Mason, J. T., Huang, C., & Biltonen, R. L. (1981) *Biochemistry 20*, 6086–6097.
McIntosh, T. J. (1978) *Biochim. Biophys. Acta 649*, 325–335.
McIntosh, T. J. (1980) *Biophys. J. 29*, 237–246.
McIntosh, T. J., McDaniel, R. V., & Simon, S. A. (1983) *Biochim. Biophys. Acta 731*, 109–114.
McIntosh, T. J., Simon, S. A., Ellington, J. C., & Porter, N. A. (1984) *Biophys. J. 45*, 41a.
Moody, M. F. (1963) *Science (Washington, D.C.) 142*, 1173–1174.
Nagle, J. F. (1980) *Annu. Rev. Phys. Chem. 31*, 157–195.
Nagle, J. F., & Wilkinson, D. A. (1978) *Biophys. J. 23*, 159–176.
O'Brien, J. S., & Rouser, G. (1964) *J. Lipid Res. 5*, 339–342.
Pearson, R. H., & Pascher, I. (1979) *Nature (London) 281*, 499–501.
Ranck, J. L., Keira, T., & Luzzati, V. (1977) *Biochim. Biophys. Acta 488*, 432–441.
Reiss-Husson, F. (1967) *J. Mol. Biol. 25*, 363–382.
Shannon, C. E. (1949) *Proc. Inst. Radio Eng. N.Y. 37*, 10–21.
Tardieu, A., Luzzati, V., & Reman, F. C. (1973) *J. Mol. Biol. 75*, 711–733.
Worthington, C. R., King, G. I., & McIntosh, T. J. (1973) *Biophys. J. 13*, 480–494.
Zaccai, G., Buldt, G., Seelig, A., & Seelig, J. (1979) *J. Mol. Biol. 134*, 693–706.

# Lateral Diffusion of Plastocyanin in Multilamellar Mixed-Lipid Bilayers Studied by Fluorescence Recovery after Photobleaching†

M. Fragata,* S. Ohnishi, K. Asada, T. Ito, and M. Takahashi

ABSTRACT: The lateral diffusion of fluoresceinyl isothiocyanate labeled plastocyanin (FITC-PCY) from spinach chloroplasts reconstituted into egg yolk phosphatidylcholine (PCh) bilayer membranes with and without stearylamine (SA) was examined by using fluorescence recovery after photobleaching. SA was included in the membranes to simulate a positively charged surface for the binding of plastocyanin, which behaves as an anion at pH 7.4 used throughout this work. As a reference, the lateral diffusion of the probe *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) was also studied in the same bilayer systems. The diffusion coefficient ($D$) of NBD-PE in PCh bilayers was found to be (4.4 ± 0.4) × $10^{-8}$ $cm^2/s$ at 296 K in agreement with results published in the literature for experiments run at comparable temperatures (287–298 K). In mixed PCh–SA (5:1) bilayers, however, $D$ is (6.4 ± 0.5) × $10^{-8}$ $cm^2/s$. On the one hand, it is noted that the diffusion of FITC-PCY in PCh and PCh–SA bilayers cannot fit the theory for a single diffusion coefficient. Plotting the data according to the expression for a multicomponent system reveals the presence of at least one fast-moving and one slow-moving species. In PCh bilayers, $D_{fast}$ ~ (4.9 ± 0.8) × $10^{-8}$ $cm^2/s$ and $D_{slow}$ ~ (8.1 ± 0.2) × $10^{-10}$ $cm^2/s$. In PCh–SA bilayers, $D_{fast}$ ~ (6.3 ± 0.7) × $10^{-8}$ $cm^2$ and $D_{slow}$ ~ (7.9 ± 0.3) × $10^{-10}$ $cm^2/s$. This shows that the $D_{fast}$ values do not differ appreciably from the diffusivities determined for NBD-PE. Another interesting point is that the slow-moving species seems to be insensitive to the presence of SA in the bilayer. On the other hand, treatment of the plastocyanin samples with dithiothreitol (DTT), a reducing agent that hinders the formation of plastocyanin dimers, has the effect of changing the relative contents of the fast and slow species, i.e., $[PCY_{fast}]/[PCY_{slow}]$, from about 3.1 (nontreated samples) to 11.5 (DTT-treated samples). This is a good indication that the fast-moving species is a monomer whereas the slow-moving species is an aggregated form. Finally, comparing the $D_{slow}$ values with a two-dimensional diffusion coefficient of ca. 2 × $10^{-9}$ $cm^2/s$ estimated by Takano [Takano, M., Takahashi, M. A., & Asada, K. (1982) *Arch. Biochem. Biophys. 218*, 369–375] for the diffusion of plastocyanin in the thylakoid membrane, we suggest that the electron donor to $P700^+$ in vivo is most probably an aggregated form of the molecule.

In green plants and algae, the electron-transport sequence between photosystem II (PS II)[1] and photosystem I (PS I) starts with a photoinduced electron transfer from a chlorophyll special pair (Katz et al., 1978; Shipman et al., 1976) to a primary acceptor and ends with the reduction of $P700^+$, the reaction-center chlorophyll of PS I (Sauer, 1975). The electron donor to $P700^+$ is plastocyanin, a "blue" copper protein that has been found to function either at the internal surface of the thylakoid membrane (Haehnel et al., 1981) between cytochrome *f* and the P700 complex (Avron & Shneyour, 1971; Haehnel et al., 1980) or in the stroma membrane (K. Asada, M. A. Takahashi, and M. Takano, personnal communication). Monomeric PCY has a single type 1 Cu atom buried in a pocket surrounded by six to seven hydrophobic residues (Colman et al., 1978; Freeman, 1981). Another important feature of the molecule is a negatively charged patch; in poplar plastocyanin, for instance, it spreads from residues 42–44 to residues 59–61 (Colman et al., 1978). Of particular interest is the suggestion that the anionic hydrophilic patch and the

† From the Department of Biophysics (S.O. and T.I.) and the Research Institute for Food Science (K.A. and M.T.), Kyoto University, Kyoto, Japan, and the Centre de recherche en photobiophysique, Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada G9A 5H7 (M.F.). *Received June 17, 1983; revised manuscript received March 16, 1984.*

[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DTT, dithiothreitol; FITC, fluoresceinyl isothiocyanate; FITC-PCY, FITC-labeled plastocyanin; FRAP, fluorescence recovery after photobleaching; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PCh, egg yolk phosphatidylcholine; PCY, plastocyanin; PS I, photosystem I; PS II, photosystem II; SA, stearylamine; cyt, cytochrome; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/84/0423-4044$01.50/0 © 1984 American Chemical Society

hydrophobic region are instrumental in the recognition of the PCY binding sites on P700 and cyt *f*, respectively (Farver et al., 1982).

To explain electron transfer to and from the metal center, the most commonly accepted mechanisms are quantum-mechanical tunneling and outer sphere collision interactions (McElroy et al., 1974; Hopfield, 1974; Moore & Williams, 1976; Holwerda et al., 1976). A characteristic of such mechanisms is a donor–acceptor orbital-overlapping step to allow electron delocalization from the metal atom of the donor to the acceptor protein. That is, donor and acceptor centers have in some way to be brought together with appropriate orientations in order for the energy levels to match. This raises the question of the functional relevance for $P700^+$ reduction of lateral and rotational diffusions of PCY at the aqueous interface of the thylakoid membrane. In a closely related system, the mitochondrial inner membrane, a correlation was found between energy transducing and lateral diffusion of membrane proteins (Höchli & Hackenbrock, 1979; Schneider et al., 1980; Sowers & Hackenbrock, 1981). A recent study of Takano et al. (1982) provides support for the view that lateral diffusion of PCY occurs in the thylakoid membrane prior to electron transfer to $P700^+$ [see also, Olsen & Pedersen (1983)]; a diffusion coefficient, or diffusivity [see, e.g., Shinitzky & Yuli (1982)], of about $2 \times 10^{-9}$ cm$^2$/s was estimated. In this work, we observed that the diffusion of FITC-labeled PCY in multilamellar lipid bilayers is characterized by at least one fast and one slow diffusion coefficient. The results show that the lower diffusivity may characterize FITC-PCY aggregates of yet undetermined size. The significance of this finding in relation to electron transfer in the thylakoids will be discussed.

## Experimental Procedures

*Chemicals.* Phosphatidylcholine was isolated from egg yolk and purified according to the method of Singleton et al. (1965). NBD-PE was obtained from Avanti Biochemicals and DTT from Wako Pure Chemicals. FITC, isomer I [see details in Steinbach (1974)], was a product of Sigma Chemical Co. (lot 21F-5014). These chemicals were used as such upon purity verification. All other products were reagent grade.

*Extraction and Purification of PCY.* Plastocyanin was extracted from spinach leaves and purified according to the method of Katoh et al. (1962). The fraction used in the experiments had a ratio $A_{597}/A_{278}$ of 0.64 and migrated as a single band on polyacrylamide gel electrophoresis. The plastocyanin concentration was determined spectrophotometrically with a molar absorbance coefficient of 4.5 mM$^{-1}$ cm$^{-1}$ at 597 nm (Milne & Wells, 1970).

*PCY Labeling with FITC.* Binding of FITC to plastocyanin was done according to Landel's method [Landel, 1976; see also, Garvey et al. (1977)]. Samples (8 mL) of a stock solution of PCY (~0.6 mg/mL) in 10 mM phosphate buffer, pH 7.6, were first dialyzed 3 times against 1 L of 0.5 M carbonate–bicarbonate buffer, pH 9.2. The PCY samples were then incubated overnight (~20 h, dark, 4 °C) in the same buffer with adequate aliquots of an ethanolic solution of FITC (~2.8 mg/mL) so that the final molar ratio of PCY to FITC was about 1:60. At the end of the incubation, the samples were dialyzed 3 times against 10 mM phosphate buffer, pH 7.4. This was followed by gel filtration with the same buffer in a Sephadex G-25 column. The fractions (~2.5–3.0 mL) containing the pure FITC-PCY were stored at about −15 °C.

*Measurement of FITC-PCY Activity.* The electron-carrier activity of FITC-labeled plastocyanin was measured as follows. Class II spinach chloroplasts were incubated in 2 M KCl at 5 °C for 15 h to delete about 80% of thylakoid-bound plastocyanin (M. A. Takahashi, M. Takano, and K., Asada, unpublished results). PS I dependent $O_2$ uptake was measured in a reaction mixture (1 mL) containing plastocyanin-depleted thylakoids (15 μg of chlorophyll), 10 mM sodium ascorbate, 0.1 mM dichlorophenolindophenol, 0.1 mM sodium azide, 0.2 mM methyl viologen, 50 mM Tris-HCl, pH 7.6, and various PCY and FITC-PCY concentrations. The reaction was carried out in a vessel of a Hansatech oxygen electrode (England) irradiated with red actinic light (λ >650 nm, 22.8 mW cm$^{-2}$) at 25 °C.

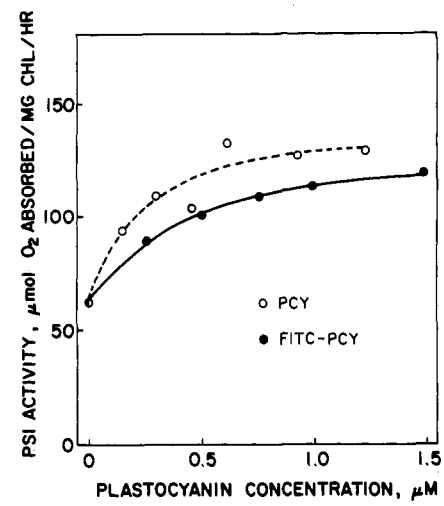


FIGURE 1: Photosystem I activity of spinach chloroplasts vs. concentration of FITC-labeled and nonlabeled plastocyanin. For reaction conditions, see Experimental Procedures.

Figure 1 shows that the addition of FITC-PCY to plastocyanin-depleted chloroplasts restored their activity of $O_2$ uptake driven by PS I. Although the maximum rates of PS I reduction by labeled and unlabeled PCY are similar, their $K_M$'s are significantly different. The results let us conclude that FITC-PCY is more loosely bound to the membrane than unlabeled PCY. It is worth mentioning in this connection that the affinity of PCY for the thylakoid membrane decreases as the p*I* of the protein becomes more acidic (Davis et al., 1980). Though this may explain the data represented in Figure 1 on account of the neutralization of the terminal $-NH_2$ group of PCY upon FITC binding, we feel that conformational changes due to the presence of FITC at the protein surface may also have to be taken into consideration.

*Treatment of PCY with DTT.* The procedure of Siegelman et al. (1976) was followed with slight modifications. Samples (0.3 mL) of FITC-PCY solutions (0.4 mg/mL) in 15 mM NaCl–10 mM phosphate buffer, pH 7.6, were incubated in the dark with DTT (final concentration 50 mM) at laboratory temperature for about 16 h followed by 5-h dialysis against several changes of a large excess of the same buffer solution. The samples were then incubated with the membranes and prepared for FRAP experiments as outlined below. The effect of unbound DTT on lipid diffusion in the membranes was tested. We observed that the diffusion of NBD-PE in DTT-treated membranes cannot be distinguished from its diffusion in nontreated membranes.

*Membrane Preparation.* The membranes were formed according to the standard procedure of Fahey & Webb (1978) slightly modified. Membranes with no protein included were prepared as follows. Samples (20 μL) of a 200 mg/mL stock solution of egg yolk PCh in chloroform–methanol (2:1) were mixed with NBD-PE (in chloroform–methanol solution) aliquots or with NBD-PE and SA (in chloroform solution) aliquots to give final molar fractions of NBD-PE to PCh and

Table I: Lateral Diffusion Coefficients ($D$) of NBD-PE[a] and FITC-PCY in Lipid Bilayers of Different Composition

| lipid composition[a] | exptl temp (K)[b] | NBD-PE[c] $D \times 10^8$ | FITC-PCY[c] $D_{fast} \times 10^8$ | FITC-PCY[c] $D_{slow} \times 10^{10}$ | ref |
|---|---|---|---|---|---|
| PCh | 287 | 4.4 ± 0.3 | | | *d* |
| | 288 | ~3.5 | | | *e* |
| | 296 | 4.4 ± 0.4 | 4.9 ± 0.8 | 8.1 ± 0.2 | this work |
| | 298 | 4 | | | *f* |
| PCh–SA (5:1) | 296 | 6.4 ± 0.5 | 6.3 ± 0.7 | 7.9 ± 0.3 | this work |

[a] See footnote 1. [b] The phase transition temperature for egg yolk PCh may differ from preparation to preparation; a value of about 249–251 K was obtained (F. Bellemare and M. Fragata, unpublished data) with a Model DSC-2 Perkin-Elmer differential-scanning calorimeter equipped with a liquid nitrogen subambient accessory and standardized with an indium sample. [c] $D$ is in $cm^2/s$; the uncertainties represent ± SD about the mean for six to eight experiments. [d] Derzko & Jacobson (1980). [e] Rubenstein et al. (1979). [f] Wu et al. (1977).

NBD-PE to SA to PCh of ca. 1:800 and 1:160:800 (the reasons for the use of SA will be discussed under Results), respectively. The mixtures were spread in flat-bottom tubes of about 1-cm diameter, and the solvent was evaporated under a weak current of nitrogen. This was followed by 2-h drying under vacuum. The dried lipids were hydrated overnight (~16 h) at laboratory temperature in 0.2 mL of 15 mM NaCl–10 mM phosphate buffer, pH 7.4. This ionic strength assured [cf. Fahey & Webb (1978)] that large lipid vesicles and/or multibilayers of diameters between 20 and 90 μm, or greater, were formed. At the end of the hydration period, the samples were centrifuged at low speed (~1500$g$) for about 3–5 min to eliminate at least one part of the vesicles much smaller than 20 μm. The vesicles were collected with a capillary tube, and a drop of the suspensions was deposited on a microscope slide and covered with a cover slip. Excess buffer solution was removed with absorbing paper. The preparations were then sealed with paraffin to avoid water evaporation and used as such in FRAP experiments.

The membranes containing FITC-PCY were prepared according to two different methods. In one of them, the membranes were first formed as described earlier, and 0.1–0.2-mL aliquots were incubated afterward with the FITC-PCY solutions in the dark at 30 °C for a period of 30 min (molar ratio of PCh to FITC-PCY ~400). This was followed by centrifugation at about 1500$g$ for 3–5 min. The precipitate was recovered and the centrifugation repeated twice. The final precipitate was suspended in 0.2–0.5 mL of 15 mM NaCl–10 mM phosphate buffer, pH 7.4. In another method, the membrane preparations were submitted to overnight incubation (~16 h) with FITC-PCY solutions (laboratory temperature, darkness) followed by the centrifugation procedures described above. The diffusivity values obtained with the two methods do not differ significantly.

*Fluorescence Recovery Measurements.* The fluorescence recovery instrument used to measure diffusion coefficients is the one described by Chang et al. (1981), which was constructed according to Axelrod et al. (1976) and Koppel et al. (1976). In this work, a light beam from an argon ion laser (Spectra Physics Model 164-06) was directed through the optics of an MPM 01K Zeiss Universal microscope (×16 objective) to the membrane preparations on the microscope stage. The photons emitted by the fluorescence probes present in the membrane were detected by a Hamamatsu R464S photomultiplier and counted in a photon-counting system. An Apple II microcomputer controlled the bleaching time, the photon-counting conditions, and other operations. It registered as well the photon counts.

Since the shape of the laser beam used in our experiments was confirmed to have a Gaussian profile, as described previously (Chang et al., 1981), with a $1/e^2$ radius equal to 3.6 μm, the data were analyzed according to the expression

$$F(t) = F(-)\nu K^{-\nu}\Gamma(\nu)P(2K|2\nu) \quad (1)$$

developed by Axelrod et al. (1976) for the fluorescence intensity $F(t)$ at time $t$ after the bleaching pulse. $\Gamma(\nu)$ is the $\gamma$ function, $P(2K|2\nu)$ is the $\chi^2$ probability distribution, and $\nu = (1 + 2t/\tau_D)^{-1}$. $\tau_D$, the characteristic diffusion time, is equal to $w^2/(4D)$, where $w$ is the $1/e^2$ radius of the focused laser beam and $D$ is the lateral diffusion coefficient. The time $\tau_{1/2}$ required to recover half of the bleach intensity is related to $\tau_D$ by the expression $\tau_{1/2} = \gamma_D\tau_D$. $\gamma_D$, a dimensionless parameter, depends upon beam shape and $K$ [cf. Figure 7 of Axelrod et al. (1976)]. The fluorescence intensity before the bleaching pulse $F(-)$ is given by

$$F(-) = qP_0C_0/A \quad (2)$$

where $q$ is the product of all quantum efficiencies of light absorption, emission, and detection, $P_0$ is the total laser power, $C_0$ is the fluorophore concentration before the bleaching pulse, and $A$ is the attenuation factor of the beam during observation of recovery. $K$, the amount of photobleaching induced by a short bleaching pulse, is obtained from

$$F(0) = F(-)K^{-1}(1 - e^{-K}) \quad (3)$$

where $F(0)$ is the fluorescence intensity immediately after the bleaching pulse. A series solution for eq 1 that is valid for all $K$ and $t$ values is given by the function

$$Q(t) = \frac{F(-) - F(t)}{F(-)} = -\sum_{n=1}^{\infty} \frac{(-K)^n}{(n+1)! + n!2nt/\tau_D} \quad (4)$$

## Results

*Lateral Diffusion of NBD-PE.* Typical fluorescence recovery curves for NBD-PE in egg yolk phosphatidylcholine multilayers prepared with SA and in its absence are presented in Figure 2. The theoretical curves were obtained from the recovery equation of Axelrod et al. (1976) by taking $K = 2.3$ for the mixture NBD-PE–SA–PCh = 1:160:800 and $K = 2.0$ for NBD–PE–PCh = 1:800. The characteristic diffusion time $\tau_D$ was found to be 0.55 and 0.80 s, respectively. For a laser beam radius of ~3.6 μm at specimen plane ($e^{-2}$ intensity point; see above), the calculated diffusion coefficients were $5.9 \times 10^{-8}$ $cm^2/s$ for bilayers prepared with SA and $4.0 \times 10^{-8}$ $cm^2/s$ without SA. A comparison of the results with data reported by other authors shows that the lateral diffusivity of NBD-PE at 23 °C in egg yolk phosphatidylcholine lamellae is in the range of values determined previously (Derzko & Jacobson, 1980; Wu et al., 1977) in bilayer systems of similar composition (Table I). The table shows in addition that NBD-PE has diffusivities up to 50% higher in PCh–SA bilayers than in membranes prepared with pure PCh. This result is interesting because it shows clearly that SA fluidifies the phosphatidylcholine bilayers at about 20 mol % used throughout the present work. Though there is not at the present time any simple explanation for the observed differences, it is possible that such effects result from generation of holes (or vacancies)

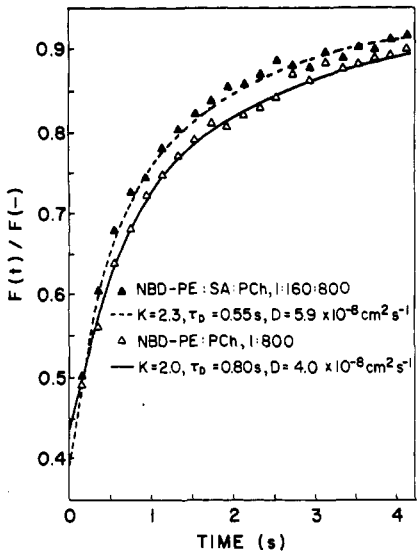


FIGURE 2: Typical fluorescence recovery curves after photobleaching of NBD-PE in phosphatidylcholine (PCh) and stearylamine–phosphatidylcholine (SA–PCh) bilayers. The triangles are experimental values, and the lines represent theoretical recovery curves for one diffusing species with $K$ equal to 2.0 (PCh) and 2.3 (SA–PCh). $F(t)$ is the fluorescence intensity after photobleaching and $F(-)$ the intensity before photobleaching.

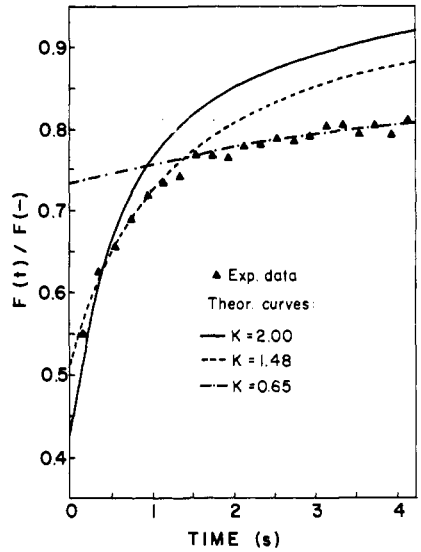


FIGURE 3: Typical time course of FITC-PCY fluorescence recovery after photobleaching in stearylamine–phosphatidylcholine bilayers. The triangles are experimental values, and the lines represent theoretical curves for one diffusing species with $K$ equal to 0.65, 1.48, and 2.00. It is seen that the curves do not fit the theory for a single diffusion coefficient. See caption of Figure 2 for the meaning of $F(t)$ and $F(-)$.

in the lipid bilayers induced by stearylamine (M. El-Kindi and M. Fragata, unpublished data).

*Lateral Diffusion of FITC-Labeled Plastocyanin.* The equations of Axelrod et al. (1976) for a Gaussian laser beam were applied to the analysis of the time course of FITC-PCY fluorescence recovery after bleaching in multibilayers prepared with PCh and binary mixtures of PCh and SA. The theoretical curves were obtained with $K$ values ranging from 0.5 (~21% bleach) to 2.0 (~57% bleach). We found that the results cannot fit the theory for a single diffusion coefficient (cf. Figure 3). A straightforward assumption is that the samples contain two or more independent diffusing species with different mobilities. To test this hypothesis, the data was first fitted with a model for two independent FITC-PCY populations (i.e., $m = 2$ in eq 5) characterized by different diffusion

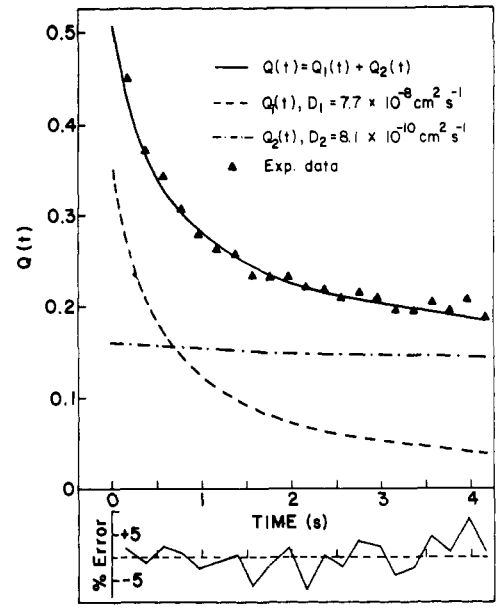


FIGURE 4: Time course of FITC-PCY fluorescence recovery after photobleaching in phosphatidylcholine–stearylamine bilayers. The triangles represent the experimental data of Figures 3 and 5. The theoretical curves were drawn according to an expression for two independent diffusing species (see eq 5). $Q(t) = [F(-) - F(t)]/F(-)$. See caption of Figure 2 for the meaning of $F(t)$ and $F(-)$. Percent error = $([Q(t)_{obsd} - Q(t)_{calcd}] \times 100)/Q(t)_{obsd}$.

coefficients ($D_1$ and $D_2$). The phenomenological expression is

$$Q(t) = -\sum_{k=1}^{m}\sum_{j=1}^{n}\frac{\alpha_k(-K)^j}{(j+1)! + j!2jt/\tau_{D_k}} \qquad (5)$$

where $\alpha_k$ is the fraction of species $k$ present in the samples and $\sum_k \alpha_k = 1$. The curve fitting was performed in a Perkin-Elmer 3600 Data Station with the Perkin-Elmer version 2.10 of Microsoft 6800 Basic. A reliable approximation was obtained by limiting the infinite sum to the first 9–12 terms of the series for $K \leq 2.7$. In the particular case represented in Figure 4, we put $K = 1.77$, $\alpha_1 = 0.695$, $\alpha_2 = 0.305$, $\tau_{D_1} = 0.42$ s, and $\tau_{D_2} = 40$ s. The presence of a fast- and a slow-moving species is promptly recognized. The calculated diffusivities for a laser beam radius of $w = 3.6$ $\mu$m (see above) are respectively $D_1 = 7.7 \times 10^{-8}$ cm$^2$/s and $D_2 = 8.1 \times 10^{-10}$ cm$^2$/s. Note in this respect that although the excellent agreement between theory and experiment is to be emphasized (average error ~2.5%; cf. Figure 4), we do not exclude however the possibility of the presence in the samples of a number of independent diffusing species larger than two.

The hypothesis of multiple independent populations of diffusing species was further corroborated by plotting the data according to a method developed recently by Yguerabide et al. (1982). In brief, these authors showed that in the absence of flow the reciprocal function

$$R(t) = F(\infty)/[F(\infty) - F(t)] \qquad (6)$$

where $F(\infty)$ is the total fluorescence intensity after full recovery at long times, is a linear function of $t$ if the laser beam profile is Gaussian and the recovery involves a single diffusion coefficient. Thus, a nonlinear $R(t)$ vs. $t$ plot characterizes an inhomogeneous set of diffusing species (see Figure 5). Note, however, that errors in the evaluation of $F(\infty)$ are as well at the origin of nonlinear results [cf. in this respect Figures 3 and 6 of Yguerabide et al. (1982)]. But, we observed that in the majority of our experiments (~90–95%) the photon counts before bleaching and at full recovery after bleaching do not

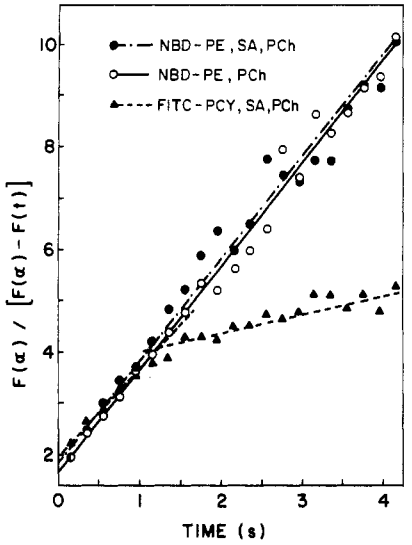


FIGURE 5: Reciprocal plots of fluorescence recovery data from NBD-PE in phosphatidylcholine and stearylamine–phosphatidylcholine bilayers and FITC-PCY in stearylamine–phosphatidylcholine bilayers. The graph shows that, in contrast to NBD-PE diffusion, FITC-PCY diffusion is characterized by at least two diffusivity values. $F(t)$ is the fluorescence intensity after photobleaching and $F(\infty)$ the total fluorescence intensity after full recovery at long times.

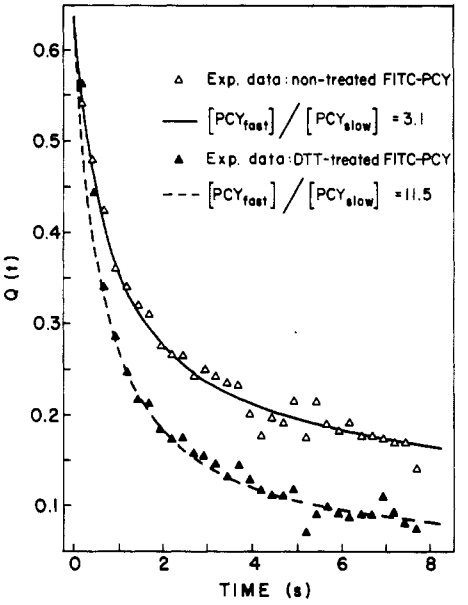


FIGURE 6: Time course of fluorescence recovery of nontreated and DTT-treated FITC-PCY in phosphatidylcholine–stearylamine bilayers. The theoretical curves were drawn according to an expression for two independent diffusing species (see eq 5). $[PCY_{fast}]$ is amount of fast-moving FITC-PCY present in the lipid bilayers (see text). $[PCY_{slow}]$ is amount of slow-moving FITC-PCY present in the lipid bilayers. DTT is dithiothreitol. $Q(t) = [F(-) - F(t)]/F(-)$. See caption of Figure 2 for the meaning of $F(t)$ and $F(-)$.

differ significantly. This excludes, therefore, spurious nonlinear effects. Hence, the data presented in Figure 5 constitute a good indication of the presence in the lipid bilayers of PCY species with different diffusivities.

In Table I we give the average diffusivities ($D_{fast}$ and $D_{slow}$) obtained from a series of experiments performed with FITC-PCY included in PCh bilayers prepared with and without SA. The table shows in particular that the $D_{fast}$ values do not differ appreciably from the diffusivities determined for NBD-PE. In this connection, it is remarked that the inclusion of SA in the bilayers was dictated by the assumption that a positively charged surface would favor the binding of the PCY anion [see in this respect Redwood & Patel (1974) and Ptak et al. (1980)]. Such methods were used previously (Kimelberg et al., 1970) with other membrane surface proteins, namely, the cytochrome *c* cation, which needs a negatively charged surface produced by cardiolipin or phosphatidic acid. The necessity for using these experimental procedures is not evident in the present experiments.

Another interesting outcome is that the slow-moving FITC-PCY seems to be insensitive to the presence of SA in the bilayers (cf. Table I). This puts the question of the localization of the protein in the lipid lamellar system. Before approaching this point, we will try first to clarify the nature of the slow-diffusing species and of its diffusion environment as well (see next section and Discussion).

*Effect of Dithiothreitol on $\alpha_1$ and $\alpha_2$.* The $\alpha$ values of eq 5 are a measure of the relative contents of fast- and slow-diffusing FITC-PCY present in the bilayers. For example, the curves represented in Figure 4 show that 69.5% of FITC-PCY is a fast-diffusing species ($\alpha_1 = 0.695$) whereas 30.5% of it has a lower mobility ($\alpha_2 = 0.305$). One can take advantage of this property to elucidate the state of plastocyanin in the membranes. To this purpose, the labeled protein was treated with dithiothreitol (DTT), a reducing agent that has been used successfully to hinder the formation of plastocyanin dimers (Siegelman et al., 1975, 1976). A typical result is displayed in Figure 6. The experimental points are averages of six (nontreated FITC-PCY) and eight (DTT-treated FITC-PCY) experiments performed, each one with different lipid bilayer samples. The data were fitted with a theory for a two-component system, i.e., puting $m = 2$ in eq 5. In summary, the theoretical curve for nontreated FITC-PCY was drawn with $K = 2.65$, $\alpha_1 = 0.76$, $\alpha_2 = 0.24$, $\tau_{D_1} = 0.55$ s, and $\tau_{D_2} = 34$ s and that for DTT-treated FITC-PCY with $K = 2.65$, $\alpha_1 = 0.92$, $\alpha_2 = 0.08$, $\tau_{D_1} = 0.48$ s, and $\tau_{D_2} = 35$ s.

From the $\alpha_1$ and $\alpha_2$ values, we calculate that the ratio $[PCY_1]/[PCY_2]$, where $[PCY_1]$ and $[PCY_2]$ are respectively the amount of species 1 and 2 present in the bilayers, is ca. 3.1 for nontreated FITC-PCY and 11.5 for DTT-treated FITC-PCY. That is, one observes an increase of species 1 concomitant with a decrease of species 2 as a result of the DTT treatment. We conclude thus that the fast-moving FITC-PCY (species 1) might be a monomer, whereas the slow-moving FITC-PCY (DTT-sensitive species 2) is probably an aggregated form of the labeled protein.

This assumption is not unreasonable. Conceptually, the basic idea stems from the self-associating properties of PCY, i.e., its capacity to form dimers and tetramers (Siegelman et al., 1975, 1976). Though the presence of aggregates in the PCY solutions was not observed by either gel chromatography or electrophoresis, we cannot exclude their occurrence with certainty on account of possible time-dependent aggregation effects. Another tenable hypothesis is that PCY aggregation is induced, or enhanced, at contact with the lipid bilayers. This view is attractive because ionization of the protein may be affected at water–lipid interfaces by local conditions such as dielectric constant (Fuoss & Kraus, 1933; Fernández & Fromherz, 1977; Ptak et al., 1980; Bellemare & Fragata, 1980; Fragata & Bellemare, 1982, 1983) and ionic strength gradients.

## Discussion

*Relationship of PCY Diffusion to Bilayer Viscosity.* We show hereinafter that the diffusivity of the fast-moving FITC-PCY is lower than the diffusion coefficient of plastocyanin in water obtained from the classical Stokes–Einstein relation [see, e.g., Einstein (1905)]:

$$D_s = k_B T/(6\pi r\eta) \quad (7)$$

where $D_s$ is the diffusion coefficient of a spherical particle of radius $r$ in an infinite isotropic continuum, $k_B$ is the Boltzmann constant, $T$ is the temperature in kelvin, and $\eta$ is the viscosity of the fluid embedding the protein. The reliability of calculations performed according to eq 7 is exemplified with the determination of the diffusivity of the hemoglobin molecule in aqueous solution. For $r \sim 30$ Å [see, e.g., Perutz (1964)], $T = 288$ K, and a water viscosity of 0.01 P, eq 7 yields $D \sim 7 \times 10^{-7}$ cm$^2$/s, whereas the experimental value is found to be $6.8 \times 10^{-7}$ cm$^2$/s (Snell et al., 1965). With PCY, we verified that, for average monomer radii of 15–19 Å (Colman et al., 1978), $T = 293$ K, and $\eta = 0.01$ P, the diffusion coefficient is about $(1.1\text{–}1.4) \times 10^{-6}$ cm$^2$/s, which is very close to the value $D^0_{20w} = 1.46 \times 10^{-6}$ cm$^2$/s obtained by analytical ultracentrifugation (Scawen et al., 1975), where $D^0_{20w}$ is the diffusion coefficient corrected for water at 293 K. Then, assuming that diffusivities of ca. $4.9 \times 10^{-8}$ cm$^2$/s of the fast-moving FITC-PCY (cf. Table I) correspond to a monomer (see afore discussed comments on DTT-treated FITC-PCY), we concluded that the diffusion of plastocyanin must not take place in the bulk solvent but at the aqueous interfaces of the lipid bilayers where $\eta$ is most probably a few orders of magnitude higher than 0.01 P.

To test this prediction, we used first the approximation of Saffman & Delbrück (Saffman & Delbrück, 1975; Saffman, 1976), which is a model for the translational diffusion of a cylindrical particle in thin fluid sheets of viscosity $\eta$ assumed to be higher than the viscosity $\eta'$ of the bounding fluid on both sides of the sheet [cf. Figure 1 of Saffman & Delbrück (1975)]. The translational diffusion coefficient ($D_t$) as a function of the viscosities and the cylinder's dimensions is given to be

$$D_t = \frac{k_B T}{4\pi\eta h}\left[\ln\left(\frac{\eta h}{\eta' r}\right) - \gamma\right] \quad (8)$$

where $h$ denotes the axis of the cylinder, $r$ is its radius, and $\gamma$ is the Euler constant (0.5772). In the following, plastocyanin is assumed to diffuse in fluid sheets constituted of the aqueous layers adjacent to the membrane. In addition, the protein is modeled as a cylinder with $h = 40$ Å and $r = 15$ Å, which is an acceptable representation of the molecule's shape, namely, a flattened cylinder with dimensions $40 \times 32 \times 28$ Å$^3$ (Colman et al., 1978). For $D_t = 4.9 \times 10^{-8}$ cm$^2$/s, $T = 296$ K, and $\eta' = 0.01$ P, the result is $\eta \sim 0.82$ P. This is a surprisingly high value comparatively to the viscosity of the lipid bilayer itself, which may range from 0.3 P (Kinosita et al., 1981) or 0.6–0.8 P (Peters & Cherry, 1982) up to several poise [see, e.g., Shinitzky & Yuli (1982) and Vaz et al. (1981)]. Furthermore, the above calculated value is also at variance with $\eta < 0.1$ P estimated by Peters & Cherry (1982). To cope with these discrepancies, the data were dealt with according to Hughes et al. approximation (Hughes et al., 1981, 1982). This is an extension of the Saffman–Delbrück model to include nonequivalent viscous drags at both ends of the fluid sheet containing the cylindrical particle. Then, $D_t$ is given by

$$D_t = \frac{k_B T}{4\pi\eta h}\left[\ln\left(\frac{2}{\epsilon}\right) - \gamma + \frac{4\epsilon}{\pi} - \frac{\epsilon^2}{2}\ln\left(\frac{2}{\epsilon}\right)\right] \quad (9)$$

where $\epsilon = (\eta'_1 + \eta'_2)r/(\eta h)$ is a dimensionless parameter, $\eta'_1$ and $\eta'_2$ are the viscosities of the bounding fluids at both ends of the diffusing cylindrical particle embedded in the fluid sheet, and the other parameters have the meaning defined above. The application of eq 9 to membrane surface proteins implies, however, a realistic knowledge of the intrinsic membrane viscosity or the viscosity at the interface polar head group/hydrocarbon core, say $\eta'_2$ if $\eta'_1$ is taken to be the bulk water viscosity. An example calculation puting $\eta'_1 = 0.01$ P, $\eta'_2 = 2$ P [see, e.g., Vaz et al. (1981)], $h = 40$ Å, $r = 15$ Å, $D_t = 4.9 \times 10^{-8}$ cm$^2$/s, and $T = 296$ K yields $\eta = 0.36$ P. This is about half the value determined with the Saffman–Delbrück equation (eq 8). In addition, 0.36 P is probably a better approximation of the surface viscosity of the bilayer [cf. in this respect Peters & Cherry (1982)]. In general, calculations according to eq 9 for a wide range of $\eta'_2$ values and in the conditions outlined above show that $\eta$ reacts rather slowly to $\eta'_2$ changes. For example, a $\eta'_2$ decrease from 2.0 to 0.3 P, the membrane viscosity limits referred to above [see Kinosita et al. (1981), Peters & Cherry (1982), Shinitzky & Yuli (1982), and Vaz et al. (1981)], gives rise to $\eta$ values ranging from 0.36 to 0.15 P, respectively.

In brief, the foregoing discussion indicates that the diffusion of FITC-PCY takes place in the lipid bilayer, but we cannot decide conclusively whether the molecule is localized completely at the bilayer surface or included partly within the membrane. This is worthy of investigation in future work on account of the molecular peculiarities of the plastocyanin outer surface polarity (see introduction).

*Size of the Electron Donor to P700$^+$.* Application of eq 8 or 9 to the slow-moving FITC-PCY is not feasible until one gets more detailed information on the degree of polymerization and the three-dimensional geometry of the presumed plastocyanin aggregate (see Results). We wish to point out, nevertheless, that this question deserves to be examined in more detail on account of the recent finding (Takano et al., 1982) that PCY diffusion in the chloroplast is characterized by a diffusivity of $\sim 2 \times 10^{-9}$ cm$^2$/s, that is to say about 2–3 times the value found for the slow FITC-PCY species, i.e., $8 \times 10^{-10}$ cm$^2$/s (cf. Table I). These matters, however, need some clarification before we attempt to draw any conclusion from the observed similarities between the diffusion coefficients.

On the one hand, $D = 2 \times 10^{-9}$ cm$^2$/s (Takano et al., 1982) is the result of a number of assumptions concerning, for instance, the chlorophyll concentration in the thylakoid membrane and the density of P700 plus PCY molecules in the stroma thylakoids. The distance between electron-transfer particles, i.e., the travel distance of PCY in the thylakoid, is then evaluated from a distribution law of Chandrasekhar (1943) and used to determine a two-dimensional diffusion coefficient ($D_2$) according to the model of Overfield & Wraight (Overfield & Wraight, 1980; see also Adam & Delbrück (1968)]:

$$D_2 = b^2/t \quad (10)$$

where $b$ denotes the space size obtained from Chandrasekhar's law minus an average PCY radius of 15 Å (Colman et al., 1978), i.e., 96 Å, and $t$ is the mean time to encounter that is given as the half-time of the P700$^+$ reaction with PCY (Takano et al., 1982), i.e., 0.53 ms. The result is $D_2 \sim 1.7 \times 10^{-9}$ cm$^2$/s, that is, approximately the value determined by Takano et al. (1982). It is emphasized, however, that a lower $D_2$ is obtained if instead of Overfield & Wraight's model (eq 10) one makes use of expressions for the mean square displacement, $\langle r^2 \rangle$, such as [see, e.g., Saffman & Delbrück (1975)]

$$\langle r^2 \rangle = 4D_2 t \quad (11)$$

Hence, it comes out that $D_2$ is $\sim 4.3 \times 10^{-10}$ cm$^2$/s, that is, quite near the diffusivities reported in Table I for the slow-moving FITC-PCY.

While the discrepancies between $D_2$ and $D_{slow}$ may be attributed to viscous drag differences of the model lipid bilayers and the thylakoid membranes, it is obvious that the results of calculations ($D_2$) are in no way comparable to the $D_{fast}$ values reported in Table I, which seem to be characteristic of monomeric PCY (cf. data on DTT-treated samples). The afore discussed arguments let us suggest that the electron donor to $P700^+$ in vivo is an aggregated form of plastocyanin.

Acknowledgments

We gratefully acknowledge Prof. Y. Morita for having let us use his Joyce-Loebl microdensitometer, Dr. J. Sochanski (Centre de Calcul, U.Q.T.R.) and M. El-Kindi for their help with the curve-fitting analyses, and R. Lanouette for the drawings of the illustrations. M.F. thanks Profs. S. Ohnishi and K. Asada and their collaborators for their hospitality and help during his stay at their laboratories, the Université du Québec à Trois-Rivières for the tenure of a sabbatical leave, and the N.S.E.R.C. Canada for a travel grant (T1812). We are grateful to the referees for their useful comments.

**Registry No.** NBD-PE, 64205-19-2; SA, 124-30-1.

References

Adam, G., & Delbrück, M. (1968) in *Structural Chemistry and Molecular Biology* (Rich, A., & Davidson, N., Eds.) pp 198–215, W. H. Freeman, San Francisco.

Avron, M., & Shneyour, A. (1971) *Biochim. Biophys. Acta 226*, 498–500.

Axelrod, D., Koppel, D. E., Schlessinger, J., Elson, E., & Webb, W. W. (1976) *Biophys. J. 16*, 1055–1069.

Bellemare, F., & Fragata, M. (1980) *J. Colloid Interface Sci. 77*, 243–252.

Chandrasekhar, S. (1943) *Rev. Mod. Phys. 15*, 1–87.

Chang, C.-H., Takeuchi, H., Ito, T., Machida, K., & Ohnishi, S. (1981) *J. Biochem. (Tokyo) 90*, 997–1004.

Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M., & Venkatappa, M. P. (1978) *Nature (London) 272*, 319–324.

Davis, D. J., Krogmann, D. W., & San Pietro, A. (1980) *Plant Physiol. 65*, 697–702.

Derzko, Z., & Jacobson, K. (1980) *Biochemistry 19*, 6050–6057.

Einstein, A. (1905) *Ann. Phys. (Leipzig) 17*, 549.

Fahey, P. F., & Webb, W. W. (1978) *Biochemistry 17*, 3046–3053.

Farver, O., Shahak, Y., & Pecht, I. (1982) *Biochemistry 21*, 1885–1890.

Fernández, M. S., & Fromherz, P. (1977) *J. Phys. Chem. 81*, 1755–1761.

Fragata, M., & Bellemare, F. (1982) *Biophys. Chem. 15*, 111–119.

Fragata, M., & Bellemare, F. (1983) *Biophys. Chem. 18*, 207–210.

Freeman, H. C. (1981) in *Coordination Chemistry* (Laurent, F. P., Ed.) Vol. 21, pp 29–51, Pergamon Press, Oxford.

Fuoss, R. M., & Kraus, C. A. (1933) *J. Am. Chem. Soc. 55*, 1019–1028.

Garvey, J. S., Cremer, N. E., & Sussdorf, D. H. (1977) *Methods in Immunology*, 3rd ed., W. A. Benjamin, Reading, MA.

Haehnel, W., Pröpper, A., & Krause, H. (1980) *Biochim. Biophys. Acta 593*, 384–399.

Haehnel, W., Berzborn, R. J., & Andersson, B. (1981) *Biochim. Biophys. Acta 637*, 389–399.

Höchli, M., & Hackenbrock, C. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1236–1240.

Holwerda, R. A., Wherland, S., & Gray, H. B. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 363–396.

Hopfield, J. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3640–3644.

Hughes, B. D., Pailthorpe, B. A., & White, L. R. (1981) *J. Fluid Mech. 110*, 349–372.

Hughes, B. D., Pailthorpe, B. A., White, L. R., & Sawyer, W. H. (1982) *Biophys. J. 37*, 673–676.

Katoh, S., Shiratori, I., & Takamiya, A. (1962) *J. Biochem. (Tokyo) 51*, 32–40.

Katz, J. J., Norris, J. R., Shipman, L. L., Thurnauer, M. C., & Wasielewski, M. R. (1978) *Annu. Rev. Biophys. Bioeng. 7*, 393–434.

Kimelberg, H. K., Lee, C. P., Claude, A., & Mrena, E. (1970) *J. Membr. Biol. 2*, 235–251.

Kinosita, K., Jr., Kawato, S., Ikegami, A., Yoshida, S., & Orii, Y. (1981) *Biochem. Biophys. Acta 647*, 7–17.

Koppel, D. E., Axelrod, D., Schlessinger, J., Elson, E. L., & Webb, W. W. (1976) *Biophys. J. 16*, 1315–1329.

Landel, A. M. (1976) *Anal. Biochem. 73*, 280–289.

McElroy, J. D., Mauzerall, D., & Feher, G. (1974) *Biochim. Biophys. Acta 333*, 261–277.

Milne, P. R., & Wells, J. R. E. (1970) *J. Biol. Chem. 245*, 1566–1574.

Moore, G. R., & Williams R. J. P. (1976) *Coord. Chem. Rev. 18*, 125–197.

Olsen, L. F., & Pedersen, J. Z. (1983) *Photobiochem. Photobiophys. 5*, 1–10.

Overfield, R. E., & Wraight, C. A. (1980) *Biochemistry 19*, 3328–3334.

Perutz, M. F. (1964) *Sci. Am. 211*, 64.

Peters, R., & Cherry, R. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4317–4321.

Ptak, M., Egret-Charlier, M., Sanson, A., & Bouloussa, O. (1980) *Biochim. Biophys. Acta 600*, 387–397.

Redwood, W. R., & Patel, B. C. (1974) *Biochim. Biophys. Acta 363*, 70–85.

Rubenstein, J. L. R., Smith, B. A., & McConnell, H. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 15–18.

Saffman, P. G. (1976) *J. Fluid Mech. 73*, 593–602.

Saffman, P. G., & Delbrück, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3111–3113.

Sauer, K. (1975) in *Bioenergetics of Photosynthesis* (Govindjee, Ed.) pp 115–181, Academic Press, New York.

Scawen, M. D., Hewitt, E. J., & James, D. M. (1975) *Phytochemistry 14*, 1225–1233.

Schneider, H., Lemasters, J. J., Höchli, M., & Hackenbrock, C. R. (1980) *J. Biol. Chem. 255*, 3748–3756.

Shinitzky, M., & Yuli, I. (1982) *Chem. Phys. Lipids 30*, 261–282.

Shipman, L. L., Cotton, T. M., Norris, J. R., & Katz, J. J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1791–1794.

Siegelman, M. H., Rasched, I., & Böger, P. (1975) *Biochem. Biophys. Res. Commun. 65*, 1456–1463.

Siegelman, M. H., Rasched, I. R., Kunert, K.-J., Kroneck, P., & Böger, P. (1976) *Eur. J. Biochem. 64*, 131–140.

Singleton, W. S., Gray, M. S., Brown, M. L., & White, J. L. (1965) *J. Am. Oil Chem. Soc. 42*, 53–56.

Snell, F. M., Shulman, S., Spencer, R. P., & Moos, C. (1965) *Biophysical Principles of Structure and Function*, Addison-Wesley, Reading, MA.

Sowers, A. E., & Hackenbrock, C. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6246–6250.

Steinbach, G. (1974) *Acta Histochem. 49*, 19–34.

Takano, M., Takahashi, M. A., & Asada, K. (1982) *Arch. Biochem. Biophys. 218*, 369–375.
Vaz, W. L. C., Kapitza, H. G., Stümpel, J., Sackmann, E., & Jovin, T. M. (1981) *Biochemistry 20*, 1392–1396.
Wu, E.-S., Jacobson, K., & Papahadjopoulos, D. (1977) *Biochemistry 16*, 3936–3941.
Yguerabide, J., Schmidt, J. A., & Yguerabide, E. E. (1982) *Biophys. J. 39*, 69–75.

# Electron Spin Resonance and Steady-State Fluorescence Polarization Studies of Lipid Bilayers Containing Integral Proteins†

David A. Pink,*,‡ Dennis Chapman, Donald J. Laidlaw,‡ and Therese Wiedmer

ABSTRACT: We derive equations that describe changes in the steady-state fluorescence polarization of the probe 1,6-diphenyl-1,3,5-hexatriene (DPH) or in the spectrum of electron spin resonance (ESR) nitroxide spin-labeled lipid probes as a function of the intrinsic molecule concentration in lipid bilayer membranes. We make use of an assumption used by us in an earlier paper. The equations are independent of any membrane model. They are valid when a DPH probe or a spin-labeled chain is equivalent to an unlabeled lipid hydrocarbon chain only as far as their general space-filling properties are concerned. We consider cases where the bilayer is either in a single homogeneous phase or in a two-phase region. We apply our equations to analyze ESR data from delipidated sarcoplasmic reticulum membranes and from egg yolk phosphatidylcholine bilayers containing $Ca^{2+}$-ATPase, and DPH data from dipalmitoylphosphatidylcholine (DPPC) bilayers containing $Ca^{2+}$-ATPase, both for $T > T_c$. The following conclusions were derived: (i) $Ca^{2+}$-ATPase oligomers are "randomly" distributed, for the concentrations studied, in the fluid phase. (ii) There is no fixed stoichiometric ratio of "boundary" lipids and oligomers. (iii) Between $24k$ and $28k$ lipid molecules are able to surround each isolated oligomer composed of $k$ $Ca^{2+}$-ATPase monomers. Finally, we apply our equations to analyze DPH studies on DPPC bilayers containing $Ca^{2+}$-ATPase for $T < T_c$. We find that the results reported are in accord with the predictions of the model. In the Appendix, we show that an analytical expression for probabilities used by us is in very good agreement with the results of computer simulation.

The question of what is measured by perturbing probes, such as nitroxide-labeled acyl chains used in electron spin resonance (ESR)[1] studies or the fluorescent probe DPH, when used in phospholipid bilayers containing integral proteins appears to still be unresolved. There is the view [e.g., see Marsh et al. (1982)] that the apparent discrepancy between $^2H$ NMR measurements and nitroxide-labeled ESR probes can be accounted for by a time-scale argument. Because such probes do report information on the static order parameter of the chain to which they are attached, it has been found necessary to bolster this with an additional picture of the state of lipid chains adjacent to an integral protein: Two competing effects have been suggested [e.g., see Jähnig (1980)] to explain results observed on the $^2H$ NMR time scale. These measurements make it appear as though lipid chains adjacent to a protein are essentially statically identical with chains in the absence of any protein, at the same temperature $T$, in some range of $T > T_c$. The suggested picture envisages hydrocarbon chains adjacent to an intergral protein to have fewer gauche bonds than those sufficiently far from the protein but to have a long-axis orientation that does not, on the average, coincide with the bilayer normal. It is argued that $^2H$ NMR will detect the combined motion but that ESR will detect only the reduction in gauche conformers. This view is not supported by recent infrared studies (Cortijo et al., 1982; Alonso et al., 1982). These studies show that for a number of intrinsic molecules,[2] the gauche "content" as measured by IR spectroscopy is essentially identical with that reported by $^2H$ NMR spectroscopy [e.g., see Jacobs & Oldfield (1981) and Seelig & Seelig (1980)]. The question as to what property is being reported by steady-state DPH fluorescence polarization or by nitroxide-labeled ESR probes thus remains unanswered.

Some years ago, a new interpretation of DPH steady-state fluorescence polarization measurements was made (Hoffmann et al., 1981). In this paper, we extend these ideas and present a number of new results. The original work came about because the observed steady-state polarization as a function of

† From the Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22908 (D.A.P. and T.W.), the Biochemistry and Chemistry Departments, Royal Free Hospital School of Medicine, University of London, London NW3 2PF, United Kingdom (D.C.), and the Theoretical Physics Institute, St. Francis Xavier University, Antigonish, Nova Scotia, Canada B2G 1C0 (D.J.L.). *Received November 9, 1983; revised manuscript received March 14, 1984.* This work was supported in part by the Natural Science and Engineering Research Council of Canada and the NATO Collaborative Research Grants Program under Grant 0409/82 (D.A.P.), by the Science Research Council and the Wellcome Trust (D.C.), and by National Institutes of Health Grant R GM26894.

‡ Permanent address: Theoretical Physics Institute, St. Francis Xavier University, Antigonish, Nova Scotia, Canada B2G 1C0.

[1] Abbreviations: ESR, electron spin resonance; NMR, nuclear magnetic resonance; DPH, 1,6-diphenyl-1,3,5-hexatriene; $T_c$, pure lipid main phase transition temperature; PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; SR, sarcoplasmic reticulum; IR, infrared; $c$, intrinsic molecule concentration defined as (number of intrinsic molecules)/(number of intrinsic molecules + number of lipids); EYPC, egg yolk phosphatidylcholine; proxyl, 2,2-dimethylpyrrolidinyl-1-oxy.

[2] In Hoffmann et al. (1981), "intrinsic molecule" refers to cholesterol, gramicidin A, or integral proteins in a lipid bilayer. Here, although we shall refer to "integral proteins" and apply our equations accordingly, the most general equations should be, in addition, applicable to the cases of cholesterol and gramicidin A in lipid bilayers composed of a single kind of lipid. Readers should note the changes to be made when such a molecule is restricted to half of the bilayer.

0006-2960/84/0423-4051$01.50/0 © 1984 American Chemical Society