Biochimica et Biophysica Acta, 461 (1977) 239–252

BBA 47331

ACIDIFICATION, BLEACHING AND OXYGEN CONSUMPTION WITH CHLOROPHYLL-CONTAINING LIPID MICROVESICLES

WILLIAM STILLWELL and H TI TIEN*

Biophysics Department, Michigan State University, East Lansing, Mich 48824 (U S A)

(Received December 30th, 1976)

SUMMARY

When chlorophyll-lipid microvesicles under aerobic conditions were illuminated with intense white light; (a) the media became more acidic, (b) the lipid microvesicles bleached from green to yellow and (c) oxygen was taken up from the bathing solution. The bleaching, which was followed spectrophotometrically, resulted in a decrease in the total chlorophyll content as well as the chlorophyll *a*:*b* ratio Some bleaching, which was slowed by the presence of electron donors, occurred in the dark. Water-soluble electron donors were shown to increase the rate of oxygen consumption with the order of effectiveness being; control = KI = ferrocyanide < hydroquinone < thiourea < cysteine < NADH < Fe^{+2} < ascorbic acid < phenylhydrazine. Chlorophyll mediated electron transfer from donors to oxygen is similar to that of the well known Krasnovsky reactions (in organic solvents and aqueous detergent suspensions) and Mehler reaction (in chloroplast suspension). Electron acceptors and β-carotene had no effect on the oxygen consumption. Lipid-soluble quinones and α-tocopherol affected the oxygen reaction to different extents. The reactions reported here are closely related to those previously described for chlorophyll in organic solvents, "quantasomes" and chloroplasts. The demonstration of these reactions in chlorophyll-lipid microvesicles is an advance in making the chlorophyll-lipid microvesicles a better model of the thylakoid membrane.

INTRODUCTION

Photochemical reactions involving chlorophylls have received much attention [1] because of their possible relevance in understanding the properties of these pigments in photosynthesis. In the past such photochemical reactions have been studied in aqueous media [2], organic solvents [3], on solid substrates [4] or at the air/water interface [5]. However, recent chloroplast research has revealed that chlorophylls (and other pigments) are located in a lamellar bilayer structure known as the thylakoid membrane [6]. Thus, a logical approach to in vitro study of the photochemical reac-

---

* To whom correspondence should be addressed

tions of chlorophyll is to use artificially constituted bilayer lipid membranes containing chlorophylls and other photosynthetic constituents. Two types of artificial bilayer lipid membrane systems have been used in such studies. The first consists of a planar bilayer lipid membrane separating two aqueous solutions where light-induced effects can be readily measured electrically [7]. The results obtained on pigmented bilayer lipid membranes of planar configuration and their use as a model for the thylakoid membrane have been reviewed [8]

The second system of bilayer lipid membranes was introduced by Bangham [9] and others [10, 11]. This system is comprised of lipid microvesicles, also known as "liposomes", in which the bilayer lipid membrane of spherical configuration encloses a volume of aqueous solution. Recently, several workers have incorporated chlorophyll *a* [12–16], chlorophyll *b* [17] and a chloroplast extract [18] into these lipid microvesicles. Of primary concern has been the method of preparations and spectral properties of the chlorophylls in lipid microvesicles A 10-nm red shift of the Soret band with respect to organic solvents was noted in the lipid microvesicles [12–18]. In addition, Mangel [18] has reported the appearance of absorbance peaks beyond 700 nm, implying the presence of chlorophyll aggregates. The spectral properties indicate that chlorophyll in lipid-microvesicles is in an environment more similar to its in vivo location in a thylakoid membrane than when dissolved in organic solvents. Upon illumination of chlorophyll-lipid microvesicles, Trosper et al. [13] demonstrated the chlorophyll-sensitized, membrane-dependent photoreduction of cytochrome *c* by hydroquinone.

Mangel [18] has shown that lipid microvesicles containing chlorophyll and carotene are photosensitive, as has been shown previously in planar bilayer lipid membranes [8]. If a redox gradient existed across these artificially constituted bilayer membranes, illumination resulted in charge transport. The quantum efficiency for such reactions are much higher than in the corresponding planar bilayer lipid membranes Oettmeier et al. [16] demonstrated that chlorophyll-lipid microvesicles can serve as a good model for photosynthetic electron transport.

Photoreduction of ubiquinone 30 by *N,N,N′,N′* tetramethylphenylenediamine (TMPD) was catalyzed by their chlorophyll-lipid microvesicles [16]. These studies were a continuation of the work started with planar bilayer lipid membranes and are the first steps in attempting to duplicate functional thylakoid membranes using chlorophyll-containing bilayer lipid membranes as a model. The work presented here is a further step toward this objective.

Light-driven, chlorophyll mediated electron transfer from electron donors to electron acceptors in both organic solvents and Triton X-100 aqueous chlorophyll dispersions have been studied by Krasnovsky [19, 20]. Among the electron donors used in Krasnovsky's models have been ascorbic acid, cysteine, phenylhydrazine, NADH, hydroquinone, and various ferrous compounds; electron acceptors include ferric compounds, quinones, methyl viologen and oxygen The Krasnovsky reactions have been extensively used to model photosystem I. In addition, Mehler [21] first reported the light dependent transfer of electrons from several electron donors (later experiments included ascorbic acid and cysteine [22]) to oxygen, producing hydrogen peroxide in chloroplast suspensions [23] In the present paper, the Krasnovsky and Mehler reactions, using various electron donors and oxygen as the electron acceptor, are shown to be operational in chlorophyll-lipid microvesicles. The effect of various electron donors, electron acceptors and membrane associated compounds on oxygen uptake and the photobleaching of the pigments in chlorophyll-lipid microvesicles are reported. Demonstration of photoreactions, including Krasnovsky and Mehler reactions in chlorophyll-microvesicles, is another advance in constructing a model system which duplicates the function of a thylakoid

MATERIALS

The spinach used in the chlorophyll preparation was obtained fresh locally. The egg lecithin used in microvesicle preparation was from Sigma. The electron donors and acceptors were purchased from Sigma (ascorbic acid, cysteine, menadione, NADH), Fisher (potassium ferricyanide, thiourea), Aldrich (benzoquinone-purified by sublimation, 1-amino-4-hydroxyanthroquinone, 2-hydroxy-1, 4-napthoquinone), K and K Laboratories (chloranil, methyl viologen), Eastman (hydroquinone, phenylhydrazine), Baker (potassium ferrocyanide) and Pfaltz and Bauer (dehydroascorbate). D, L-α-tocopherol was purchased from Sigma and β-carotene from ICN. The water soluble chlorophyllin was from Pflatz and Bauer. Catalase was Sigma C-30 and had an activity of 20 000 units/mg

METHODS

Chlorophyll preparation. A chlorophyll extract was made from fresh spinach by the method previously reported [8]. As calculated by the method of Mackinney [24], the extract contained chlorophyll *a*, chlorophyll *b* and several yellow xanthophylls-carotenes with the ratio 1.0 : 0.72 : 0.02 The extract was spotted on Silica Gel thin layer sheets (Polygram Sil G – Brinkmann Instruments, Inc.) and developed in the solvent petroleum ether-acetone, 7 : 3 [25]. Traces of pheophytin and allomerized chlorophyll were also noted Similar chlorophyll *a* : *b* ratios were obtained by scraping the individual bands off the TLC sheets and measuring the absorbance in H_2O/acetone, 2 : 8 at 663 and 645 nm.

Lipid microvesicle preparation. Lipid microvesicles were prepared by drying a chloroform solution of egg lecithin (300 mg) and the chlorophyll extract (approx. 10 mg of chlorophyll per experiment) under nitrogen, in the dark, on the inside of a 50 ml round bottom flask. 30 ml of the test solution (usually 100 mM KCl, and 10 mM of either tris or potassium acetate at pH 7 5 or 5 0, respectively, to which water soluble electron donors or electron acceptors were added) and 4 glass beads were added and the solution was vigorously mixed in the dark on a vortex mixer for 15 min The pigmented lipid microvesicles were then sonicated in the dark for 1.5 min on the top power setting of a Bronson model W140D sonicator. The chlorophyll-lipid microvesicles were stored in the dark. 10-ml samples were removed and added to 140 ml of the test solution and mixed thoroughly. This diluted lipid microvesicle preparation was usually used in the illumination experiments. A 60 ml sample of these lipid microvesicles contained 40 mg of egg lecithin and about 1.3 mg of chlorophyll.

Illumination. 60 ml of the dilute chlorophyll-lipid microvesicles were placed in the chamber of a YSI Model 5302 Macro Bath (Yellow Springs Instrument Co.). The bath was kept in a dark box which was surrounded by a bank of 5 lights. (See below.) The temperature was controlled at 25 °C±0.2 by a constant temperature circulating water bath. The actual chlorophyll-lipid microvesicle temperature was measured on a YSI 581C Digital Thermometer with a YSI model 701 Thermilinear Probe (Yellow Springs Instrument Co.) Throughout the experiment the microvesicles were constantly mixed with a magnetic stirrer. 60 ml of the pigmented-lipid microvesicle preparation was also added to a totally dark chamber located outside of the box and was kept at the same temperature as the chamber inside the box by the same circulating water bath The dark-control microvesicle temperature was also monitored by a YSI Thermilinear Probe Prior to the experiments both dark and light chlorophyll-lipid microvesicle preparations were either purged with nitrogen for 15 min to give anerobic conditions or else were bubbled with air for 5 min to air saturate the microvesicle suspension. The pigmented microvesicles inside the box were sealed in their chamber by a YSI Model 533 oxygen probe and lucite plunger All air bubbles were carefully removed from the solution The microvesicles were then ready for irradiation. The box was surrounded by 5 lights (a 500-W CZX DAB Sylvania projection lamp at 20 cm, a 150-W DFG GE projection lamp at 30 cm and 3 Sylvania F4T5/D florescent lights at 8 cm) Direct heating of the chlorophyll-lipid microvesicles was reduced by the circulating water bath as well as the intervention of 2 cm water heat filters between the lamps and the sample.

Acidification experiments. At various times equal samples were taken from the light and dark chlorophyll-lipid microvesicles. The pH of both samples was determined by either a Beckman Expandomatic pH meter or else a Sargent-Welch Model NX Digital pH meter The dark sample was then titrated with a known dilute acid until the pH matched that of the light sample. The micromoles of protons produced in the light reaction was then calculated.

Bleaching experiments. Upon aerobic illumination the chlorophyll-lipid microvesicles slowly bleached from green to yellow. At various times of illumination, samples of the chlorophyll-lipid microvesicles were extracted with petroleum ether and analyzed for their chlorophyll *a* and chlorophyll *b* content as reported above. For rapid routine analysis a standard curve was made. Chlorophyll-lipid microvesicles were removed at various times of irradiation and the absorbance at 660 nm measured. The absorbance was shown to decrease as the pigmented microvesicles bleached. At the same illumination times that the microvesicles absorbance was determined, the chlorophyll content was accurately determined after petroleum ether extraction A plot of absorbance at 660 nm of the chlorophyll-lipid microvesicles vs. chlorophyll content yielded a straight line for each spinach extraction batch. From this curve a quick measurement of the absorbance of microvesicles at 660 nm gave an exact determination of chlorophyll content.

Oxygen consumption At time zero, in the dark, the chlorophyll-lipid microvesicles were saturated with air to give the 100 % air saturation level on the YSI Model 53 oxygen monitor. The chamber was sealed and all air bubbles carefully removed. The amount of oxygen remaining in the sample was measured with a YSI Model 533 Oxygen Probe continuously throughout the period of illumination (usually 100 min) The μmol of oxygen in the sample was calculated from Murray and Riley [26].

RESULTS

When aerobic suspensions of chlorophyll-lipid microvesicles were illuminated with intense white light: (a) the media became more acidic, (b) the microvesicles bleached from green to yellow, and (c) oxygen was consumed by the microvesicles (Fig 1). These three properties, acidification, bleaching and oxygen consumption were studied in detail.

Acidification. Upon illumination with white light, under aerobic conditions, a slight acidification of the chlorophyll-lipid microvesicle suspension was noted (Fig. 1 – Curve A). This ΔpH-acidic was measured at pH 7.5, 6.25 and 5.0 in both unbuffered solutions and solutions slightly buffered with Tris, potassium phosphate and potassium acetate, respectively. Potassium iodide was shown to inhibit this acidification A very large acidification (about 4 times that of the unmodified chlorophyll-lipid microvesicles) was noted at pH 7.5 in the presence of ascorbic acid. This large ΔpH was not inhibited at all by potassium iodide and appears to be a different reaction than the one producing a small ΔpH in the absence of ascorbic acid. No acidification could be measured when the chlorophyll-lipid microvesicles were kept in the dark for several hours or were sealed under nitrogen and illuminated. Also, the egg lecithin microvesicles without chlorophyll resulted in no acidification upon illumination. From these experiments it was concluded that photooxidation of the chlorophyll-lipid microvesicles resulted in production of protons. The reaction depended on the presence of air, light and chlorophyll.

Bleaching. Upon illumination with white light the chlorophyll-lipid microvesicles rapidly bleached from green to yellow (Fig. 1). This bleaching was followed spectrophotometrically as well as by thin layer chromatography. During the bleaching

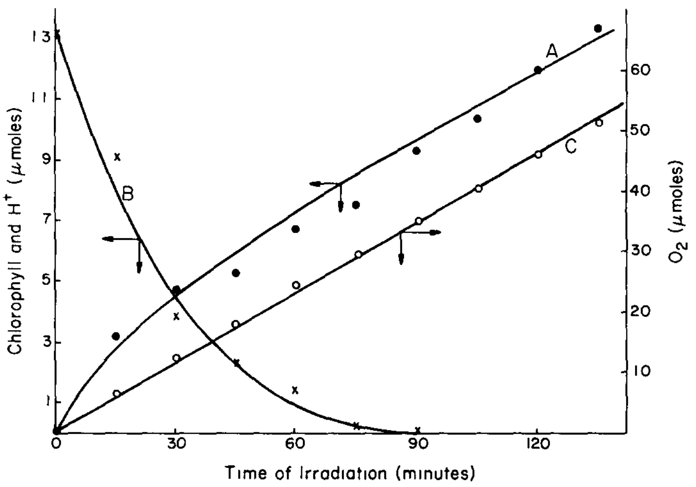

Fig. 1 Acidification (Curve A), chlorophyll bleaching (Curve B) and oxygen consumption (Curve C) when chlorophyll-lipid microvesicles were aerobically irradiated under intense white light. Microvesicles were made from egg lecithin and the chlorophyll extract as described, in 0.1 M KCl unbuffered solution adjusted to pH 7.5. 60 ml of vesicles in the irradiated sample initially contained 40 mg of egg lecithin and 1.18 mg of chlorophyll

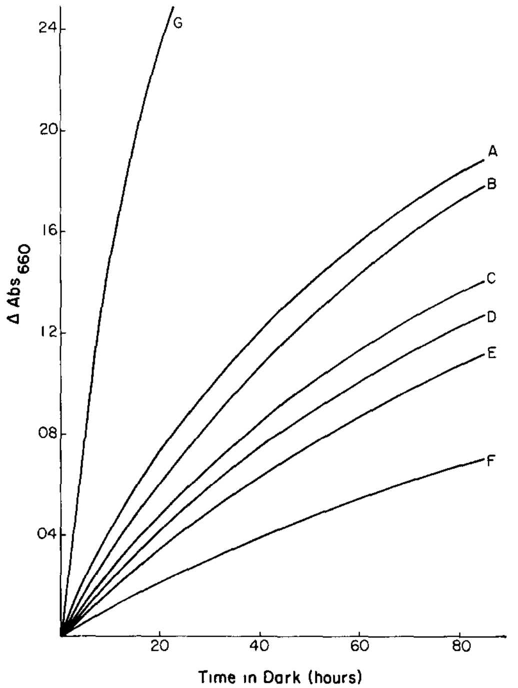

Fig 2 Bleaching of chlorophyll-lipid microvesicles in the dark Microvesicles were made as reported in either 100 mM KCl, 10 mM Tris, pH 7.5 buffer or 100 mM KCl, 10 mM potassium acetate, pH 5 0 buffer 300 μl of microvesicles were added to 6 0 ml of buffers to which 2 mM concentration of the following electron donors were included (A) no electron donor added, pH 7 5, (B) ferrocyanide, pH 7 5, (C) cysteine, pH 7 5, (D) NADH, pH 7 5, (E) thiourea, pH 7 5, (F) ascorbic acid, pH 7 5 and (G) no electron donor, pH 5 0 The absorbance change from time zero at 660 nm is reported vs the time the microvesicles were kept in the various buffers

chlorophyll *a* to chlorophyll *b* ratio continuously decreased. The photobleaching could not be reversed by the later addition of ascorbate When sealed under nitrogen, little bleaching could be detected upon illumination, again implicating a photooxidation. Aerobically, in the dark, a slow bleaching occurred (Fig. 2). This bleaching was noticeably slowed by the presence of electron donors (Fig 2). Later addition of ascorbic acid reversed the dark bleaching by about 25 % The dark bleaching was followed at pH 5 0 and also pH 7.5 Chlorophyll in microvesicles is much more stable to the dark oxidation at the higher pH (Fig. 2)

Oxygen consumption. Upon illumination in white light the chlorophyll-lipid microvesicles rapidly absorbed oxygen from the media. This oxygen consumption was dependent on the amount of chlorophyll in the microvesicles (Fig. 3). Illumination of egg lecithin microvesicles without chlorophyll resulted in the consumption of very little oxygen (Fig 3) Upon ageing in the dark, chlorophyll-lipid microvesicles increased their ability to absorb oxygen upon subsequent illumination. Chlorophyll-lipid microvesicles at pH 7.5 were more resistant to oxidation than were identical chlorophyll-lipid microvesicles at pH 5.0 (Fig. 4). At pH 7.5 chlorophyll-lipid microvesicles remained very much the same in terms of oxygen consumption, even after ageing 23 5 hours in the dark while at pH 5 0, a noticeable change occurred in 3 45

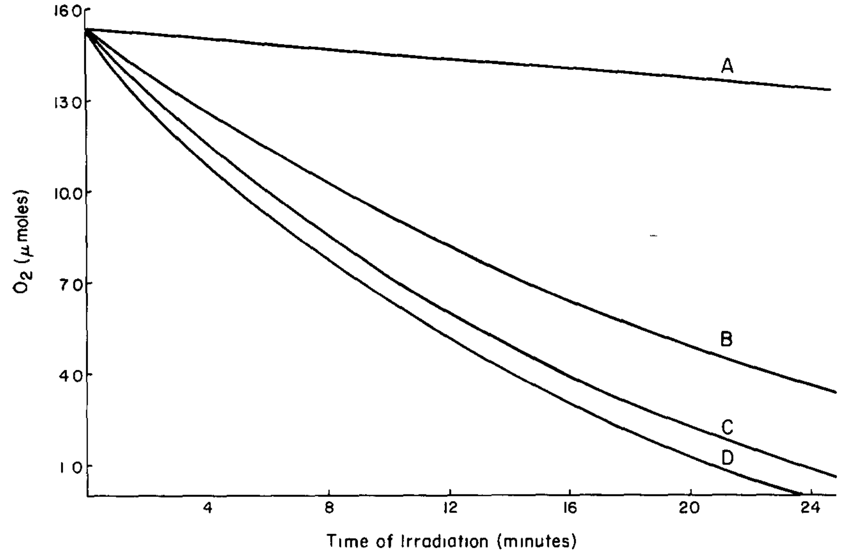

Fig 3 Oxygen consumption upon irradiation of chlorophyll-lipid microvesicles of various chlorophyll content. Egg lecithin microvesicles with chlorophyll (chlorophyll-lipid microvesicles) and without (E.L -microvesicles) were made in 100 mM KCl, 1.0 mM Tris, 0.5 mM $MnCl_2$ buffer at pH 7.5 The microvesicles were mixed in the following proportions and then the rate of oxygen consumption measured

Expt	E L -microvesicles	Chlorophyll-lipid microvesicles	Buffer
A	15	0	45
B	10	5	45
C	5	10	45
D	0	15	45

All experiments contained 40 mg of egg lecithin per 60 ml The chlorophyll content varied from 0 to 5 mg of chlorophyll

hours. One of the major objectives of the experiments presented here was to study the photoreduction of oxygen by several electron donors sensitized by chlorophyll-containing lipid-microvesicles (Krasnovsky and Mehler reactions). The rate of oxygen reduction in chlorophyll-lipid microvesicles in the presence of various electron donors (Fig. 5), electron acceptors (Fig. 6) and membrane associated components (Fig. 7) is reported Electron transfer from a series of donors to oxygen was measured and the relative rates reported in Table I. The rate of oxygen reduction in the presence of electron donors resulted in the following series: control (no added electron donor) = KI = ferrocyanide < hydroquinone < thiourea < cysteine < NADH < Fe^{+2} < ascorbic acid < phenylhydrazine. As the chlorophyll-lipid microvesicles absorbed oxygen, a concomitant bleaching of the pigments was noted. The rate of bleaching as a function of oxygen consumption is reported in Fig 8.

Water-soluble chlorophyll. The effect of water-soluble chlorophyllin on acidification, bleaching, and oxygen consumption in the presence and absence of egg lecithin microvesicles was measured. Water-soluble chlorophyllin is known to give large

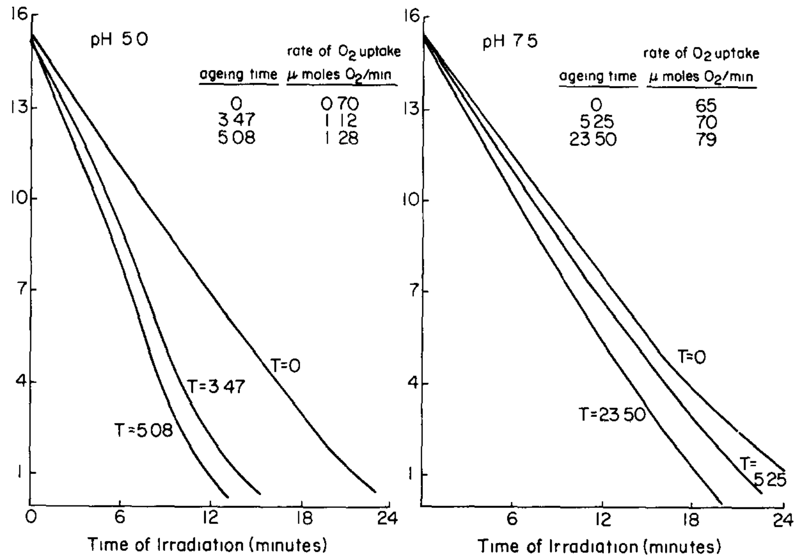

Fig. 4 The effect of ageing chlorophyll-lipid microvesicles at pH 5.0 and 7.5 on the ability to consume oxygen in the light. Microvesicles were made in 100 mM KCl/10 mM potassium acetate, pH 5.0 buffer or 100 mM KCl/2 mM Tris, pH 7.5 buffer. Microvesicles contained 50 mg of egg lecithin and about 1.7 mg of chlorophyll in each 60 ml sample. The microvesicles were stored in the dark and were tested for oxygen consumption at time 0, 3.47, and 5.08 h after preparation at pH 5.0 and at time 0, 5.25 and 23.5 h at pH 7.5. The initial rate of oxygen consumption upon illumination for the various chlorophyll-lipid microvesicles is reported in the insert.

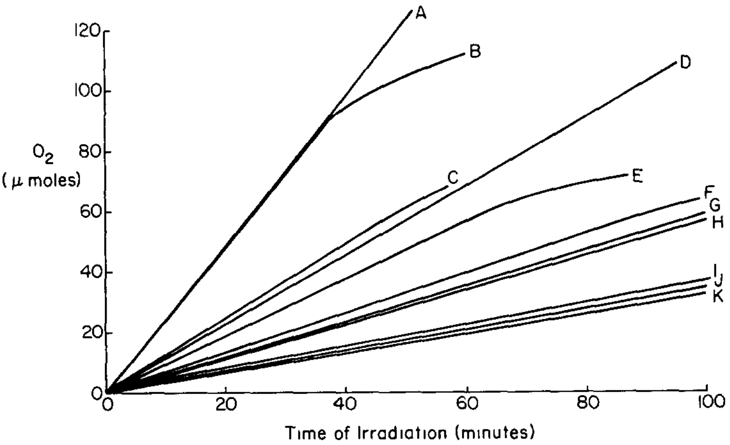

Fig. 5 The effect of electron donors on the rate of oxygen consumption when chlorophyll-lipid microvesicles were irradiated with white light. Microvesicles were made in 100 mM KCl/10 mM Tris, pH 7.5 buffer (the Fe^{+2} experiment was run in 100 mM KCl/10 mM potassium acetate, pH 5.0 buffer because Fe^{+2} is not soluble at pH 7.5). Each 60 ml experiment contained 40 mg of egg lecithin and 1.17 mg of chlorophyll. Electron donors tested were (A) 10 mM ascorbic acid, (B) 1 mM phenylhydrazine, (C) 1 mM ascorbic acid, (D) 1 mM Fe^{+2} (pH 5.0), (E) 1 mM NADH, (F) 1 mM cysteine, (G) 1 mM thiourea, (H) 1 mM hydroquinone, (I) no addition control, (J) 1 mM ferrocyanide, and (K) 10 mM KI.

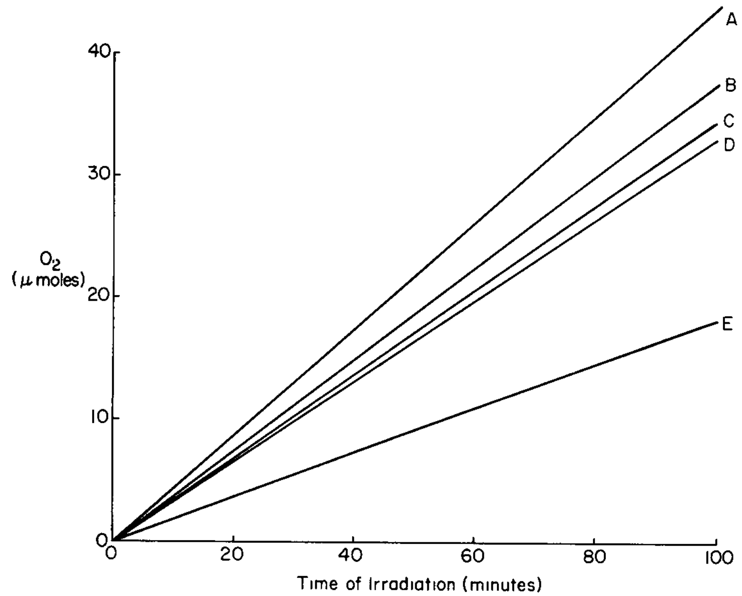

Fig 6 The effect of electron acceptors on the rate of oxygen consumption when chlorophyll-lipid microvesicles were irradiated with white light Microvesicles were made in 100 mM KCl/10 mM Tris, pH 7.5 buffer Each 60 ml microvesicle sample contained 40 mg of egg lecithin and 1.21 mg of chlorophyll Electron acceptors were (A) 1 mM dehydroascorbate, (B) 1 mM methyl viologen, (C) no addition control, (D) 1 mM ferricyanide and (E) 1 mM benzoquinone

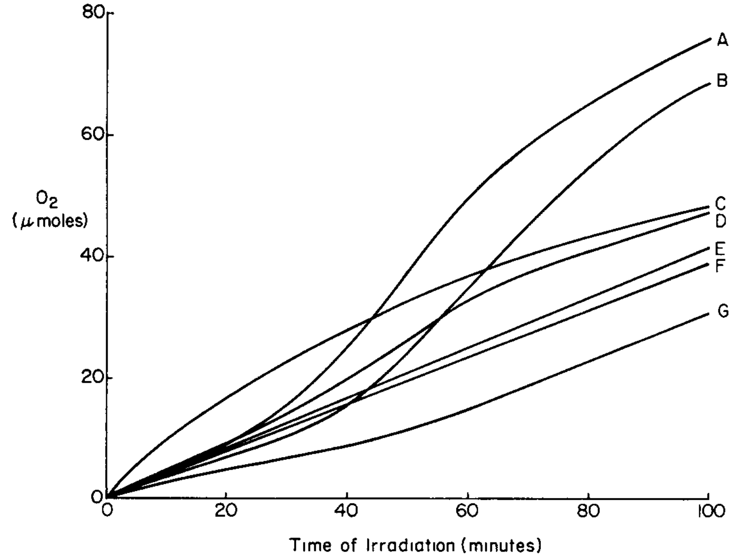

Fig. 7 The effect of lipid soluble, membrane associated components on oxygen consumption when chlorophyll-lipid microvesicles were irradiated with white light Microvesicles were made as usual, however, the following lipid soluble components were added to the egg lecithin, chlorophyll, chloroform solution before drying (A) 2-hydroxy-1,4-napthoquinone (0 05 mM), (B) chloranil (0 05 mM), (C) α-tocopherol (0 05 mM), (D) menadione (0 05 mM), (E) β-carotene (0.05 mM), (F) no addition control, and (G) 1-amino-4-hydroxyanthroquinone (0 05 mM) The irradiated chlorophyll-lipid microvesicles with lipid soluble components contained 40 mg of egg lecithin, 1 37 mg of chlorophyll and 0 0067 mM of each membrane component per 60 ml

TABLE I

Effect of water-soluble electron donors and acceptors on the rate of oxygen consumption when chlorophyll-lipid microvesicles were irradiated with white light The initial oxygen uptake rate was determined from Figs 7 and 8

Compound	O_2 consumption (μmol/min)
Electron donors	
Control (no addition)	0 37
KI (10 mM)	0 34
Ferrocyanide	0 36
Hydroquinone	0 48
Thiourea	0 50
Cysteine	0 66
NADH	0 95
Fe^{+2} (pH 5 0)	1 16
Ascorbate	1 24
Phenylhydrazine	2 46
Electron acceptors	
Benzoquinone	0 18
Ferricyanide	0 33
Methyl viologen	0 38
Dehydroascorbate	0 43

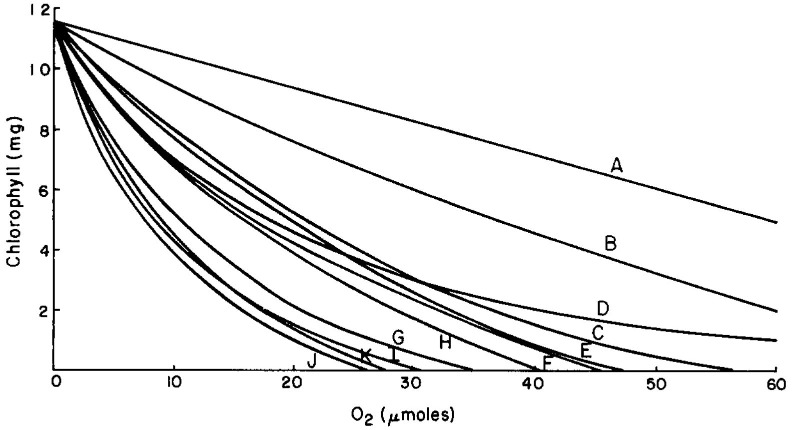

Fig 8 Chlorophyll degradation vs oxygen consumption in the presence of various electron donors when chlorophyll-lipid microvesicles were irradiated with white light Microvesicles were made in 100 mM KCl/10 mM Tris, pH 7 5 buffer (except for Fe^{+2} which was in 100 mM KCl, 10 mM potassium acetate at pH 5 0) Each 60 ml experiment contained 40 mg of egg lecithin and 1 17 mg of chlorophyll Electron donors were (A) 10 mM ascorbic acid, (B) 1 mM phenylhydrazine, (C) 1 mM ascorbic aicd, (D) 1 mM Fe^{+2} (pH 5 0), (E) 1 mM NADH, (F) 1 mM cysteine, (G) 1 mM thiourea, (H) 1 mM hydroquinone, (I) no addition – control, (J) 1 mM ferrocyanide and (K) 10 mM KI

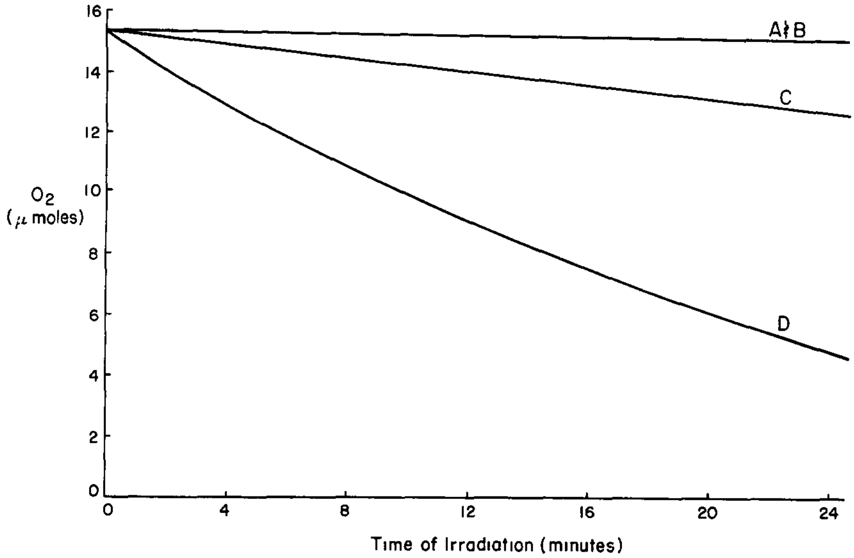

Fig. 9. Oxygen uptake when water-soluble chlorophyllin was illuminated in white light. Egg lecithin microvesicles (containing no chlorophyll) and normal chlorophyll-lipid microvesicles were made in 100 mM KCl/1 mM Tris, pH 7.5 buffer. Solutions of E.L.-microvesicles, chlorophyll-lipid microvesicles and E.L.-microvesicles plus water-soluble chlorophyllin were made as follows

Expt	EL-microvesicles (ml)	Tris buffer (ml)	Tris buffer + water soluble chlorophyllin (ml)	Chlorophyll-lipid microvesicles (ml)
A	–	5	55	–
B	5	55	–	–
C	5	–	55	–
D	–	55	–	5

Water-soluble chlorophyll concentration was 3.33 mg/60 ml of solution. The egg lecithin was 50 mg/60 ml of solution while the chlorophyll content of the chlorophyll-lipid microvesicles used in D was 1.67 mg/60 ml of solution

photopotentials in planar bimolecular lipid membrane systems [27]. The water-soluble chlorophyllin, however, proved to be very inert. Neither a ΔpH nor bleaching could be measured with or without egg lecithin microvesicles upon illumination. A slight oxygen consumption was measured (Fig. 9). Upon illumination, oxygen consumption was noticeably increased in the presence of egg lecithin microvesicles. Light-driven electron transfer from ascorbic acid to oxygen in the presence of water-soluble chlorophyllin could be measured, however, only at about 5 % of the rate for chlorophyll-lipid microvesicles.

DISCUSSION

The main objective of these experiments has been to demonstrate that reactions (acidification, bleaching and oxygen consumption) occurring in vivo and in other photosynthetic model systems can also occur in pigmented lipid microvesicles. In the experiments reported here, illumination of chlorophyll-lipid microvesicles with white light resulted in acidification of the media (Fig. 1). The production of pH gradients upon illumination of chloroplasts has been well documented [28] and is believed to be the driving force for photophosphorylation.

Bleaching of photosynthetic pigments in organic solvents [3, 29], in "quantasomes" [30] and in vivo [31] have been studied for many years. (In this paper "quantasomes" are used only as an example of an intermediate step in complexity of the chlorophyll environment between that of simple liposomes and that of the thylakoid membrane. We are not implying any physiological function for the "quantasomes".) In the experiments reported here a comparable oxidative photobleaching was measured in the chlorophyll-lipid microvesicles (Fig. 8). Upon bleaching, the amount of chlorophyll as well as the ratio of chlorophyll *a* to chlorophyll *b* decreased, indicating the more labile nature of the chlorophyll *a* to oxidation. The rate of bleaching was shown to be partly inhibited by benzoquinone. A much slower aerobic dark bleaching, similar to that reported for "quantasomes" [30], was measured for the chlorophyll-lipid microvesicles (Fig. 2). As in "quantasomes", the dark bleaching in microvesicles was inhibited by electron donors and was partly reversed (up to 25 %) by the later addition of ascorbic acid.

It has been previously demonstrated that chlorophyll is most stable to photooxidation in vivo [31] and it is less stable in "quantasomes" [30] and very unstable in organic solvents [29]. The stability of chlorophyll to bleaching depends on the environment that chlorophyll is in. It is pH and oxygen sensitive [29, 30, 32]. The chlorophyll-lipid microvesicles used in the experiments reported here were more stable to photobleaching at pH 7.5 than at pH 5.0 (Fig. 4) and were very stable when illuminated under nitrogen. The chlorophyll-lipid microvesicles were shown to be more resistant to photooxidation than was chlorophyll in organic solvents but was less stable than chlorophyll *a* in "quantasomes" or in vivo.

Krasnovsky has suggested that the photobleaching of chlorophyll is less in chloroplasts than in organic solvents because of the close association of chlorophyll to lipids and proteins in vivo [19]. In addition, one of the suggested roles of carotene has been as a protecting agent for the photooxidation of chlorophyll [33]. Since the chlorophyll extract used in the synthesis of the chlorophyll-lipid microvesicles in the experiments reported here contained a considerable amount of carotene, the increased stability of chlorophyll to photobleaching in microvesicles is understandable. However, an additional experiment was run to test if the phospholipids used in making the chlorophyll-lipid microvesicles could have added to the stability of chlorophyll. The rate of bleaching of chlorophyll was shown to be markedly decreased in the presence of egg lecithin and dipalmotylphosphatidylcholine (Table II).

A further increase in stability of chlorophyll-lipid microvesicles towards photooxidation was noted if a $CHCl_3/CH_3OH$ extract of spinach containing some protelipids was used instead of the normal petroleum ether/CH_3OH extract. In terms of modeling a thylakoid with relation to chlorophyll stability, the following sequence is suggested:

$$\begin{array}{c}\text{Chlorophyll in}\\\text{organic solvent}\\\text{(least stable)}\end{array} \xrightarrow[\text{carotene}]{\text{phospholipids}} \begin{array}{c}\text{lipid}\\\text{microvesicles}\end{array} \xrightarrow{\text{lipoproteins}} \text{“quantasomes”} \xrightarrow[\text{extract}]{\text{aqueous}} \begin{array}{c}\text{thylakoid}\\\text{(most stable)}\end{array}$$

Pigmented lipid microvesicles were shown to catalyze the transfer of electrons

TABLE II

The effect of egg lecithin and dipalmitoylphosphatidylcholine on the rate of bleaching of chlorophyll in chloroform The samples were irradiated with a 500W lamp at 20 cm The change in absorbance at 665 nm per minute for the various samples as reported

Expt	Egg lecithin (mg)	Dipalmitoylphosphatidylcholine (mg)	Initial $\Delta A_{66\,5/\mathrm{min}}$
A	0	0	0 087
B	5 05	–	0 066
C	25 05	–	0 052
D	–	5 05	0 044
E	–	25 25	0 022

from a series of electron donors to oxygen in light. These Krasnovsky reactions are shown in Fig. 5 and Table I. The donors varied from KI and ferrocyanide which has no effect on oxygen consumption to ascorbic acid, Fe^{+2} (pH 5) and phenylhydrazine which were the most active. With the exception of benzoquinone, water soluble electron acceptors had no effect on the rate of oxygen consumption (Fig. 6). The effect of the hydrophobic compounds quinones, β-carotene and α-tocopherol on oxygen consumption is shown in Fig. 7. Additional β-carotene had no effect on the oxygen uptake, however, a considerable quantity of carotene was contained in the chlorophyll extract. With chlorophyll-lipid microvesicles containing α-tocopherol (a known antioxidant) an initial rapid uptake of oxygen occurred which slowed upon prolonged irradiation The quinones tested all demonstrated an initial slow oxygen consumption followed by a rapid oxygen reaction and a slow period (Fig. 9). The significance of this anomalous behavior is unknown.

Illuminated chlorophyll-lipid microvesicles were demonstrated to reduce oxygen with various electron donors in Mehler type reactions (Fig 5). Included in this list of Mehler electron donors tested in the chlorophyll-lipid microvesicles were ascorbic acid and cysteine. Sine H_2O_2 is predicted by the Mehler reaction to be a product of photoreduction of oxygen, the addition of catalase to the chlorophyll-lipid microvesicles should release oxygen trapped as H_2O_2, if any H_2O_2 is present [22] In fact, the addition of catalase had no effect on the amount of oxygen appearing in the illuminated chlorophyll-lipid microvesicles when ascorbic acid was used as the electron donor and so it was concluded that free H_2O_2 was not produced in the reaction.

Water-soluble chlorophyllin was tested for its ability to: (a) acidify the media, (b) bleach, and (c) take up oxygen, when irradiated. Brune and San Pietro [35] have used chlorophyllin *a* in the photoreduction of viologen dyes (a Krasnovsky reaction). In the present system, however, water-soluble chlorophyllin was shown to be very ineffective in acidification, bleaching or oxygen consumption Even in the presence of ascorbic acid, oxygen consumption was very small compared to the chlorophyll-lipid microvesicle system. Although chlorophyllin alone or egg lecithin microvesicles (with no chlorophyll) alone did not take up oxygen from the media, when the two components were mixed a noticeable increase in oxygen consumption could be measured (Fig. 9). This implies that part of the oxygen consumption reaction as measured here may be a surface phenomenon of chlorophyllin and the lipid bilayer of the microvesicles.

In summary, several observations previously made on organic solutions of chlorophyll, "quantasomes" and chloroplasts have now been demonstrated in the chlorophyll-lipid microvesicles Electron transfer similar to that in the Krasnovsky reactions and the Mehler reaction is readily exhibited by the chlorophyll-lipid microvesicles Pigment bleaching in this model is also similar to that reported in organic solutions and "quantasomes". Even the production of protons may be related to pH gradients known to have significance in vivo These observations are an additional step in modeling a functional thylakoid membrane using chlorophyll-lipid microvesicles.

ACKNOWLEDGEMENTS

The work presented here was accomplished through a United States Public Health Service Grant 14971.

REFERENCES

1 Seely, G. R (1966) in The Chlorophylls (Vernon, L P and Seely, G R , eds) p 523, Academic Press, New York
2 Csatorday, K , Lehoczki, E and Szalay, L (1975) Biochim Biophys Acta 376, 268–273
3 Krasnovsky, A A (1969) Progress in Photosynthesis Research II (Metzner, H , ed), pp. 709–727
4 Dam, R J , Kongslie, K F and Griffith, O H (1975) Photochem Photobiol 22, 265–268
5 Leblanc, R M , Galinier, G , Tessier, A. and Lemieux, L (1974) Can. J Chem 52, 3723–3727
6 Branton, D (1969) Ann Rev Plant Physiol 20, 209–243
7 Tien, H T. (1968) Nature 219, 272–274
8 Tien, H. T (1974) Bilayer Lipid Membranes (BLM)/Theory and Practice p 546, Marcel Dekker, Inc New York
9 Bangham, A. D (1968) Progr Biophys Mol Biol 18, 31–95
10 Paphadjopoulos, D (1973) in Biological Horizons in Surface Sicence (Prince, L M and Sears, D F , eds), pp 159–225
11 Huang, C -H (1969) Biochem 8, 344–351
12 Chapman, D and Fast, P G (1968) Science 160, 188–189
13 Trosper, I , Raveed, D and Ke, B (1970) Biochim Biophys Acta 223, 463–465
14 Tomkiewicz, M and Corker, G (1975) Photochem Photobiol 22, 249–256
15 Ritt, E and Walz, D (1976) J Memb Biol. 27, 41–54
16 Oettmeier, W , Norris, J. R and Katz, J J (1976) Z Naturforsch 31c, 163–168
17 Nicholls, P , West, J and Bangham, A D (1972) 8th FEBS Meeting, Abstract No 26
18 Mangel, M (1976) Biochim Biophys Acta 430, 459–466
19 Krasnovsky, A A (1972) Biophys J 12, 749–763
20 Krasnovsky, A A (1965) Photochem Photobiol 4, 641–655
21 Mehler, A H (1951) Arch Biochem Biophys 33, 65–77
22 Allen, J F and Hall, D O (1973) Biochem Biophys Res Commun 52, 856–862
23 Krogman, D W (1961) in Light and Life (McElroy, W D and Glass, B , eds) pp 615–630, Johns Hopkins Press, Baltimore
24 Mackinney, G (1941) J Biol Chem 140, 315–322
25 Sherma, J and Lippstone, G S (1969) J Chromat 41, 220–227
26 Murray, C N and Riley, J P (1969) Deep-Sea Res 16, 311–320
27 Tien, H T (1977) Brookhaven Symp Biol 28, 105–130
28 Gregory, R P F (1977) Biochemistry of Photosynthesis, Wiley, New York
29 Goedheer, J C (1958) Biochim Biophys Acta 27, 478–490
30 Sauer, K and Calvin, M. (1962) Biochim Biophys Acta 64, 324–339
31 Thomas, J. and Nijhuis, H. H (1968) Biochim Biophys Acta 153, 868–877
32 Colmano, G (1961) Biochim Biophys Acta 47, 454–457
33 Claes, H and Nakayama, T O M (1959) Z Naturforsch 14b, 746–747
34 Stanier, R Y (1958) U S At Energy Comm BNC 512, 43–53
35 Brune, D and San Pietro, A (1970) Arch Biochem Biophys 141, 371–373