate between riboflavin quenching and oxygen quenching, and the singlet oxygen species have been reported to be quenched by ions.[32] The authors thank the referee for drawing our attention to this point.

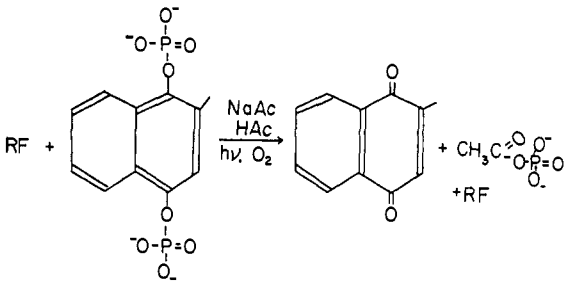

Figure 17.

Finally, the implication of the present system as a model for the bioquantum conversion will be described. Previously, attempts to trap the transient metaphosphates with added orthophosphate were not successful,[2] due to the instant hydrolysis. We have carried out preliminary experiments in anhydrous acetic acid containing sodium acetate. On the basis of the increase in absorbance at 350 nm, it appears that the reaction occurs as shown in Figure 17. Paper chromatography[33] of the photolysis mixtures of menadiol diphosphate in anhydrous acetic acid with sodium acetate and orthophosphate has shown the presence of pyrophosphate. More data are needed for elucidating the mechanism of the phosphorylation reaction. The implications of the photodephosphorylation as well as these preliminary observations are obvious. Acetyl phosphate and pyrophosphate are so-called "high-energy" phosphate compounds due to their relatively high free energy of hydrolysis. Consequently, the present photochemical system is a significant bioquantum conversion model in which the light energy originally absorbed by riboflavin can be converted into chemical potentials in the form of the phosphate ester bond of biochemical importance. The mechanism of the above model process in different organic solvents is being investigated, and further details will be published in the near future.

Acknowledgments. This work was generously supported by the Robert A. Welch Foundation (Grant No. D-182). The technical assistance by William Kurtin and computational assistance by the staff of the Texas Technological College Computer Center are greatly appreciated. We are indebted to Professor C. S. Foote for making a preprint available to us prior to publication.

(32) J. S. Arnold, R. J. Browne, and E. A. Ogryzlo in "Symposium on Chemiluminescence," Advanced Research Projects Agency, Office of Naval Research and U. S. Army Research Office Durham, N. C., 1965, p 35.

(33) The chromatography was carried out in *i*-PrOH–H_2O (80:20); trichloroacetic acid (5 g) and orthophosphate and pyrophosphate were detected according to the method of Hanes and Isherwood.[34]

(34) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).