THE EFFECT OF OXYGEN ON THE AMPLITUDE OF PHOTODRIVEN ELECTRON TRANSFER ACROSS THE LIPID BILAYER-WATER INTERFACE

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ABSTRACT The surprisingly small effect of oxygen on photoelectron transfer in pigmented lipid bilayers is traced to a short lifetime of the excited states. Decreasing the oxygen concentration by >100-fold decreases the half saturating concentration of acceptor by only threefold and has no effect on the maximum photovoltage observed at acceptor saturation. This holds true for both magnesium octaethylporphyrin and chlorophyll with both ferricyanide and methyl viologen as acceptors. Since oxygen quenches excited states at near the encounter limit, the lifetime of reactive state must be short, <100 ns. About 100-fold higher concentrations of acceptor are required to quench the fluorescence (in liposomes) than to saturate the photoeffect. Thus the reactive state is most likely the triplet. The short life of the excited state is caused by concentration quenching, i.e., their reaction with ground state molecules. The increase of photovoltage with increasing pigment concentration shows that this quenching in a condensed form of the pigment produces ions that lead to the observed photovoltage by interfacial reaction of the anion with acceptor.

INTRODUCTION

A study of the effect of O_2 on photoreactions in pigmented bilayers was needed to clarify a fundamental question. Almost all known electron transfer photoreactions in solution are quenched by O_2 because they originate from long-lived triplets. Why then do we see efficient photoeffects in bilayers in the presence of air? The phenomenological answer was obtained early: The interfacial electron transfer occurs before (<100 ns) the excited states can be quenched by O_2, even if the quenching reaction is encounter limited (Hong and Mauzerall, 1976). The rapidity of the electron transfer raised the possibility that the singlet state was active in this process. We have analyzed the effect of O_2 by studying its competition with other acceptors. We answer the specific questions: (*a*) Is the O_2 effect competitive or noncompetitive with added acceptor? (*b*) Does O_2 quenching form any ionic products of its own? (*c*) Which electronic state of the pigment is active in electron transfer? (*d*) Do monomer or aggregated forms of the pigment form ions?

Earlier studies on the O_2 effect (Lutz et al., 1974) concerned the decrease in photoconductivity seen with O_2 under continuous illumination, but in the absence of added acceptor. The results are very complex. Our preliminary studies concerned the effect of O_2 on the well-characterized hyperbolic relation between acceptor concentration and photoeffect (Ilani and Mauzerall, 1981*b*), but were limited to a 10-fold variation in O_2 concentration (Ilani and Mauzerall, 1981*a*). The results indicated a surprisingly inefficient competition between O_2 and acceptors. A method using glucose oxidase to lower the O_2 concentration ≫100-fold was developed. It is confirmed that the O_2 effect is small. The reason is the very short lifetime of the pigment excited states. Few stabilized ions are formed (<10% of maximum yield from acceptors) from the O_2 quench. It is concluded that the triplet state is chiefly responsible for the photoeffect at normal ($\sim 10^{-3}$ M) concentrations of acceptor, but the singlet state may contribute at higher concentration (10^{-1} M). The short lifetime of the excited states is caused by concentration quenching that leads to the formation of pigment cations and anions. The reaction of the aqueous acceptor with the pigment anion produces the observable interfacial photovoltage. The evidence for these statements is the subject of this paper.

EXPERIMENTAL

The pigments, acceptors and methodology are as described before (Ilani and Mauzerall, 1981*b*; Losev and Mauzerall, 1983). The pigment (0.6–7.2 mM) is dissolved in egg lecithin-cholesterol-decane (3%, 0.8% wt/vol) and the bilayer is formed by brush in a 1.6 mm hole of a Teflon septum. The aqueous solution is usually 0.1 M NaCl, 10 mM K-phosphate, pH 6.8. Acceptor is added to one side to the desired concentration. The response to a <1 μs flash-lamp-pumped dye (Rhodamine 6G) laser, 1–10 mJ, at 590 nm was measured via calomel electrodes. The photovoltage was measured via a 10^{10} Ω impedance amplifier (3A7; Tektronix, Inc., Beaverton OR), digitized (Biomation 805; Biomation Inc., Santa Clara, CA) and read out on a recorder. The photovoltage was measured (0.2 μs/channel) at the maximum of the recorded effect, typically 1–3 μs following the laser flash. For constant

decay times, membrane capacitance (5–7 nF) and apparatus response, this maximum is exactly proportional to the integral over the light flash of the charge displacement across the lipid water interface (Hong and Mauzerall, 1976).

In preliminary experiments the O_2 concentration was decreased (increased) by bubbling the solutions in the membrane chamber with N_2 (O_2), the whole being in a glove bag. A 10-fold change in O_2 concentration was achieved by the objective criteria of an O_2 electrode immersed in the solution. The detailed results are sensitive to diffusion, convection, etc. The use of glucose oxidase allowed much greater depletion of O_2 (Benesch and Benesch, 1953). A study was made of appropriate concentrations of the reactants necessary for rapid (<5 min) deoxygenation. Control experiments showed that neither glucose, glucose oxidase, nor superoxide dismutase (SOD) interfered with the photoreaction at the concentrations usd. Catalase was usually not added since it appeared to be inhibited in the presence of ferricyanide. It will be shown (Liu, T. M., and D. Mauzerall) that H_2O_2 affected only the kinetics of the photovoltage decay by oxidizing ferro- to ferricyanide. The following protocol was developed. The buffer was increased to 10 mM to counteract the acidity of the product, gluconic acid. After forming the membrane, the solution was deoxygenated by adding glucose oxidase (50 μg/ml; Sigma Chemical Co., St. Louis, MO) and glucose (10 mM) and stirring. A wait of 10 min, without stirring, produced an O_2 level below detection by the O_2 electrode (<2% of ambient). Measurements of the triplet state via delayed luminescence showed by the observed half-life that the level of O_2 was $<10^{-3}$ of ambient. The very slow diffusion of O_2 over macroscopic distance, the absence of convection (thermal equilibration and adequate time for stirring vortices to dissipate), and continual sinking of any entering O_2 allow one to work macroscopically anaerobic out in the open. SOD was used to check the effect of O_2^- in the aqueous phase. No effect was observed. It was observed that solutions of glucose and glucose oxidase slowly decolorized ferricyanide after becoming anaerobic.

Liposomes containing pigment were prepared by incubating the mixture at 37°C for 30 min and sonicating under N_2 (Racker, 1973), followed by filtration through a Sephadex column. Fluorescence was measured on a fluorimeter (MPF-2A; Perkin-Elmer Corp., Instrument Div. Norwalk, CT); delayed luminescence (Feitelson and Mauzerall, 1982) and fluorescence lifetimes were measured using instruments built in our laboratory.

RESULTS

To quantitate the O_2 effect in pigmented bilayers, the effect of O_2 on the dependence of the photosignal on the acceptor concentration was determined. It has been shown that the acceptor saturation curves are hyperbolic and thus characterized by two parameters: V_{max}, the photovoltage extrapolated to infinite concentration of acceptor, and K, the reciprocal of the concentration of acceptor required to reach one half V_{max} (Ilani and Mauzerall, 1981*b*), i.e., $V_{obs} = V_{max}\, KA/(1 + KA)$. It is found that O_2 does not affect V_{max} but increases K by a factor of 2–3. The preliminary results obtained by N_2 or O_2 bubbling (Ilani and Mauzerall, 1981*a*) are shown in Table I. A more delicate test on a single membrane and using the more thorough deoxygenation by glucose oxidase is shown in Fig. 1. In these experiments the photoresponse to three consecutive additions of acceptor are recorded. The solutions on both sides of the membrane are then made anaerobic by addition of glucose oxidase. The photoresponse is recorded again and similarly the effect of two further additions of acceptor are recorded. The acceptor concentrations are chosen such that valid determinations of K are available. The results (Fig. 1) show that the slope

TABLE I
EFFECT OF O_2 ON THE SATURATION CONSTANTS FOR DIFFERENT ACCEPTORS WITH MgOEP

Electron acceptor	O_2 enrichment*	K	O_2 depletion*	K
		mM^{-1}		mM^{-1}
$Ru(NH)_3^{+3}$	3	0.5	0.2	0.95
$Fe(CN)^{-3}$	3.5	1.6	0.25	2.2
Anthraquinone-2-sulfonate	3.5	21	0.4	35

*O_2 tension relative to air saturated solution.

($1/K$) is decreased 2–3-fold by O_2 but the intercept, V_{max}, is not. The results are similar with both pigments, magnesium octaethylporphyrin (MgOEP) and chlorophyll *a* (chl *a*). Since these data refer to the same membrane, there can be no question of the extraneous influence of variable membrane parameters. In fact, however, determination of V_{max} and K by complete concentration curves on separate membranes gave the same result (data not shown).

If the glucose-glucose oxidase was added only to the side opposite that containing the acceptor, one-fifth (chl *a*-methylviologen [MV]) to one-half (chl *a*-ferricyanide, MgOEP-MV) of the effect found on symmetrical addition was observed. Thus, a considerable gradient of O_2 across the membrane was established by this procedure. Although of interest in its own right, all further experiments refer to symmetrical deoxygenation.

The experiments were repeated for various concentrations of pigment in the lipid-bilayer–forming solution. The data are given in Table II, and V_{max} is plotted vs. pigment concentration in Fig. 2.

The reactivity of the singlet states of the pigment was directly measured by observing the fluorescence yield of chl-containing liposomes in the presence of increasing concentrations of acceptor (Fig. 3). The concentrations of acceptor required to half-saturate the quenching of fluorescence was 50 times that required to saturate the photoeffect for anthraquinone sulfonate and 200 times that for ferricyanide (Ilani and Mauzerall, 1981*b*) (if we ignore the questionable but reproducible 20% quenching at a much lower value; this may be caused by irreversible oxidation of some chl). These results indicate that it is the triplet state that caused the photoreaction in the lipid bilayer at usual acceptor concentrations. At high concentrations of acceptor, the singlet state may contribute to the interfacial charge transfer. The concentration of anthraquinone-2-sulfonate required to half-quench the fluorescence is rather low ~1 mM. We believe the cause is the same as that for the even lower concentration required by the photocharge transfer process (Ilani and Mauzerall, 1981*b*): this amphoteric compound is concentrated in the interfacial region relative to the aqueous solution. The conclusion is confirmed by the lack of fluorescence quenching by MV (Fig. 3). The dication may even be repelled by the choline dipole surface field (Ilani and Mauzerall, 1981*b*). The

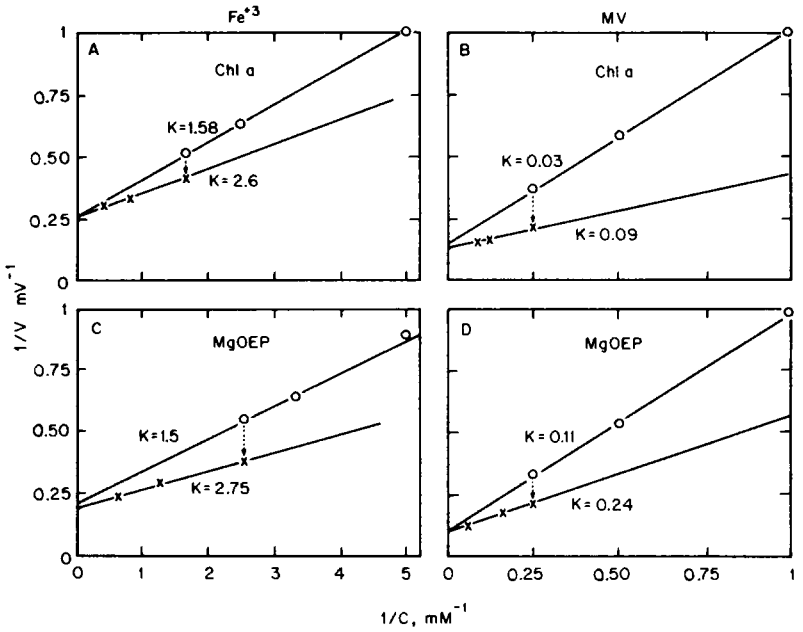

FIGURE 1 The reciprocal of the observed photovoltage is plotted vs. the reciprocal of acceptor concentration for two pigments, chl *a* (*A* and *B*) and MgOEP (*C* + *D*), and two acceptors, ferricyanide (*A* and *C*) and MV (*B* and *D*). The pigment concentration was 5 mg/ml (chl *a*) and 4 mg/ml (MgOEP). Other conditions were as given in the experimental section. Three additions of acceptor were measured, then at the dotted line the solutions were deoxygenated by adding glucose oxidase, followed by remeasurement and two further additions of acceptor. The full scale of the ordinate on each graph corresponds to the reciprocal of *A*, 1.4 mV; *B*, 0.4 mV; *C*, 1.8 mV, and *D*, 0.9 mV. The full scale of the abscissa on graph *B* is 0.2 mM^{-1}. Deoxygenation always results in an increase of the photovoltage and a decreased slope of the resulting curve, but in little or no change in the voltage extrapolated to infinite acceptor concentration.

quenching at 1 M concentration may be only a salt effect since even sodium chloride quenches to the same extent. The known quencher iodide is similarly poorly active, as is ascorbate. The observation that more than one-half of the pigment fluorescence could be quenched (Fig. 3) indicates that there is more pigment on the outside of the liposomes than inside (Ford and Tollin, 1982) and/or that some leakage of acceptor occurred.

The fluorescence yield of chl in these liposomes was quenched as the chl/lipid ratio increased but the lifetime shortened only to 4 ns (Table III). This indicates that the fluorescence originates in nonaggregated or very weakly aggregated chl, i.e., the system is heterogeneous. This fluorescence could even come from partially oxidized chl. Concentration quenching of chl is known to occur in concentrated solution (Losev, 1978) or near monolayer coverage on latex particles (Cellarius and Mauzerall, 1966). The experiment was carried out at various ratios of chl/lipid since the chl content of the bilayer is thought to be about one-tenth that of the membrane-forming solution (Steinemann et al., 1971; Cherry et al., 1971), which is 2 mg chl/10 mg lipid in decane-cholesterol. Even at the ratios used to obtain the data shown in Table III (0.025–0.25 mg chl/10 mg lipid) the fluorescence of chl is highly quenched.

The triplet state was monitored by the delayed luminescence of the pigment. This has been shown to be a very sensitive measure of these triplets (Feitelson and Mauzerall, 1982). The delayed luminescence of chl in a deoxygenated suspension of liposomes (50 μg chl/10 mg lipid) was half-quenched by ~5×10^{-4} M $K_3Fe(CN)_6$, very similar to that required to half-saturate the photovoltage (Fig. 1). Deoxygenated suspensions of chl-containing liposomes showed a 25–50-fold decrease in delayed luminescence for a 10-fold increase in chl/lipid ratio (Table III). The

TABLE II
EFFECT OF PIGMENT CONCENTRATION ON ACCEPTOR SATURATION CONSTANTS WITH AND WITHOUT O_2

Pigment (mM)	K (mM^{-1}) with O_2	K (mM^{-1}) no O_2	K (no O_2)/ K (with O_2)
MgOEP	*mV*		
7.2	0.11	0.24	2.2
2.4	0.15	0.42	2.8
0.8	0.075	0.24	3.2
Chl *a*	*mV*		
5.5	0.03	0.09	3
1.8	0.06	0.19	3.1
0.6	0.03	0.17	5.7
Chl *a*	ferricyanide		
5.5	1.6	2.6	1.6
1.83	1.4	3.1	2.2
0.6	1.4	3.1	2.3

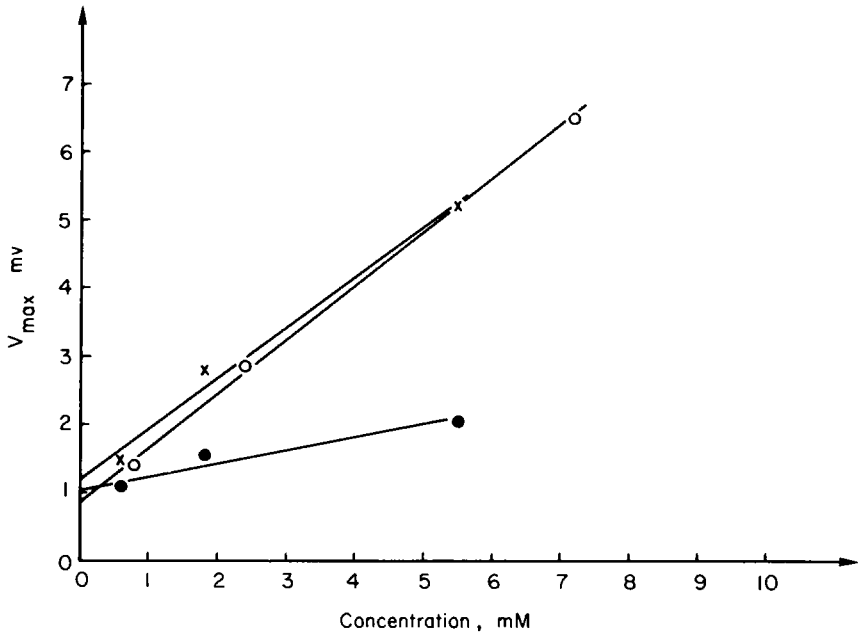

FIGURE 2 V_{max} is plotted vs. concentration of pigment in the membrane-forming solution. The various pigment-acceptor pairs are: O, MgOEP-MV; x, chl *a*-ferricyanide; and ●, chl *a*-MV. Other conditions are as given in the experimental section. Deoxygenation did not affect the values of V_{max} that were obtained from plots similar to Fig. 1.

observed lifetimes were distributed from 10 to 100 μs, i.e., the system again shows heterogeneity. Thus the triplet state is also highly quenched at the usual chl concentrations in the lipid bilayer. The observation of lifetimes nearly independent of decreasing yields in both fluorescence and delayed light is characteristic of aggregating systems where only the less-aggregated tail of the distribution of sizes of aggregates remains unquenched and thus observable (see below).

It was found that samples of oxidized chlorophyll gave

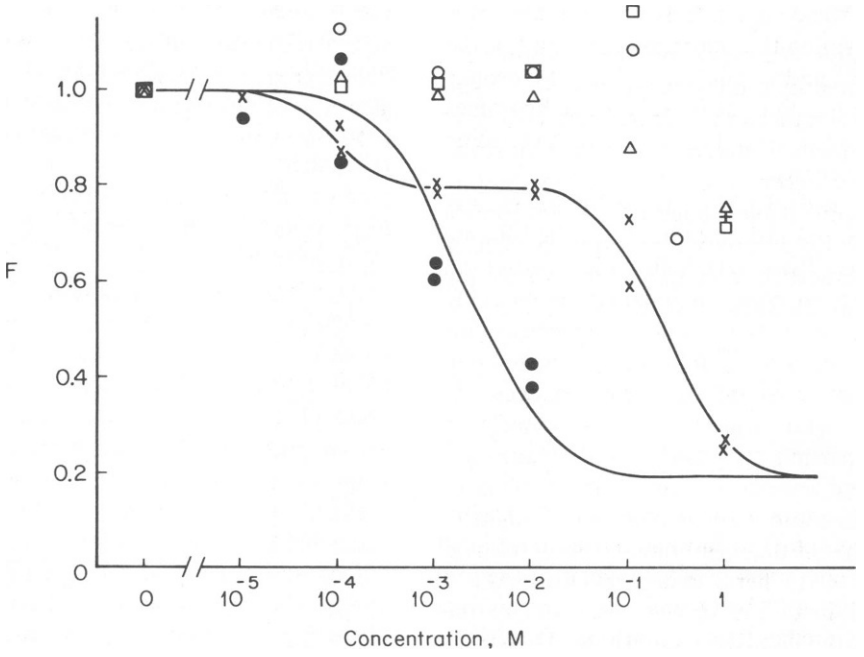

FIGURE 3 The relative fluorescence yields of chl-containing liposomes are plotted vs. the logarithm of the outside acceptor concentration. The various acceptors are: ●, anthraquinone-2-sulfonate; x, ferricyanide; O, ascorbate; △, iodide; □, methyl viologen; +, sodium chloride. The solid lines are hyperbolic saturation curves, $K = 0.5\ mM^{-1}$ for anthraquinone-2-sulfonate (AQS) and $(1/5)K = 10\ mM^{-1}$, $(4/5)K = 0.005\ mM^{-1}$ for ferricyanide, with 80% of the chl fluorescence quenchable. The double points for ferricyanide and AQS are the results of measurements at one-tenth the usual chl concentration, which was 5 mg/ml of membrane forming solution or 2 mg chl/10 mg lipid.

TABLE III
FLUORESCENCE HALF-LIFE AND DELAYED LUMINESCENCE FROM Chl *a* IN LIPOSOMES

Chl (μg/10 mg lipid)	Fluorescence half-life	Delayed luminescence per microgram of chl intensity, arbitrary units	
		(40 μs)	(150 μs)
	ns		
25	4.3	4.8	1.0
50	—	2.4	0.3
125	3.7	0.5	0.1
250	3.7	0.1	0.04

Delayed luminescence was measured after deoxygenation of the liposome suspension with glucose-glucose oxidase. An N_2 laser was used as actinic source, together with a gated photomultiplier. The delayed luminescence is quenched by O_2. The observed half-lives were nonexponential and distributed from 10 to 100 μs. That of chl in dilute organic solvents is 300 μs. The fluorescence was measured with a 300 ps pulse of light, high speed PM and digitizer. The half-life of chl *a* in ether was measured to be 5.2 ns.

considerably more delayed luminescence than pure samples. Since some oxidation of chlorophyll doubtlessly occurs in the bilayers, quantitative analysis of the delayed luminescence is set aside. This reservation applies also to the measurements of fluorescence but is less crucial since chl *a* fluorescence yield is equal to or greater than that of oxidized chl. In fact a ratio of fluorescence yield to delayed luminescence may be a useful way to unravel the state of chl in bilayers.

DISCUSSION

The effect of the presence of O_2 on the photovoltages developed in pigmented lipid bilayers adjacent to a single solution of acceptors is rather small. Decreasing the oxygen concentration >100-fold only decreases the half-saturating concentration (K) of acceptor by 2–3-fold and has no effect on the maximum photovoltage (V_{max}) obtained by extrapolating to infinite acceptor concentration (Fig. 1, Table I). If O_2 competes with acceptor for the excited state, then following Ilani and Mauzerall (1981*b*):

$$V = V_{max}\frac{P^* KA}{KA + 1} \quad (1)$$

$$K = \frac{k_3}{k_1 O_2 + k_2}, \quad (2)$$

where k_3 is the rate constant for reaction of P* (excited pigment) with A (acceptor) to form stabilized interfacial ions that produce the observed photovoltage, k_1 is the rate constant for quenching of P* by O_2 and $1/k_2$ is the lifetime of P*. The equations predict that V_{max} will be independent of O_2 at infinite concentration of acceptor, as is observed. The fact that complete O_2 depletion led to an increase of about threefold in K suggests that $k_1 O_2 \simeq 2k_2$ at atmospheric levels of O_2, i.e., 6×10^{-4} M in water. Since quenching of excited states by O_2 occurs near the encounter limit, i.e., $k_1 \sim 10^{10}\ M^{-1}\ s^{-1}$, then $k_2 \sim 3 \times 10^6\ s^{-1}$. This value is too slow to be identified with the reciprocal lifetime of the singlet state (10^9–$10^8\ s^{-1}$), but is also much faster than that of the triplet state ($3 \times 10^3\ s^{-1}$ for chl *a*, $30\ s^{-1}$ for MgOEP). These inferences are verified by direct measure of these excited states in liposomes. While there may well be differences between interfacial reactions of liposomes and that of planar lipid bilayers, the results are sufficiently clear that these relatively minor differences will not affect the conclusions. The availability of macroscopic liposomes (Mueller et al., 1983) may allow the comparison of these similar bilayer systems in the future. It is found that the concentration of acceptor required to half-quench the fluorescence of chl *a* is ~100-fold higher than that required to half-saturate the photoeffect (Fig. 3). Thus the singlet state is not the reactive state at the usual millimolar concentrations of acceptor. The decreased yield of delayed luminescence with increasing pigment concentration (Table III) shows that the shortened lifetime of the triplet state is caused by concentration quenching. The observed lifetimes of both the singlet and the triplet (Table III) are strong evidence for a heterogeneous distribution of aggregate size of the pigment in the lipid bilayers. As one increases the concentration of pigment, the yield of observable prompt and delayed fluorescence decreases strongly, but the observed lifetimes change by lesser factors. Increasing the concentration of the substance increases the fraction of condensed form but has little effect on the distribution of monomers or small, loose aggregates. Eigenberg et al. (1982*a*) have shown by differential thermal analysis that chl in distearoylphosphatidylcholine can form a heterogeneous system of differing phases. Nuclear magnetic resonance (NMR) evidence was obtained for at least two forms of chl, at least one of which involved strong interaction between phospholipid head groups and chl *a* (Eigenberg et al., 1982*b*). Thus one can think of the pigment in the bilayer as being in two forms: the condensed form wherein luminescence is quenched, and the loose form wherein the lifetimes are only somewhat shortened and typically distributed in value. The increase of V_{max} with increasing pigment concentration (Fig. 2) shows that the condensed form is the source of most of the intrafacial ions (photovoltage) formed at high pigment concentration. The finite photovoltage remaining on linear extrapolation to zero pigment concentration represents that voltage arising from the monomeric or loosely aggregated forms of the pigment. Concentration quenching of porphyrin triplet states has been shown to be an efficient source of pigment anions and cations in solution (Ballard and Mauzerall, 1980). Thus the absorption of light in the condensed form of the pigment produces pigment cations and anions. The latter react (≤ 1 μs) at the interface with the acceptor to form intrafacial ions that produce the observed photovoltage. The escape of the ions from the first formed pair is favored by the initial triplet spin orientation of the radicals (Mauzerall and Ballard, 1982). It may be further

enhanced by the sharp changes of electric field present in the polar region of the bilayer-water interface (Ilani and Mauzerall, 1981*b*). The rather indifferent variation of K with pigment concentration (Table III) indicates that the photovoltage is formed largely via the condensed form of the pigment or that the reaction rates (and thus K) of acceptor with P^- and P^* are similar.

What of the formation of ions via the O_2 quenching reaction? In the absence of other acceptors or donors, no photovoltage is seen because of the vectorial nature of the interfacial electron transfer, and the presence of equal concentrations of O_2 on both sides of the membrane. It is thus similar to neutral quinones (Krakover et al., 1981). The fraction of ions formed from O_2 quenching can be readily estimated by adding sufficient donor to one side such that the lifetime of the pigment cation is reduced to <1 μs. This experiment has repeatedly been shown to form $<10\%$ of the V_{max} observed with ionic acceptors. This is in agreement with photoconductivity measurements in various solvents, where the ion yield from the reaction of triplet ZnOEP with O_2 is $<1\%$ of that with ZnOEP itself, i.e., that reaction of concentration quenching (Ballard and Mauzerall, 1980). Other photosignals observed on a very slow time scale, $\sim$100 ms, will be shown to be caused by O_2^- (Woodle, M., and D. Mauzerall, manuscript in preparation). A kinetic treatment of the different reaction paths of condensed and loose pigments and of the O_2 effect verify the above conclusions.

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