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PHOTOINDUCED CHARGE SEPARATION IN LIPOSOMES CONTAINING CHLOROPHYLL *a*

I. PHOTOREDUCTION OF COPPER(II) BY POTASSIUM ASCORBATE THROUGH LIPOSOME BILAYER CONTAINING PURIFIED CHLOROPHYLL *a*

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Summary

Photosensitivity of a dispersion of phosphatidylcholine bilayer liposomes containing purified chlorophyll *a* was examined. The reduction of Cu(II) in the solution outside liposomes was observed upon illumination with visible light under anaerobic condition by means of ESR. The rate of photoreduction was significantly increased by a reductant, potassium ascorbate, localized in the solution of the opposite side of the membrane. The action spectrum of the reduction agreed with the absorption spectrum of chlorophyll *a* in the dispersion. The amount of bleached chlorophyll *a* was negligible compared with that of reduced Cu(II).

These facts lead to the conclusion that the photoinduced redox reactions at both the membrane-solution interfaces are coupled with each other through the bilayer of each liposome.

Kinetic analysis of the reactions based on a possible reaction scheme was carried out and some of the kinetic parameters were determined.

Introduction

Recently many experiments were carried out on photochemical properties of pigments incorporated phospholipid bilayer membranes, in connection with

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PS I and PS II in living photosynthetic systems. In early stages of these studies, the photoresponse of bilayer lipid membranes containing chloroplast extracts whose composition in terms of lipids and pigments was not accurately known was examined mainly in the presence of a redox potential gradient across the membranes [1]. Mangel and coworkers [2,3] studied the essential components for the photoinduced charge transport across the bilayer and found that carotene as well as chlorophyll was necessary in both bilayer lipid membranes and liposome systems. From these results they concluded that the chlorophyll chromophores act as a photosensitizer while carotenes play a role of an electron carrier.

In this paper, we demonstrate the results which conflict with the proposed role of carotenes, showing that the redox reactions involving excited chlorophyll *a* (Chl *a*) which occur at both the membrane-solution interfaces can be coupled to each other through the liposome bilayer, even in the absence of a particular electron carrier such as β-carotene.

A kinetic analysis of the photoinduced couple reactions is also carried out, based on a possible reaction scheme involving chlorophyll *a* cation radical formation.

Experimental

Materials. Phosphatidylcholine was extracted from egg yolk and purified by the method of Pangborn [4], through the formation of phosphatidylcholine-cadmium complex. The purity of the preparation was checked by thin-layer chromatography. Chl *a* was extracted from fresh spinach leaves with methanol and purified by the repeated precipitations [5] followed by chromatographic procedures using a sugar column [6]. The purity of the preparation was estimated to be more than 98% by the absorbances at 660 nm and 642.5 nm, referring to the equations given by Cormar and Zscheile [7]. KCl, $CuCl_2$ and ascorbic acid and Tris-(hydroxymethyl)aminomethane (Tris) of analytical grade were used.

Preparation of phosphatidylcholine-Chl a liposomes. Phosphatidylcholine, colyophilized with Chl *a* of a desired composition from benzene solution under vacuum, was suspended in an aqueous solution from which oxygen had been removed by bubbling with Ar gas for 30 min. The suspension was ultrasonically irradiated with a sonicator (Otake Seisakusho OT-5205) for 1 h at 20 kHz and 4°C under Ar atmosphere. Undispersed phospholipids were removed by centrifugation at 17 000 × *g* for 10 min. Phosphatidylcholine concentration in the lipisome dispersion was determined by Fiske-Subbarow method [8] and expressed in terms of lipid phosphorus in mol/l. Chl *a* concentration in the dispersion was estimated from the absorbance at 669.8 nm using the molar extiction coefficient of $6.2 \cdot 10^4$ (l/mol) [9].

Measurements of photoreduction of Cu(II). Photoreduction of Cu(II) existing in the ambient aqueous phase of the liposome dispersion was measured by means of ESR at 25°C. Each liposome in the examined dispersion contained potassium ascorbate of a desired concentration in its inner compartment. To prepare this type of liposome system, sonication was carried out in an oxygen-free aqueous solution (0.1 M KCl, 1.5 M Tris-HCl at pH 7.50) containing

potassium ascorbate of a given concentration. Removal of potassium ascorbate from the external aqueous phase was accomplished by rapid passage of the resulting dispersion over a column of Sephadex G-50 which had been previously equilibrated with the oxygen-free solution of 1.5 M Tris-HCl and 0.1 M KCl at pH 7.50 and 4°C. A desired amount of $CuCl_2$ dissolved in the buffered solution was added to the elute and the resulting solution was transferred to an ESR cell with the inner diameter of 1.1 mm.

These procedures were carried out under Ar atmosphere, in the dark at 4°C. The concentration of Chl *a* in each sample solution was kept constant, $1.6 \cdot 10^{-4}$ mol/l, thus the phosphatidylcholine concentration in the liposome dispersion was varied to change the local concentration of Chl *a* in a liposome. Since a proportionality between the ESR intensity at 3320 gauss and the concentration of Cu(II) was confirmed to be held under the present experimental conditions, the time dependence of the Cu(II) reduction was directly monitored by the intensity variation at 3320 gauss. A 550 W Xenon lamp with both an infrared cut-off and ultraviolet cut-off glass filters (Toshiba IRQ 80, UV-39) was used throughout the experiments and light intensity was varied by means of neutral density filters up to 1.3 W/cm^2. A set of interference filters was used as needed.

Results and Discussion

Photosensitivity of the liposomes

Typical photoreduction process of Cu(II) in the external aqueous phase of the liposomes containing 0.75 M potassium ascorbate in their inner compartments is shown in Fig. 1, together with bleach of Chl *a* in the corresponding dispersion. The data were obtained in a dispersion of the liposome at a Chl *a*/

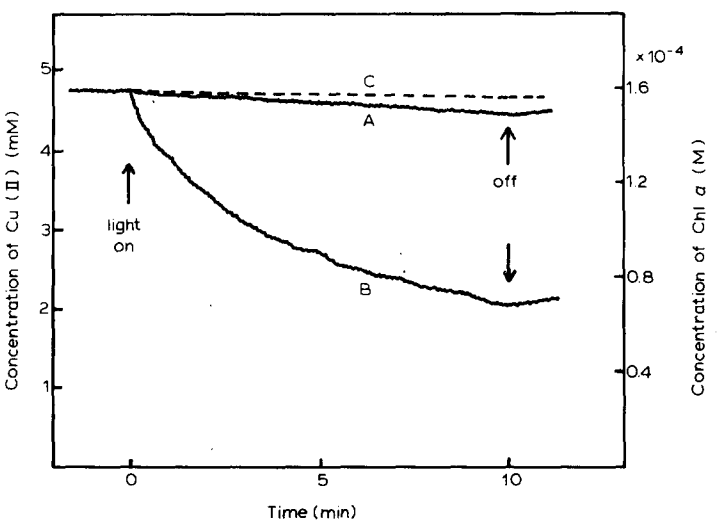

Fig. 1. Time dependence of Cu(II) and Chl *a* concentrations in the liposome dispersion upon illumination with the filtered light at 1.3 W/cm^2. (A) Time dependence of Cu(II) concentration in the absence of potassium ascorbate; (B) the corresponding curve in the presence of 0.75 M potassium ascorbate, and (C) bleach of Chl *a* in the case of (B).

phosphatidylcholine molar ratio of 1/400 under the filtered light at 1.3 W/cm^2. Reduction of Cu(II) was scarcely observed in the dark, but it was enhanced enormously by the light illumination. On the contrary, in the absence of potassium ascorbate the light illumination had little effect on the Cu(II) reduction. If a certain amount of potassium ascorbate was added to the external aqueous phase containing Cu(II) instead of the internal solution, almost the equivalent amount of Cu(II) was reduced immediately irrespective of the illumination. These results clearly demonstrate that potassium ascorbate contained in the inner compartment of each liposome is not released to the external aqueous phase during a successive experiment under the conditions studied, thus it is concluded that the Cu(II) reduction illustrated in Fig. 1 occurred under the influence of potassium ascorbate localized at the opposite side of the liposome bilayer. A gradual decrease in the absorbance of the liposome dispersion at 669.8 nm upon illumination indicated the bleach of Chl *a*, but the amount of the bleached Chl *a* was negligible compared with the reduced Cu(II) under the same condition. Fig. 2 compares the action spectrum of the photoreduction rate of Cu(II) at the initial stage with the absorption spectra of Chl *a* in the liposomes and of Cu(II) in the buffered solution. The action spectrum has two peaks around 440 and 670 nm corresponding to the absorption bands of Chl *a*, but with no similarity to the absorption spectrum of Cu(II). These results lead to the conclusion that a liposome bilayer containing Chl *a* is photosensitive and when it is subjected to a redox potential gradient established by Cu(II) at one surface of the membrane and potassium ascorbate at the other surface, coupling of redox reactions across the membrane does occur upon illumination with the light absorbed by Chl *a*. Such a property of the liposome system in the present work is similar to that of black lipid bilayer membranes [2] and liposomes containing chlorophyll and carotenoids [3].

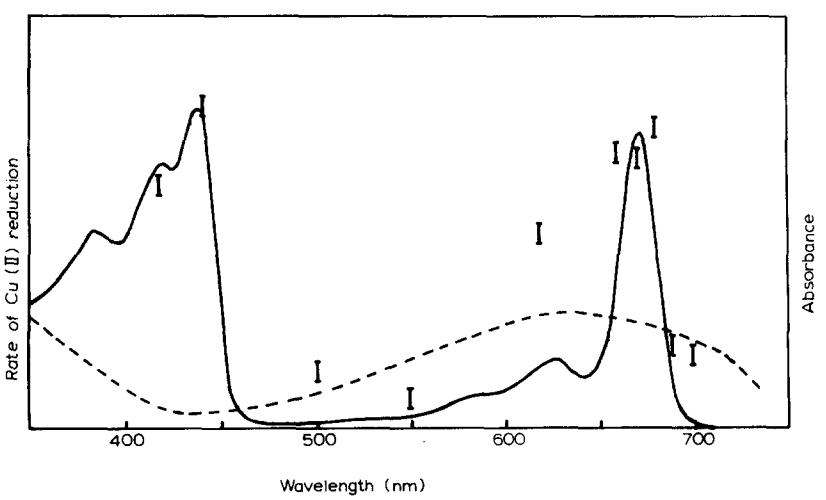

Fig. 2. Comparison of the action spectrum of Cu(II) reduction with the absorption spectra of Chl *a* and Cu(II) in the liposome dispersion. [Cu(II)]$^\circ$ = 4.46 mM; concentration of potassium ascorbate, 1.0 M; [Chl]/[phosphatidylcholine] = 1/400. Error bar: rate of Cu(II) reduction at the wavelength. ———, absorption of Chl *a* in the liposome; - - - - - -, absorption of Cu(II) in the buffered solution (0.1 M KCl, 1.5 M Tris-HCl at pH 7.50).

However, a distinct inconsistency between the present results and the previous observations should be mentioned here. The initial rates of Cu(II) reduction obtained in the present study are plotted against the light intensities in Fig. 3 with and without β-carotene. It is quite apparent that the data are well fitted to a single straight line, irrespective of the presence of β-carotene. On the contrary, the previous results mentioned above suggested that the presence of not only chlorophyll but carotenoid in the bilayer were essential for the photosensitivity of the membrane [2,3]. It is not yet possible to give a reasonable explanation for the inconsistency with regards to carotenoid.

Analysis of the photoreduction rate of Cu(II)

Fig. 4 shows the time dependence of Cu(II) concentration under the filtered light illumination based on the first-order reaction kinetics. The data were obtained in the dispersion of the liposomes containing 0.75 M potassium ascorbate in their inner compartments at different initial concentrations of Cu(II) in the external solution. Similar results were obtained for other potassium ascorbate concentrations. It is noted that the data deviate from the first-order kinetics at an early stage of the reaction particularly when the concentration of potassium ascorbate is low or that of Cu(II) is high. These behaviors may provide insight into the mechanism of the reactions. Tomkiewicz and Corker

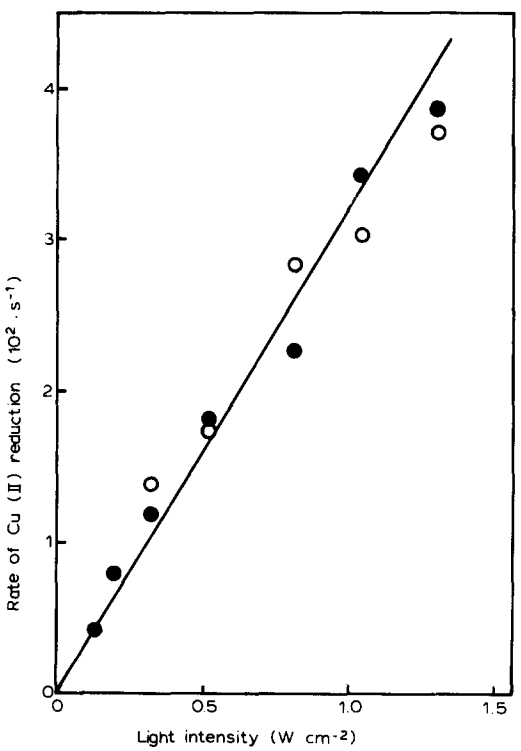

Fig. 3. The initial rate of Cu(II) reduction as a function of the light intensity in the presence and absence of β-carotene. $[Cu(II)]^{\circ}$ = 4.76 mM; concentration of potassium ascorbate, 0.75 M; [Chl]/phosphatidylcholine = 1/400. ●, without β-carotene; ○, with β-carotene.

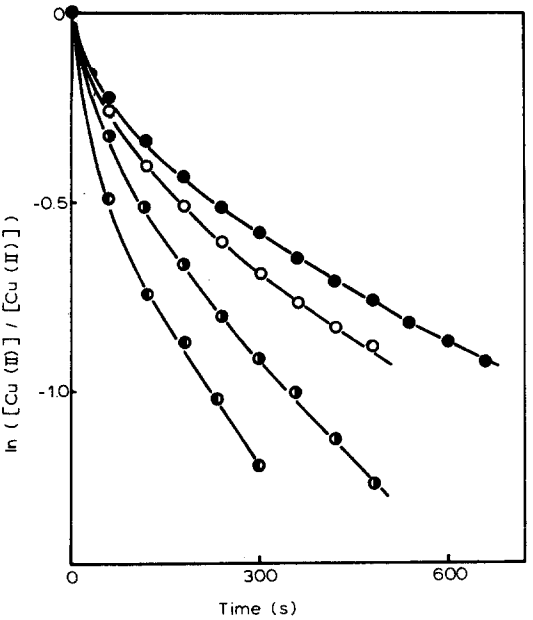

Fig. 4. Plots of ln([Cu(II)]/[Cu(II)]°) against illumination time with different [Cu(II)]° at 0.75 M of potassium ascorbate. ●, [Cu(II)]° = 12.1 mM; ○, 9.09 mM; ◑, 4.46 mM; ◐, 2.27 mM. The light intensity is 1.3 W/cm^2.

[10] observed the photoinduced formation of chlorophyll cation radical in the dispersion of lipisomes containing Chl *a* in the presence, but not in the absence, of a hydrophilic electron acceptor such as $K_3Fe(CN)_6$ at liquid N_2 temperature and −20°C. Upon a continuous illumination, the cation radical concentration increased with time and was saturated. This saturation behavior was explained by the back electron transfer from the reduced acceptor to the cation radical. Oettemeier et al [11] reported similar results on the photoinduced formation of chlorophyll *a* cation radicals in the presence of an electron acceptor having access to the lipid membranes, like Fe^{3+}-pyrophosphate. These results suggest the electron transfer from Chl *a* in the excited state to an acceptor molecule. Thus, in the present system it may be considered that the electron transfer from an excited Chl *a* to Cu(II) is the primary process of the photoinduced Cu(II) reduction by potassium ascorbate across the bilayer membrane. Here, we primarily follow the reaction scheme proposed by Tomkiewicz and Corker [10]

$$\mathrm{Chl} + h\nu \xrightarrow{I} \mathrm{Chl}^* \tag{1}$$

$$\mathrm{Chl}^* \xrightarrow{k_1} \mathrm{Chl} \tag{2}$$

$$\mathrm{Chl}_0^* + \mathrm{Cu(II)} \xrightarrow{k_2} \mathrm{Chl}_0^+ + \mathrm{Cu(I)} \tag{3}$$

$$\mathrm{Chl}_0^+ + \mathrm{Cu(I)} \xrightarrow{k_3} \mathrm{Chl}_0 + \mathrm{Cu(II)} \tag{4}$$

$$\mathrm{Chl}_0^+ + \mathrm{AS(R)}_i \xrightarrow{k_4} \mathrm{Chl}_0 + \mathrm{AS(O)}_i \tag{5}$$

Here, Chl^* and Chl^+ are excited Chl *a* and chlorophyll *a* cation radicals, respectively. Chl_0 represents the Chl *a* molecule localized in membrane surface facing the ambient aqueous phase outside the liposome. $AS(R)_i$ and $AS(O)_i$ are potassium ascorbate in the reduced and oxidised forms both trapped in the inner compartment of each liposome. k_i represents a rate constant of each reaction. The explanation of these equations is as follows. Chl *a* molecules are excited by absorbing light at the both membrane-solution interfaces (Eqn. 1) and relax to the ground state (Eqn. 2), but a part of the excited Chl *a* molecules outside the liposomes are oxidized by Cu(II) to form the Chl *a* cation radicals (Eqn. 3). The back reaction may occur at the external surface (Eqn. 4). The reaction scheme described by Eqns. 1—4 is essentially the same to that of chlorophyll cation radical formation [10]. In the present system, however, the experimental data strongly suggest the existence of another additional process represented by Eqn. 5 in the presence of potassium ascorbate inside each liposome, although the molecular mechanism has not been clarified yet.

In our heterogeneous system in which the chromophores of Chl *a* present only at the lipid-water interfaces and redox species only in the aqueous phases, the kinetics of the reactions would not be described by equations for simple second-order reaction, because the reactions occur at the lipid-water interfaces and their rates depend upon the spatial distribution of the redox species. For the Reactions 3 and 5, we can include the rates of the diffusions of Cu(II) and AS(R) in k_i by using large excess compared with Chl^* and of Chl^+, respectively. While for the Reaction 4, we should examine the behavior of Cu(I) more precisely, because, in the light reaction, Chl^+ and Cu(I) are produced simultaneously in a microscopically close vicinity to each other, consequently if the back electron transfer between them is sufficiently rapid compared with the diffusion process of Cu(I), the reaction rate has no relation with the concentration of Cu(I) in the bulk solution. Thus in order to analyze the data for the photoreduction process in the present system precisely, we should take the diffusion process of Cu(I) into consideration. To avoid mathematical complexity, however, we limit our discussion to the following two extreme cases: (1) diffusion of Cu(I) is extremely rapid compared with the electron transfer between Chl^+ and Cu(I) at liposome surfaces. (2) The diffusion is sufficiently slower than the electron transfer.

(1) In the first case, difference in the Cu(I) concentration between the liposome surfaces and the bulk solution is negligibly small, so that the reaction described by Eqn. 4 as well as Eqns. 3 and 5 may be considered to obey the second-order kinetics.

In addition to this assumption, we use the steady-state approximation for Chl^* and Chl^+ during illumination:

$$d[Chl_0^*]/dt = I - k_1[Chl_0^*] - k_2[Chl_0^*][Cu(II)] = 0 \quad (6)$$

$$d[Chl_0^*]/dt = k_2[Chl_0^*][Cu(II)] - k_3[Chl_0^+][Cu(I)] - k_4[Chl_0^+][AS(R)_i] = 0 \quad (7)$$

where I is the rate of light absorption in the system, (einstein/s). Under these approximations together with the assumption of $k_1 >> k_2[Cu(II)]$, the time

course of Cu(II) reduction is described by

$$d[Cu(II)]/dt = -k_2[Chl_0^*][Cu(II)] + k_3[Chl_0^+][Cu(I)]$$
$$= -\frac{k_2}{k_1} I[Cu(II)]\left[1 - \frac{k_3[Cu(I)]}{k_3[Cu(I)] + k_4[AS(R)_i]}\right] \quad (8)$$

Integration of Eqn. 8 with the initial conditions: $[Cu(II)] = [Cu(II)]^\circ$, $[Cu(I)] = 0$; $[AS(R)_i] = [AS]^\circ$ and $[AS(O)_i] = 0$, yields

$$\frac{k_1}{k_2 I}\left[1 + \frac{k_3}{k_4(1-bx)}\left[x - \frac{\ln(1-bx(1-\xi))}{\ln \xi}\right]\right] = -\frac{t}{\ln \xi} \quad (9)$$

where ξ, b and x represent $[Cu(II)]/[Cu(II)]^\circ$, the volume ratio of the external aqueous phase to the total internal aqueous phase of liposomes and $[Cu(II)]^\circ/[AS]^\circ$, respectively.

(2) In the second case, since Cu(I) in the bulk solution will not contribute to Eqn. 4, Eqns. 7 and 8 should be, respectively, modified as follows:

$$d[Chl_0^+]/dt = k_2[Chl_0^*][Cu(II)] - k_3[Chl_0^+] - k_4[Chl_0^+][As(R)_i] = 0 \quad (10)$$

$$d[Cu(II)]/dt = -k_2[Chl_0^*][Cu(II)] + k_3[Chl_0^+] \quad (11)$$

These two equations, with Eqn. 6, lead to,

$$\frac{k_1}{k_2 I}\left[1 + \frac{k_3}{k_4(1-bx)[AS]^\circ}\left[1 - \frac{\ln(1-bx(1-\xi))}{\ln \xi}\right]\right] = -\frac{t}{\ln \xi} \quad (12)$$

instead of Eqn. 9.

In the examination of Eqns. 9 and 12 with experimental data, we are concerned with the early stage of the reaction, where the limiting forms of the equations can be used. The second term in the bracket of the left hand side of Eqn. 9 and that of Eqn. 12 are, respectively, expanded in power of $(1-\xi)$ to give

$$\frac{k_1}{k_2 I} + \frac{k_1 k_3 x}{2k_2 k_4 I}(1-\xi) + O[(1-\xi)^2] = -\frac{t}{\ln \xi} \quad (13)$$

$$\frac{k_1}{k_2 I}\left(1 + \frac{k_3}{k_4[AS]^\circ}\right) + \frac{k_1 k_3 bx}{2k_2 k_4 [AS]^\circ I}(1-\xi) + O[(1-\xi)^2] = -\frac{t}{\ln \xi} \quad (14)$$

Eqn. 13 predicts that the intercept (α) of a plot of $-t/\ln \xi$ against $(1-\xi)$ is independent of $[Cu(II)]^\circ$ and $[AS]^\circ$ and the initial slope (β) of the plot is proportional to x, while Eqn. 14 suggests that α decreases with increasing $[AS]^\circ$ and β depends on $[AS]^\circ$ as well as x. In Fig. 5 $-t/\ln \xi$ is plotted against $(1-\xi)$ for several different $[Cu(II)]^\circ$ at 0.75 M of $[AS]^\circ$. In Fig. 6, the corresponding plots are shown for the data with various $[AS]^\circ$ at 4.55 mM of $[Cu(II)]^\circ$. One can see from both the figures that α of a straight line is independent of $[Cu(II)]^\circ$ and of $[AS]^\circ$ within the experimental error as expected by Eqn. 13, but not by Eqn. 14. Fig. 7 shows a plot of the values of α/β of the lines in Figs. 5 and 6 against x at various $[AS]^\circ$. It is clearly demonstrated that the plots fall on a single straight line irrespective of $[AS]^\circ$, satisfying the prediction of Eqn. 13, but not Eqn. 14. These results suggest that the reaction

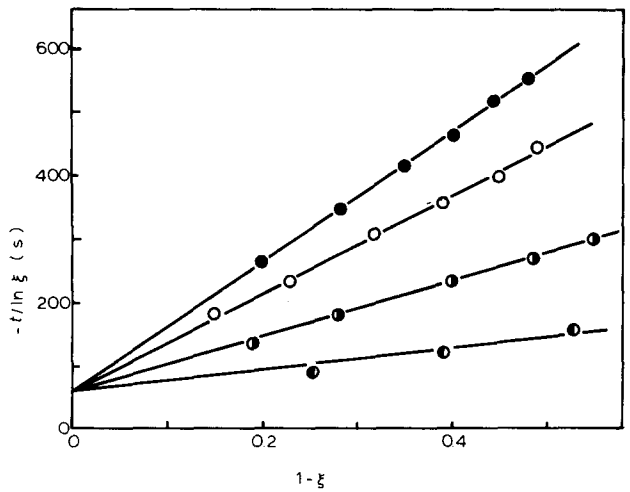

Fig. 5. Plots of $-t/\ln \xi$ against $(1 - \xi)$ with different $[Cu(II)]^\circ$ at 0.75 M of potassium ascorbate. [Chl]/[phosphatidylcholine] = 1/400. ●, $[Cu(II)]^\circ$ = 12.1 mM; ○, 9.09 mM; ◐, 4.55 mM; ◑, 2.27 mM.

represented by Eqn. 4 is the electron transfer control process, consequently the time course of the photoreduction of Cu(II) in the present system can be described by the second-order kinetics for the reaction of Eqn. 4 as well as Eqns. 3 and 5.

Using the value of α in Figs. 5 and 6, $(k_2/k_1)I$ was determined to be $1.7 \cdot 10^{-2}$ s^{-1}. The light absorption rate, I, in the system was evaluated to be $3.9 \cdot 10^{-2}$ einstein $\cdot$ $l^{-1} \cdot s^{-1}$ by the chemical actinometry using ferrioxalate [12]. Thus, we obtain 0.44 l $\cdot$ mol^{-1} for k_2/k_1.

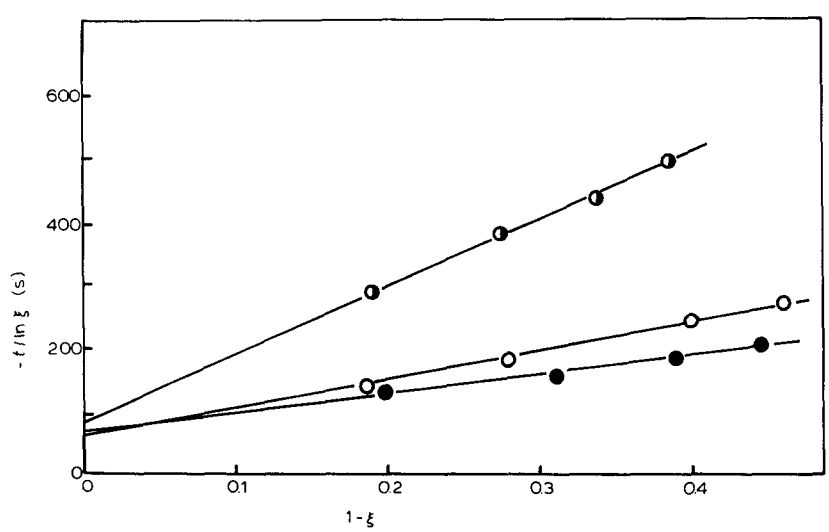

Fig. 6. Plots of $-t/\ln \xi$ against $(1 - \xi)$ with different concentrations of potassium ascorbate at 4.55 mM of $[Cu(II)]^\circ$.[Chl]/[phosphatidylcholine] = 1/400. Potassium ascorbate concentrations of: ◐, 0.5 M; ○, 0.75 M; ●, 1.0 M.

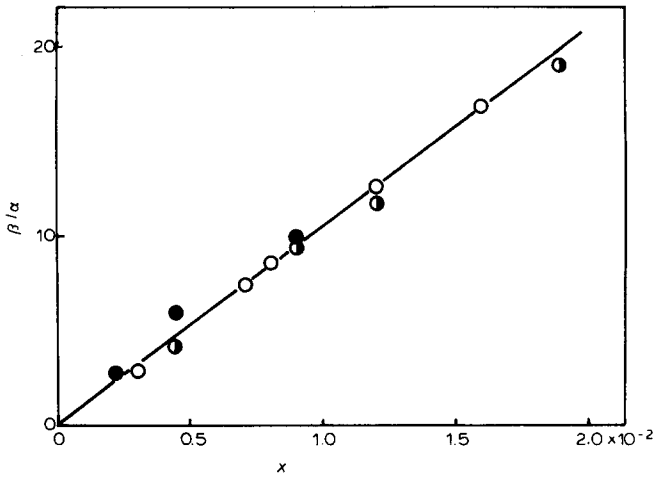

Fig. 7. Plot of β/α of the lines shown in Figs. 5 and 6 against x. Notation is the same as in Fig. 6.

It may be conceivable to equate $1/k_1$ to the mean life-time of Chl* in the liposome dispersion without Cu(II). In the present system it was determined as 7.6 ns which leads to $5.8 \cdot 10^7$ l · mol^{-1} · s^{-1} for k_2. This value suggests that the primary electron transfer from Chl* to Cu(II) is near to the diffusion control process. While the slope of the straight line in Fig. 7 gives $k_3/k_4 = 2100$ suggesting that the reduction rate constant of Chl_0^+ by potassium ascorbate through lipid bilayer of the liposomes is three order of magnitude smaller than that by Cu(I) in the solution facing Chl_0^+.

The mechanism of the transport of the reducing force from potassium ascorbate to Chl_0^+ across the bilayer membrane remains an unsolved problem at present.

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