J. Membrane Biol. 8, 333 – 356 (1972)

Photoresponse of Chlorophyll-Containing Bileaflet Membranes and the Effect of Phycocyanin as Extrinsic Membrane Protein

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Received 9 September 1971

Summary. Artificial bileaflet membranes were formed from extracts of chloroplasts. Gradients of a redox potential were created across the membranes by adding various concentrations of ceric-cerous ions, ferric-ferrous ions, and ascorbic acid to the aqueous solutions on either side of the membrane. When a membrane interposed between solutions of different redox potential was irradiated with light, a potential difference of up to 50 mV was recorded. Analysis of the photoresponse allowed its separation into two components: a photoelectromotive driving force dependent upon the redox potential gradient, and a photoconductive pathway dependent upon the amount of light absorbed by the membranes. There appeared to be a limit to the photocurrent that could be drawn from a membrane at a particular intensity of irradiation; i.e., it did not increase indefinitely with increase of the redox potential gradient. Conductance of the photoconductive pathway was independent of temperature. Phycocyanin added to the aqueous solution participated in the photoresponse in a unidirectional manner that suggested facilitation of electron transport from membrane to acceptors in the aqueous solution.

Thin lipid artificial membranes were shown to be sensitive to light irradiation if appropriate pigments were included in the membranes [13, 15, 16] or if light-absorbing solutes were added to the aqueous solutions [11, 17]. The photosensitivity expressed itself in a form of current or voltage response to light irradiation. In most cases, the response was well sustained as long as irradiation proceeded, but in a significant exception a type of transitory response was observed [17]. When light absorbing solutes were in the aqueous solution [11], the photoresponse was attributed to an ionic process. When the pigments constituted part of the membrane, the response was considered to be an electronic one [15]. This study involves the latter type of membranes and tends to support the idea that electron movements across the membrane are responsible for the observed photosensitivity.

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The aims of this study were: (a) to reproduce and confirm the results reported by Tien and collaborators [13, 15] of studies on chloroplast extract membranes; (b) to analyze the photoeffect in terms of a simplified model which resolves the response into two parts, i.e., a photodriving force and a photoconductive pathway; and (c) to investigate the possible interaction of biliproteins with the chloroplast lipid membrane.

The essence of the photoresponse observed in these membranes is the flow of negative current from the more reduced side to the more oxidized side of the membrane (or vice versa for positive current). If the phenomenon is an electronic one this would mean that the flow of electrons in the system is contrary to what is found in a chloroplast in the sense that photoillumination of the membranes allows a dissipation of a prexisting redox energy. Phycocyanin interacts with the lipid layer in a fashion which directs electron flow into the phycocyanin-containing side of the membrane. In a selected experimental arrangement it was possible to get "electron flow" into the more reduced phycocyanin side of the membrane, i.e., in a photosynthetic-like fashion. The relevance of this study to primary processes in photosynthesis is dealt with briefly in the discussion.

Materials and Methods

Preparation of Chloroplast Extract Solution

Leaves of fresh spinach (25 to 30 g) were washed several times in distilled water, and chopped in 0.5 liter of 0.5 M sucrose and 0.05 M $NaHCO_3$ in a Waring Blendor. The gross precipitate was removed by light centrifugation ($3{,}000 \times g$ for 10 min). The supernate was centrifuged at $20{,}000 \times g$ for 20 min and the precipitate was extracted several times with a 2:1 solution of petrol ether/methanol. About 0.5 liter of the petrol ether/methanol extract was collected and dried by flash evaporation. The dried extract was dissolved in a solution of approximately 5 ml of *n*-octane and kept in a cold (4 °C), dark place for up to 12 weeks. Before use, 20% by volume of *n*-butanol was added to the aliquot of the *n*-octane solution. The butanol/octane solution (1:5) was used as a membrane-forming mixture containing 40 to 80 mg/ml of dry material. The absorption spectrum of a 1:400 dilution of such a solution is shown in Results.

Experimental Setup

A small amount of the membrane-forming solution was applied with a fine brush to a hole (1 mm in diameter) punched in a Teflon cell according to the technique described by Mueller, Rudin, Tien and Westcott [12]. The hole served as the point of communication between the aqueous solutions inside and outside the Teflon cell (*see* Figs. 1 and 2 for a diagram of the experimental arrangement and electrical circuitry).

The resistance and capacitance of the communication between outside and inside solutions were monitored on an a-c bridge at 500 cps (Wayne-Kerr Universal Bridge). As soon as a layer of octane-butanol solution was deposited on the hole, the conductivity

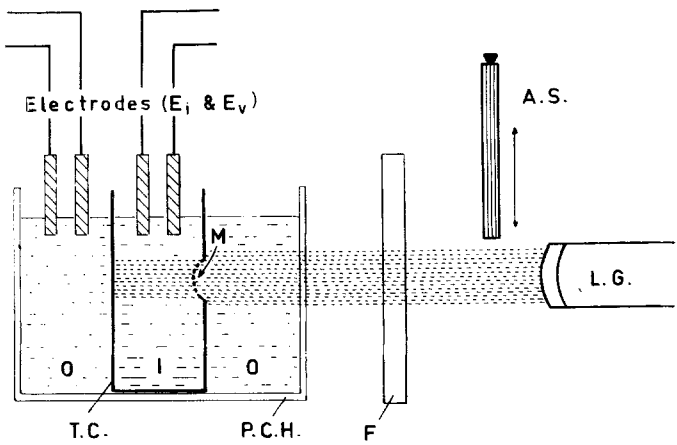

Fig. 1. Scheme of experimental set-up. L.G., light guide; A.S., aluminum foil used as shutter; F, light filter; P.C.H., Perspex cell holder; O, outside; I, inside; T.C., Teflon cell; M, membrane; S.L., solution level. One pair of electrodes (E_v) used for measuring potential and capacitance; second pair (E_i) used for applying current

of the system approached zero. Because of a spontaneous thinning of the layer, the capacitance rose in the following 3 to 15 min until it reached a steady value. Current could be applied to the membrane through a pair of electrodes (E_i in Fig. 2) connected in series with precision high megohm resistances (The Victoreen Instrument Co., Cleveland, Ohio) and a variable voltage source. The membrane potential was measured through another pair of electrodes connected to an input of a cathode follower (input resistance $> 10^{11}$ Ω). The output of the cathode follower was connected to a servo-recorder (Heath Model EU 14) and an oscilloscope. Unless otherwise specified the voltage polarity refers to the inside solution. The electrodes used were Ag—AgCl immersed in 3 M KCl.

The optical system consisted of a fiberglass illuminator (containing a 500 Watt tungsten lamp and a filter which absorbs the infrared part of the spectrum) and light guide (Edmund Scientific); band-path interference filters and other types of light filters as specified in Results; aluminum foil which served as a shutter; and a Model G 5 Radiometer (Yellow Spring Instrument Co.) with a thermistor bolometer as a probe (No. 6651).

The diameter of the outlet of the light guide was 1 cm. The center of the light beam was directed at the center of the hole in the Teflon cell. The relatively large diameter of the light beam ensured uniform illumination of the hole in the Teflon cell (*see* Fig. 1). The total light intensity at the level of the membrane was 120 to 150 mW/cm^2. The relative intensity of the light filtered through the various band-path interference filters is shown in Results (Fig. 10).

The primary aqueous solution was 0.1 M potassium acetate buffer, pH 5.0. Solid $FeCl_3$, $FeCl_2$, or ascorbic acid was added to the solution to the desired concentrations. Ceric sulfate was available as a 0.1 M solution in 1 N sulfuric acid. A stock solution of 0.1 M potassium acetate and 2 mM ceric sulfate, adjusted to pH 5.0 was prepared. The ceric sulfate stock solution was used for preparing the specified ceric ion-containing solutions used in this study. Phycocyanin from *Anabaena variabilis* was purified as previously reported [10] and lyophilized from distilled water. The dry protein was added to the acetate buffer solution. A Cary 14 recording spectrophotometer was used for measuring the absorption spectra of some of the solutions.

Results

Proposed Working Model of Membrane

The model in Fig. 2 is used as a tool for analyzing the photoeffects in the membrane. The symbols are: C_m, membrane capacitance; R_m, membrane resistance. The membrane is connected through one pair of electrodes E_i to an external variable voltage source V_e and an external resistance R_e. Another pair of electrodes E_v can be connected either to an a-c bridge or the input of a cathode follower which measured the voltage V_m across the membrane. When the membrane is irradiated with light, the series elements of V_p, R_{pi}, and R_{pm} are assumed to be added to the membrane where V_p is the electromotive force of the photoeffect, R_p is the internal resistance of the photobattery with R_{pi} representing the resistance at the membrane-water interface, and R_{pm} is the resistance through the membrane proper. The reciprocal of resistance terms $1/R$ will be designated by the corresponding conductance terms K.

Another alternative simple model is to put the photodriving force in series with the membrane conductance. However, a 'series' model is untenable because of the observation that spontaneous increase in membrane conductance led invariably to a decrease in the photovoltage response. As will be evident from the 'Discussion', a possible physical meaning of the suggested 'parallel' model is as follows: The dark conductance of the membrane is ionic in nature. During illumination an electronic conductive pathway is formed through the membrane. Thus, it is only during illumina-

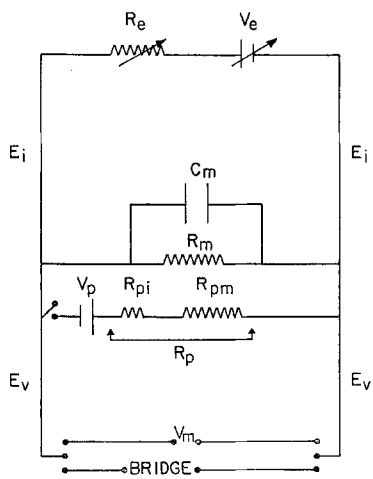

Fig. 2. Electrical model of the membrane system. The switch in the model is closed on light irradiation and opened on stopping the irradiation. Symbols are explained in text

tion that existing redox potential gradient (i.e., the driving force) is allowed to dissipate through the membrane.

For the purpose of an analysis, it is assumed that R_m and C_m are light-independent quantities. This assumption was verified for membrane capacitance by shining light on the membrane while the membrane was connected through the E_v electrode to the Wheatstone bridge. The hypothesis that the membrane resistance is not affected by light could not be verified since a reduction in membrane resistance would be expected upon irradiation resulting from shunting by the R_p element. The only inconsistency with such an assumption would be the observation that the membrane resistance increases in response to irradiation. This type of response was observed only during the early stages of membrane formation. The increase in membrane resistance under those circumstances was irreversible and disappeared in a short time.

Determination of the Electromotive Force of the Photoresponse and Its Internal Resistance

Two tests of the membrane model are described in this section. We define the photovoltage response ΔV as $V_{mp} - V_m$ where V_{mp} and V_m are the steady-state voltages measured across the membrane in the presence and absence of light irradiation, respectively. If V_e is zero, R_e acts only as an external shunt to the membrane. As shown in the Appendix (*Case A*), the relation between the reciprocal of the photoresponse ($1/\Delta V$), and K_e should yield a straight line which has the slope of R_p/V_p and passes through the point $(-K_m, 1/V_p)$ (Fig. 3*B*). In principle, such a curve specifies R_p and V_p if K_m is known. In many experiments, however, $1/V_p$ is very close to zero so that relatively small uncertainties in the values of K_m or in the exact position of the $1/\Delta V$ *vs.* K_e curve may lead to large variation in the values of R_p and V_p even though the ratio R_p/V_p is firmly established. Therefore, another method is necessary for more accurate establishment of the individual values of R_p and V_p and can be accomplished by measurement of the photoresponse ΔV as a function of the membrane potential V_m. V_m can be varied by manipulating V_e and R_e; i.e., by passing various currents through the membrane. The results of such measurements are shown in Fig. 3*C* (▵—▵). As shown in the Appendix (*Case B*), this curve should cut the abscissa at a point $V_m = V_p$ and the slope of such a curve $d\Delta V/dV_m$ is equal to $1/(1 + R_p/R_i)$ where R_i is the equivalent resistance of both the membrane resistance R_m and the external shunt resistance R_e; i.e., $1/R_i = 1/R_m + 1/R_e$. This derivation is based upon the assumption that R_m (and

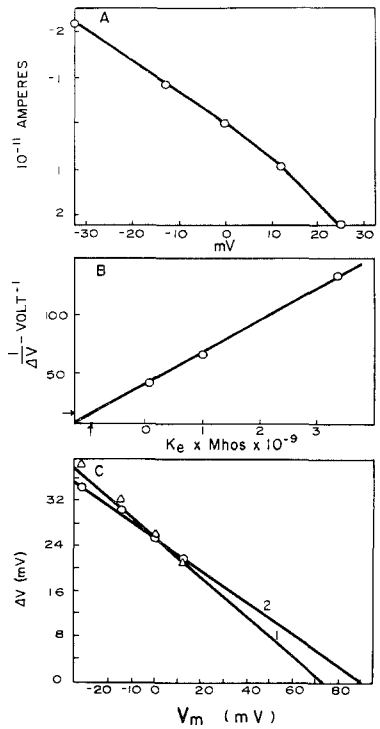

Fig. 3. Electrical measurements and photoresponse analysis of a typical membrane. Outside solution contains 1 mM ceric ions. Membrane capacitance, 2,500 pF. (*A*) Current-voltage relationship in the absence of irradiation. Slopes at upper and lower parts of the curve correspond to 1,500 and 1,140 MΩ, respectively. (*B*) Reciprocal of photovoltage response $1/\Delta V$ at $V_m=0$ as function of the conductance of an external shunt. Arrows indicate positions of $-K_m$ and $1/V_p$ on abscissa and ordinate, respectively. The calculated values of R_p and V_p from this curve are 2,100 MΩ and 70 mV, respectively. (*C*) ΔV as a function of V_m. ▵—▵, measured ΔV; ○—○, corrected values as explained in the text. The calculated values of R_p and V_p from these curves are 2,200 to 2,900 MΩ and 73 to 90 mV, respectively

therefore R_i) is independent of membrane potential. In many membranes this is not the case. As shown in Fig. 3*A*, the current-voltage curve is not linear over the whole range of voltage encountered. Yet the resistance R_m does not vary by more than 10% from the average. It is possible to take into consideration the fact that R_m changes in the course of the photoresponse ($V_m \rightarrow V_{mp}$) by subtracting the corresponding term $V_e \Delta R_i/R_e$ from ΔV as shown on the left-hand side of Eq. (16) of the Appendix. As shown in Fig. 3*C*, the photoresponse is about 25 mV and there is, on the average, a change of 120 MΩ per 25-mV change in membrane potential. R_e is 10,000 MΩ, and V_e for the four points from left to right is −250 mV,

-100 mV, 0, and $+100$ mV, respectively. Therefore, the correction terms ($V_e \Delta R_i / R_e$) are -3.0, -1.2, 0, and $+1.2$ mV, respectively. The corrected values are shown by the circles in Fig. 3*C*. Curves *1* and *2* are drawn through the measured and corrected values, respectively. As can be seen, they yield values of R_p and V_p which are in good agreement with the value determined by the method shown in Fig. 3*B*. Thus, for this particular membrane, V_p (ceric ions containing side negative) is between 70 and 90 mV and R_p is between 2,200 and 2,900 MΩ.

The Significance of V_p and K_p

The fact that the polarity of the photoresponse is dependent upon the asymmetry with respect to the presence of oxidizing cations [15] suggests that V_p is determined by the difference between the redox potential of the solutions on the two sides of the membrane. It would be appropriate to assume that the photons impinging on the membrane are responsible only for opening the relevant conductive pathway K_p. The experimental evidence for such a hypothesis is described in this section.

K_p and V_p at Various Light Intensities. The photoresponse dependence upon intensity in the inset of Fig. 4 shows the same characteristics as found by Nguyen-thuong-Van and Tien [13]. There is an approximate linear relationship between photoresponse and light intensity which tends to level off at high intensities. Analysis of the photoresponse at high and low intensity of irradiation of the same membrane reveals that the primary difference between the two must be in the R_p term (Fig. 4). The results are consistent with the assumption that V_p is independent of light intensity.

K_p and V_p at Various Increments of Redox Potential across the Membrane. The dependence of the polarity of the photoresponse upon the side on which $FeCl_3$ or $Ce(SO_4)_2$ was present as stated clearly by Tien and his collaborators [13, 15] was confirmed by this study. If both sides of the membrane were exposed to identical solutions that had a defined redox potential value, the photoresponse at zero potential was absent or negligible. On the other hand, a photoconductive effect was observed; namely, the direction of the photoresponse ΔV was dependent upon the polarity of membrane potential V_m.

A semi-quantitative analysis of the relation between the oxidation-reduction potential difference and V_p is shown in Fig. 5. As indicated in the legend, before adding any ascorbic acid to the outside solution, there was only a small photoresponse at zero membrane potential and this was of

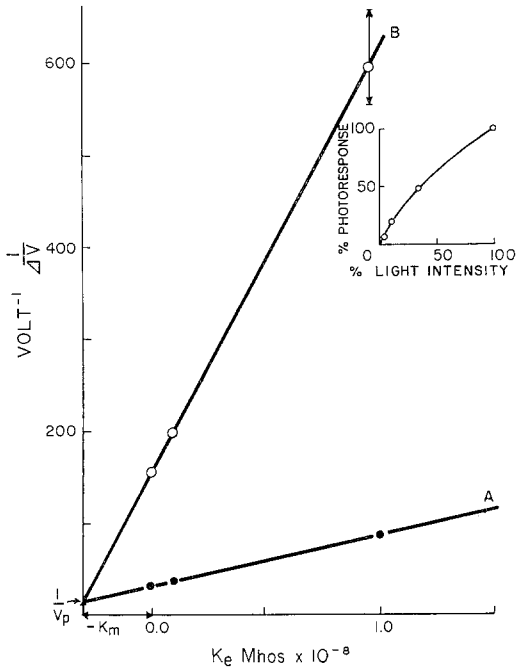

Fig. 4. *Ordinate:* The reciprocal of the photovoltage response as a function of the conductance of an external shunt at *A*, 100% light intensity and *B*, 10% light intensity. The value of V_p for both curves is 60 mV, whereas the values of R_p are 300 and 2,700 MΩ for curves *A* and *B*, respectively. The points on curves *A* and *B* are from recording tracing having a sensitivity of 10 and 4 mV per inch, respectively. The only significant uncertainty is in the uppermost point where the voltage response was only 1.7 mV with limits of 1.55 to 1.8 mV. Membrane capacitance, 3,200 pF; inside solution contains 1 mM $FeCl_3$. *Inset:* Photoresponse as a function of light intensity

opposite polarity to the response observed after the addition of ascorbic acid. It is clear from the analysis that V_p increases as more ascorbic acid is added to the outside solution. The values of V_p after the second and third addition of ascorbic acid could not be determined to a very high accuracy because of the fact that at higher levels of oxidation-reduction potential differences, the relation of ΔV *vs.* V_m clearly deviates from linearity. In spite of the uncertainty which this behavior introduces in the determination of V_p and R_p, it is clear that the limits of V_p after the second addition of ascorbic acid (between 135 and 225 mV) were well above the V_p after the first addition, which was only 80 ± 10 mV. On the other hand, the corresponding limits of R_p were 3,600 and 7,400 MΩ, a range which covers R_p determined after the first addition of ascorbic acid (5,700 MΩ). After the third addition, the determined value of approximately 250 mV and 6,000 MΩ is near the lower limit for V_p and R_p. The upper limit is not defined because

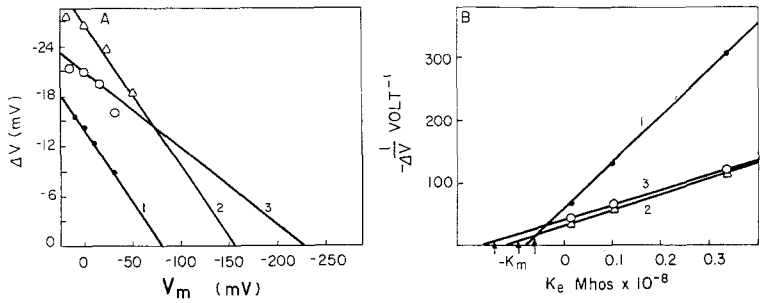

Fig. 5. Photoresponse analysis for a membrane exposed originally to identical media on both sides. The medium contained 1 mM $FeCl_3$ and 0.5 mM $FeCl_2$. The initial photoresponse was less than 1 mV, inside positive. Curves *1*, *2*, and *3* represent analysis of photoresponse, inside negative, after three consecutive additions to the outside solution of ascorbic acid to a concentration of about 0.3, 0.6, and 1.2 mM, respectively. The volume of ascorbic acid solution added to the external medium was very small and it did not affect the membrane area as determined by the constancy of the capacitance. The membrane resistance determined from current-voltage curves was 1,380, 1,020, and 695 MΩ for curves *1*, *2*, and *3*, respectively. The corresponding $-Km$'s are indicated by arrows in (*B*). Membrane capacitance, 1,900 pF. The light irradiated on the membrane was filtered through a 2–60 Corning filter (transmittance curve of that filter shown in the inset of Fig. 14). The intensity at the level of the membrane was in that case 50 to 80 mW/cm^2. This procedure was used to avoid changes in intensity of light reaching the membrane because of variations in the absorbance of the external ferric solution (absorbance of ferric chloride solution shown in Fig. 12). (*A*) ΔV as a function of V_m. Values of V_p corresponding to the drawn curves are -80, -160, and -230 mV and values of R_p are 5,700, 4,400, and 6,400 MΩ for curves *1*, *2*, and *3*, respectively. (*B*) Reciprocal of photoresponse as a function of the conductance of an external shunt. Values of V_p calculated from these curves are -80, approximately -200, and approximately -250 mV. Values of R_p are 6,000, 5,400, and 6,000 MΩ for curves *1*, *2*, and *3*, respectively. The uncertainties of V_p (in mV) and R_p (in MΩ) are: curve (*1*), V_p 80 ± 10 $R_p = 5{,}700 \pm 800$; curve (*2*), $135 < V_p < 225$, $3{,}600 < R_p < 7{,}400$; and curve (*3*), $200 \leq V_p$, $5{,}500 \leq R_p$

the $1/\Delta V$ *vs.* K_e curve cuts the ordinates at $K_e = -K_m$ close to zero so that any value of V_p above 250 mV could be consistent with this curve. Since the ΔV *vs.* V_m curve shows a very pronounced discontinuity around $V_m = 0$, it is of little help in ascertaining a value for R_p or V_p in an independent way. (Lines 2 and 3 in Fig. 5*A* represent only rough approximations of the data. The uncertainties about point of intersection of these curves with abscissa at $\Delta V = 0$ represent uncertainties in V_p which are indicated in the legend. The crossing between curves *2* and *3* in Fig. 5*A* occurs because of the decrease in membrane resistance R_m during the experiment as given in the legend. As shown in Eq. (12) of the Appendix the slope of the curve ΔV *vs.* V_m decreases with decrease in R_m.) However, it is clear from Fig. 5*B* that assigning any value higher than 250 mV to the V_p after the third addition of ascorbic acid entails a proportionate increase in R_p.

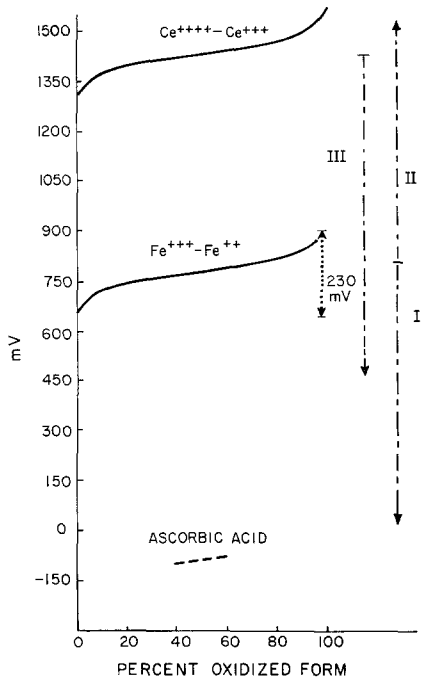

Fig. 6. A diagram of the redox potential of three redox substances used in this study. Line I denotes the redox potential gradient across the membrane in the experiment shown in Fig. 5, curve *3*. Lines II and III represent redox potential gradients in experiments described in the text. The arrow side of the lines indicates an undefined redox potential value. The dotted line represents a hypothetical gradient across a membrane where 99% of a particular substance (e.g., iron ions) will be in the oxidized form on one side of the membrane and 99% of it will be in the reduced form on the other side of the membrane

The results of these experiments suggest that the maximum "effective" electromotive force of the photoresponse V_p cannot be more than 250 mV. If it is higher, it is much less efficient because it is associated with a concomitant increase in the resistance of the photobattery. Since the oxidation-reduction potential difference between $Fe^{3+} - Fe^{2+}$, and ascorbic-dehydroascorbic acid is about 0.7 V (Fig. 6), the above finding implies that only a fraction of the difference between the oxidation-reduction potential across the membrane is effective as the driving force of the photoresponse. This observation may lead to the conclusion that a gradient of a particular substance in its oxidized or reduced form is the driving force of the photoresponse. A gradient of a particular redox substance can not be affected significantly by increasing the redox potential increment unless it occurs around a specific level on the redox potential scale. (*See* comment on

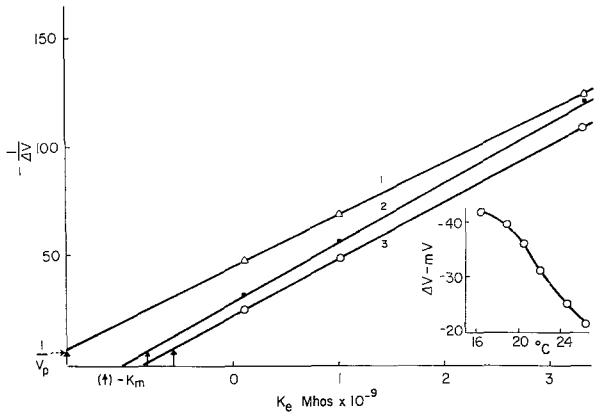

Fig. 7. Analysis of photoresponse at three temperatures. Inside solution contained 2 mM $FeCl_3$; membrane capacitane, 2,100 pF. *Ordinate:* reciprocal of photoresponse. *Abscissa:* conductance of an external shunt. The following values apply to curves *1*, *2*, and *3*, respectively: (a) temperature, 26, 22, and 19 °C; (b) membrane resistance (in MΩ), 620, 1,200, and 1,700; (c) V_p (in mV), −140, −140, and −130; (d) R_p (in MΩ), 3,400, 3,700, and 3,400. Note that the major effect of temperature is on membrane resistance. There is very little effect, if any, on V_p or R_p. *Inset:* Photovoltage response as a function of temperature

dotted line in legend of Fig. 6.) Yet, our observations on the photoeffect of the chloroplast extract membrane indicate that a redox potential increment around the ceric-cerous level is as effective as that around the ferric-ferrous level. Thus, a V_p of 200 mV and R_p of 6,000 MΩ were observed for a system: 1 mM ceric, membrane, 0.75 mM ferric + 0.25 mM ferrous (line II, Fig. 6). Similar values were measured in the case of a membrane interposed between 1 mM ceric + 1 mM cerous on one side and 1 mM cerous and 1 mM ferrous on the other side (line III, Fig. 6).

Thus, the limit of the effective photoconductive driving force cannot result from limits on the gradient of a particular substance across the membrane. It would seem rather that there is a limit to the photocurrent that can be supplied by the membrane at a particular intensity of irradiation. In view of these experiments, the limit is about 5×10^{-12} amps or in the order of 10^{-9} to 10^{-10} amps/cm^2. An explanation of such a limit is offered by a theoretical model of electron transport across membranes which will be presented in a separate paper [8].

Effect of Temperature. The inset in Fig. 7 shows the relationship between photoresponse and temperature and confirms a similar observation by Nguyen-thuong-Van and Tien [13]. The analysis of this relationship establishes that the major effect of temperature is on membrane conductance (Fig. 7). As expected, there is no change in V_p. The interesting finding is

that photoconductance K_p is essentially unaffected by the temperature change even though the membrane conductance varies by almost 300%. This finding implies that the activation energy of photoconductance is small or zero and suggests that the photoconductance pathway is not ionic in nature (*see* Discussion).

Time Course of Photoresponse

Hitherto, the analysis of the photoresponse in terms of the model of Fig. 2 was applied to the steady-state characteristics of the system. The transient response according to the model should depend upon the time constant of the membrane and upon the time course of the developing conductive pathway $K_p(t)$. If K_p were an instantaneous event in comparison with the time constant of the membrane (a plausible assumption), the model would predict that the time constant of the "on" response would be shorter than that of the "off" by a factor of $(K_m+K_p)/K_m$ and that the time constants of the membrane and of the "off" response would be identical. Many measurements of these parameters did not substantiate these simple expectations. The time constant of the "off" response was indeed always longer than that of the "on" while the ratio between the two was larger than that expected from values of K_m and K_p determined from the steady-state properties of the system. Also, the time constant of the "on" response was slightly larger or equal to that of the membrane (Figs. 8, 9). Thus, the

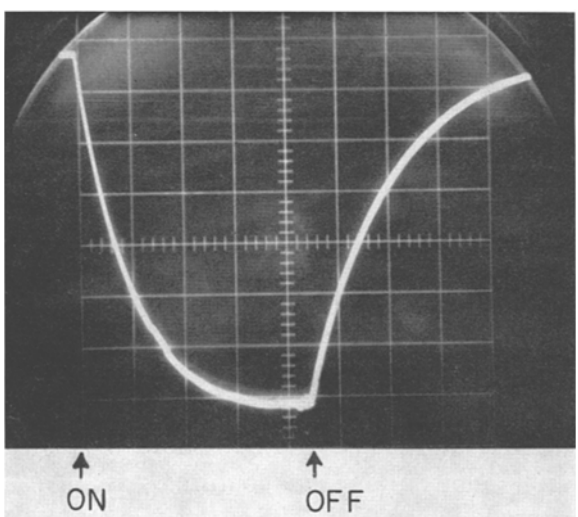

Fig. 8. A typical oscilloscopic trace of a photoresponse. *Abscissa* (*time*): 5 sec, each division. *Ordinate* (*voltage*): 5 mV, each division. Outside solution contains 1 mM $Ce(SO_4)_2$ and the membrane capacitance is 1,400 pF

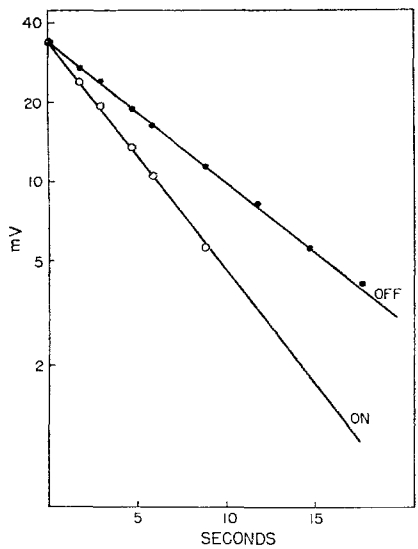

Fig. 9. Analysis of the oscilloscope trace shown in Fig. 8. *Abscissa:* time in seconds. Ordinate: $[V_m(t) - V_m(\infty)]$ in mV on logarithmic scale. Time constants of (a) membrane, 4.8 sec (not shown); (b) "on" response, 5.05 sec; (c) "off" response, 8.0 sec. Time constant of membrane determined by analysis of an oscilloscopic trace of a voltage response to the application of a square pulse of current in the absence of light irradiation

electric model of Fig. 2 is not compatible with the observations on the transients of the photoresponses. A modified electric circuit which arises from a theoretical model for electronic movements across the membrane [8] can account qualitatively for these findings. The essence of the modified model is the presence of a photoresistance pathway through the interface (R_{pi} in Fig. 2) which is shunted by an interfacial capacitance (not shown in Fig. 2). The time constant of the interfacial portion of the membrane may account for the larger time constants of the "on" and "off" photoresponses.

Action Spectrum of Photoresponse

Various band-pass interference filters were interposed between the outlet of the light guide and either the membrane or a thermistor bolometer. It was obvious from many measurements on various days and at various distances between the light guide and the bolometer that the relative intensity of light through the different filters was quite constant (Fig. 10). The maximum intensity was obtained at 5,440 Å wavelength and the intensity of light coming out through the other filters was expressed as a fraction of the intensity at 5,440 Å. The photoresponse ΔV as observed at each particular

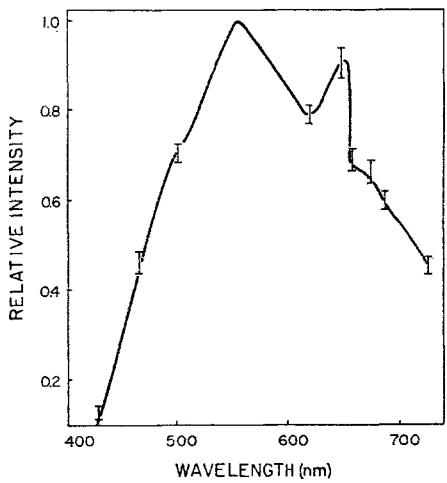

Fig. 10. Relative intensity of light passing through the several band-pass interference filters used in this study. The intensity of 5,440 Å (between 8 and 12 mW/cm^2) was arbitrarily taken as 1.0. Bars represent spread of measurements at various times and distances between light guide outlet and bolometer probe. The spectrum of the unfiltered incident light is only roughly similar to the curve since the latter reflects also the particular transmittance properties of the filters

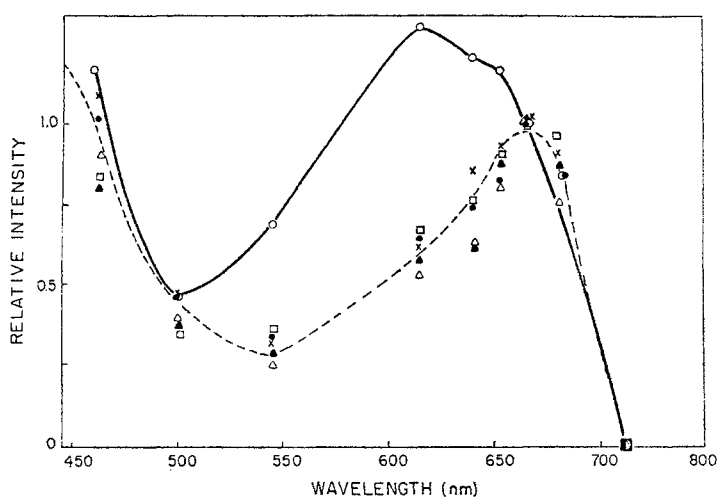

Fig. 11. Action spectrum of photoeffects. Ordinate, relative photoresponse intensity in arbitrary units. The intensity at each wavelength was determined by dividing the photoresponse ΔV by the relative intensity of the incident light. To render spectra from many experiments presentable on the same scale, the intensity at 665 nm was set arbitrarily as 1.0. ×----×, membrane capacitance, 4,500 pF, outside 1 mM Ce^{4+}; ▲----▲, membrane capacitance, 1,200 pF, outside 1 mM Ce^{4+}; ▲----▲, membrane capacitance, 2,000 pF, inside 1 mM Fe^{3+}; ●----●, membrane capacitance, 2,500 pF, inside 1 mM Ce^{4+}; □----□, membrane capacitance, 3,200 pF, inside Fe^{3+}; ○—○, same membrane as in □----□, but after adding to inside solution phycocyanin (about 0.12 mg/ml); optical density at 620 nm of inside solution, 0.79

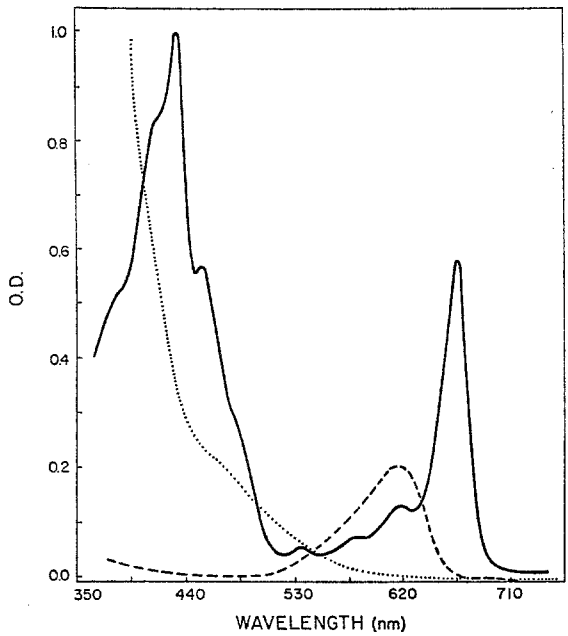

Fig. 12. Adsorption spectra of some of the solutions used in this study. ———, 1:400 dilution of chloroplast-extract solution in 1:5 butanol/octane; -------, phycocyanin solution about 0.03 mg/ml in 0.1 M potassium acetate buffer, pH 5.0; ·····, $FeCl_3$ (about 1 mM) in potassium acetate, 0.1 M, pH 5.0. (The spectrum of the Fe solution shifts to the right with aging of the solution)

wavelength, was corrected by dividing it by the appropriate relative intensity as read from Fig. 10.

The spectrum of the corrected photoresponse (Fig. 11) represents results of experiments on membranes exposed to Ce^{4+} ions on the inside or outside, and to Fe^{3+} ions on the inside. When Fe^{3+} ions were present on the outside, the blue peak disappeared, most probably because of the absorption of the short wavelength light by the outside solution (dotted line, Fig. 12). These spectral studies reveal a very definite similarity between the spectrum of the photoresponse and that of the absorption of the solution from which the membrane was prepared (Fig. 12). This observation is also in keeping with the data of Nguyen-thuong-Van and Tien [13].

Effect of Phycocyanin

Phycocyanin is one of the biliproteins that occurs in blue-green algae as aggregates known as phycobilisomes between the chlorophyll-containing photosynthetic membranes (thylakoids) in the so-called stroma spaces of the cell [1, 4].

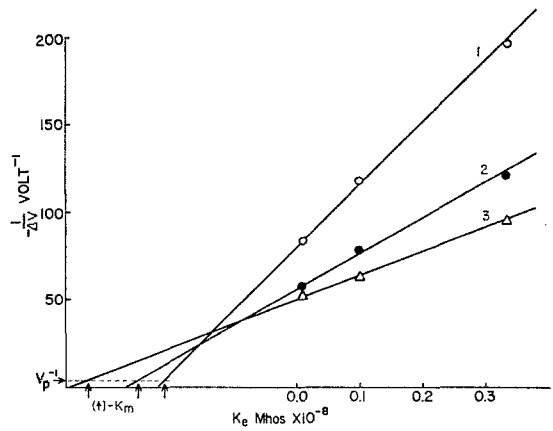

Fig. 13. Analysis of photoeffects in a membrane 20 min before (curve *1*), 5 min after (curve *2*), and 45 min after (curve *3*) addition of phycocyanin to a concentration of about 0.15 mg/ml to the inside solution. The inside solution contains 1.0 mM $FeCl_3$ and the outside solution contains about 0.1 mM of ascorbic acid. Membrane capacitance, 3,100 pF. Membrane broke 60 min after addition of phycocyanin. *Ordinate:* reciprocal of photoresponse. *Abscissa:* conductance of an outside shunt. Because of nonlinearity of current-voltage curve, the exact membrane conductance is within $\pm 10\%$ of the indicated values (arrows). V_p for the three curves is about -200 mV. R_p values in MΩ are 7,200, 4,400, and 2,800 for curves *1*, *2*, and *3*, respectively

Adding phycocyanin to one side of the thin chloroplast-extract membrane invariably led to a decrease in membrane resistance R_m and eventually to disruption of the membrane. This is a rather common effect of protein on artificial bileaflet membranes [6]. In membranes of high capacitance ($\sim$5,000 pF), the decrease in resistance was very fast and therefore was associated with decrease in photovoltage response, as explained in the second paragraph of Results. In cases of such rapid decrease in photoresponse, it was impossible to make any analysis of the photoeffect after adding phycocyanin. However, in membranes of lower capacitance, the decrease of resistance caused by phycocyanin was slower (*see*, e.g., values of K_m in Fig. 13) and the analysis could be carried out.

The most relevant and sensitive indication of the participation of phycocyanin in the photoresponse was a study of the photovoltage spectrum. Phycocyanin could be added only to the inside solution, since its addition outside would have altered the intensity and spectrum of light reaching the membrane. In principle, phycocyanin could be added to the more oxidized or more reduced side of the membrane. Since the oxidized side in these studies contained either Fe^{3+} or Ce^{4+} ions, there were four permutations for the phycocyanin experiments. Of these, only one gave a definite positive

response in its ability to affect the spectrum (continuous line, Fig. 11) and to increase the photoconductance K_p (Fig. 13), namely, the one in which phycocyanin was added to the Fe^{3+}-containing side of the membrane. Thus, it seems quite clear that phycocyanin when present on the oxidized side of the chloroplast extract membrane contributes to the photoresponse. On the other hand, adding phycocyanin to the reduced side did not cause any discernible effect on the spectrum or on K_p. Also, the addition of phycocyanin to the oxidized side of the Ce^{4+}-containing system had no effect. However, since Ce^{4+} caused a definite and immediate precipitation of the protein, the lack of effect in the latter case is not necessarily meaningful.

These experiments suggest that phycocyanin can contribute to the photoeffect in a one-way manner; in the direction which promotes negative current (probably electron) transfer from the membrane to the aqueous solution. This observation could be verified quite clearly in a membrane exposed initially to 1 mM $FeCl_3$ on both sides. Since the redox potential under these conditions is not defined, a minimal reduction of Fe^{3+} on one side of the membrane can result in a measurable photoresponse. As indicated in Fig. 14, the polarity of the photoresponse was outside negative. When phycocyanin was introduced inside, it led to a decrease in photoresponse. The analysis shown in this figure indicates that excitation of phycocyanin had an opposite effect to that of the "chloroplast-extract-membrane" excitation alone. This is evident from the fact that after adding phycocyanin (hatched columns) the photovoltage response was higher with a 2–60 filter compared to the response with a 3–66 filter even though the latter transmitted more light.

It is evident that the increment in photoeffect resulting from the presence of phycocyanin is not secondary to increased light excitation of the membrane caused by energy transfer. Such a contribution must be negligible also on an *a priori* consideration since the optical densities of the phycocyanin and the chloroplast-extract solutions differ by two orders of magnitude. The phycocyanin solution in a 1-cm cell had on OD of approximately 1.0 while the chloroplast extract had an affective OD in a 1-cm cell of 400. (The optical density of the bileaflet membrane is probably even higher than that of a bulk solution of comparable thickness, since chlorophyll as an amphophyll will be more concentrated at interfaces.) Phycocyanin molecules effective in energy transfer to the membrane must be within about 80 Å of the solution adjacent to the membrane. The membrane is at least 40 Å thick so that the ratio of phycocyanin to chlorophyll effective optical densities is approximately 1:200.

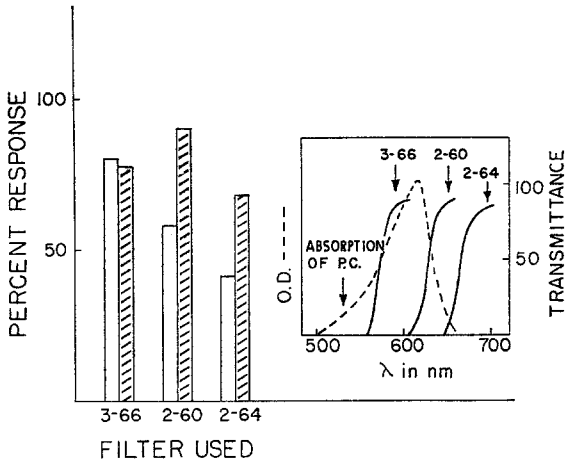

Fig. 14. Analysis of the effect of phycocyanin on the magnitude of the photoresponse observed as a function of spectra of the incident light. The membrane is exposed on both sides to $FeCl_3$, 1 mM, in potassium acetate 0.1 M, pH 5.0. Even though the solutions are identical, there is a photoresponse of about 11 mV, outside negative. Three minutes after adding phycocyanin (P.C., about 0.15 mg/ml) to inside solution the response was reduced to about 7 mV before any change in membrane resistance was noticed. The photoresponse in the absence of any light filters is taken as the 100% response (i.e., 100% equals 11 mV before and 7 mV after adding phycocyanin). The percent of photoresponse after using three types of filters before (clear columns) and after (hatched columns) adding phycocyanin is shown. The transmittance curves of the filters used are shown in the inset, together with the absorption spectra of phycocyanin (dashed line). Note that after adding phycocyanin, the photoresponse with a 2–60 filter gave a higher response than with a 3–66 filter, even though the latter obviously transmitted more light. Membrane capacitance, 1,700 pF

It is very unlikely that phycocyanin acts by changing the redox potential of the ferric ions because: (1) The phycocyanin concentration is about 10^{-6} M, whereas that of ferric chloride is 10^{-3} M. (Under these conditions ferric does not cause changes in phycocyanin absorbance or aggregation [7]); (2) If ferric chloride concentration is affected by phycocyanin, one would have to assume it occurs only when phycocyanin is excited, since exclusion of the part of the spectrum which excites phycocyanin leads to a "normal response" as can be judged from Figs. 11 and 14; (3) The main effect of phycocyanin is on the photoconductance K_p and not on driving force V_p (Fig. 13).

In the discussion it is suggested that phycocyanin reduces the energy barrier for electron movement from the chromophores in the membrane to acceptors in the aqueous phase. A specific interaction between ferric ions and phycocyanin chromophores is also inferred from the ferric quenching effect on phycocyanin fluorescence [7].

Discussion

There are two cardinal questions that are important for the interpretation and significance of the experiments described in this study. (1) Is the photoresponse an electronic process or is it ionic in nature? (2) Does the photoresponse occur in the thin or thick portions of the membrane or in both? Although neither one of the following arguments is in itself conclusive evidence for unequivocal answers to the above questions, together they constitute a plausible case for the interpretation that electron movement is responsible for the observed photoresponse and that this electron movement occurs primarily in the thin section of the partition between the two aqueous solutions.

(1) The fact that a substantial photoresponse can be obtained only if a redox potential gradient exists across the membrane and that it is obtained equally well at gradients which occur around the ferric-ferrous or around the ceric-cerous redox potentials, are strongly suggestive that the process is an electronic one.

(2) The fact that the photoconductivity is independent of temperature in contrast to the membrane dark conductance (Fig. 7) is also more consistent with an electronic process at least as far as current at the membrane-water interface is concerned. It is highly improbable that ions likely to be produced by the photoillumination such as chlorophyll$^-$ [14] could move from their position in the membrane-water interface without requiring a considerable amount of activation energy. On the other hand, electron exchange across the membrane-water interface could occur by quantum-mechanical tunnelling through an energy barrier similar to electron movements at electrode interfaces [5]. Such a process would be independent of temperature.

(3) Phycocyanin is not soluble in octane solution and therefore it can affect the photoresponse only at the membrane-water interface. Since phycocyanin augments the photoresponse only when present on the oxidized side of the membrane, it is not participating in the photoresponse through energy transfer. It is necessary to assume that excitation of phycocyanin facilitates the relevant photocurrent at the interfacial level. The photoresponse cannot be, therefore, a property of the bulk lipid-chlorophyll solution only.

(4) If the photoresponse was a property of a bulk thick solution one would expect that the response would be sensitive also to the direction of illumination. (It is quite possible that the small photoresponses observed

in the absence of redox potential difference across the membrane, as indicated in the legend of Fig. 5, are caused by gradients of excited molecules in the thick portion of the membrane.)

(5) The driving force for the photoresponse is clearly the redox potential gradient which exists between the two interfaces of the membrane, whereas the photoconductance is dependent upon the amount of light absorbed (Fig. 4). Since for thick bulk membrane the light absorbed per unit light path is in the limit equal to that for the bileaflet membrane (and for increasing thickness decreases with light path through the membrane), it is clear that the photocurrent (driving force multiplied by photoconductance) will decrease with increase of thickness of membrane; i.e., the thinner the membrane the more photoactive it will be. This reasoning may be invalid for a thin bileaflet membrane in two ways: (a) It is possible to argue that the lack of chlorophyll molecules between the two interfaces may impede current transport through the "bulk" hydrophobic layer, whereas, chlorophyll or other molecules present in the bulk solution can "approach more easily" the amphophyll layers at the interfaces. In this case only a membrane thicker than a bileaflet would be photoconductive; still a thin layer would obviously have less resistance than a thick one. (b) Another more plausible possibility is that the presence of carotenoid, xantophyll or detached chlorophyll molecules can facilitate electron transport between the chromophores of the two interfaces, and in that case a bileaflet region would be much more suitable for electron transport compared to a region where the two interfacial monolayers are separated by bulk octane solution.

Contrary to the notion that the photoresponse occurs primarily in the thin bileaflet membrane, we could not observe a consistent positive correlation between the membrane capacitance and photoconductance (K_p). It is possible that this was because of some undefined chemical differences between membranes formed at different days and from different bulk solutions. It is also possible that light absorbed by thick portions of the membrane could increase irradiation of the thin membrane by fluorescence. The quantum yield of chlorophyll-a fluorescence in vitro can be as high as 22% or 35% [2, 9]. On the other hand, Forster type energy transfer [3] is not likely to be a factor since the quantum efficiency of the photoresponse (electron transported per quantum absorbed) even for the thin membrane is very low.

The possible relevance of the photoresponse in artificial bileaflet membrane to the understanding of the primary processes in photosynthesis will be discussed in detail in an article on a theoretical model for electron

transport through bileaflet membranes containing chlorophyll [8]. The idea is that electrons can be tunnelled from donors into vacant orbitals in the chromophore portion of the membrane and vice versa. On the other hand, it is suggested that electron transport across an interface could not occur by passing over the energy barrier unless an appropriate enzyme was present to reduce the energy barrier between the chromophore in the membrane and an acceptor in the aqueous solution. It is suggested that the mechanism of action of phycocyanin in the artificial membranes of this study is through reduction of the energy barrier for electron movement across the interface. The reduction of the energy barrier to a level comparable to that of the excited electron would facilitate electron transport from membrane to the aqueous solution but not vice versa. This explains the observed unidirectional effect of phycocyanin. In a photosynthetic membrane such a role is accomplished by an enzyme which enables electron movement into an acceptor belonging to a redox pair of low redox potential (more negative). The flow of electrons from a donor belonging to a redox pair of high (positive) redox potential into the vacant orbitals of the chromophores will occur by the tunnelling mechanism.

It should be emphasized that the artificial bileaflet membrane formed from an octane solution of a crude lipid extract of chloroplast does not necessarily function as an in vivo chloroplast. The fact that it is possible to elicit in this system a sustained photoresponse and to modify its nature by a proteineous photopigment is significant, even though it does not behave like an intact thylakoid membrane. However, the implication of this paper is that in the natural chloroplast membrane, which does not contain octane and in which the chlorophyll, carotenoid and other pigments are much more densely packed, the quantum efficiency (electron transport per quantum absorbed) would be much higher than that observed in the artificial bileaflet system; yet, the basic electronic processes may be the same in both membranes.

Appendix

Steady-State Properties of the Circuit Shown in Fig. 2[1]

There are three routes for currents in the circuit: through R_e, through R_m (since at steady state $dV/dt=0$ everywhere, there will be no current flow through the capacitance C_m), and through R_p. The sum of these currents must be zero:

1 The reciprocal of the resistance term $1/R_j$ will be designated by the corresponding conductance term K_j.

Case A: $V_e=0$. Therefore,

$$V_{mp}/R_e+V_{mp}/R_m+(V_{mp}-V_p)/R_p=0. \tag{1}$$

V_{mp} is the membrane potential when the membrane is irradiated. In the absence of irradiation, V_p is disconnected from the membrane and thus, $V_m=0$. Therefore, in Eq. (1) V_{mp} is equal to the photoresponse $\Delta V=V_{mp}-V_m$. Thus, from Eq. (1):

$$\Delta V/R_e+\Delta V(1/R_m+1/R_p)=V_p/R_p. \tag{2}$$

Rearranging and substituting K's for $(1/R)$'s, one obtains:

$$(R_p/V_p)K_e+(R_p/V_p)K_m+1/V_p=1/\Delta V. \tag{3}$$

Eq. (3) shows that plotting $(1/\Delta V)$ against K_e should yield a straight line with a slope of R_p/V_p. Also, at a point in the plot where $K_e=-K_m$, $1/\Delta V=1/V_p$.

Case B: $V\neq 0$. The condition of $\Sigma I=0$ yields, in the case of the open switch:

$$(V_m-V_e)/R_e+V_m/R_m=0 \tag{4}$$

and in the case of the closed switch,

$$(V_{mp}-V_e)/R_e+V_{mp}/R_m+(V_{mp}-V_p)/R_p=0. \tag{5}$$

Defining $1/R_i=1/R_m+1/R_e$, Eqs. (4) and (5) yield Eqs. (6), (7), and (8), respectively.

$$V_m=R_i(V_e/R_e)=V_e/(R_eK_i). \tag{6}$$

$$V_{mp}=(V_e/R_e+V_p/R_p)/(K_i+K_p). \tag{7}$$

$$V_{mp}=K_iV_e/R_eK_i(K_i+K_p)+V_pK_p/(K_i+K_p). \tag{8}$$

Introducing Eq. (6) into Eq. (8) results in:

$$V_{mp}=V_mK_i/(K_i+K_p)+V_pK_p/(K_i+K_p). \tag{9}$$

Thus,

$$V_{mp}-V_m=\Delta V=V_m[K_i/(K_i+K_p)-1]+V_pK_p/(K_i+K_p), \tag{10}$$

or

$$\Delta V=-V_mK_p/(K_i+K_p)+V_pK_p/(K_i+K_p), \tag{11}$$

or

$$\Delta V=-V_m/(R_p/R_i+1)+V_p/(R_p/R_i+1). \tag{12}$$

Eq. (12) shows that plotting ΔV against V_m yields a straight line with a slope: $-1/(R_p/R_i+1)$ and that at $V_m=V_p$, $\Delta V=0$.

It should be noted that Eq. (12) is derived from two steady-state equations requiring that the sum of the currents in the three pathways be zero.

The meaning of the resistance R in each equation is the difference ratio of voltage and current and not the differential dV/dI. If R_m varies with membrane potential, it will have a certain value in the case of a membrane potential V_m [Eq. (6)] and a different one in the case of membrane potential V_{mp} [Eq. (7)]. Let us assume that the membrane resistance at V_m is equal to $R_m + \Delta R_m$ which leads also to a change of R_i to $R_i + \Delta R_i$. Therefore, Eq. (6) will read as follows:

$$V_m = (R_i + \Delta R_i)\, V_e/R_e = V_e/K_i R_e + \Delta R_i\, V_e/R_e. \tag{13}$$

Introducing Eq. (13) instead of Eq. (6) into Eq. (8) will result in:

$$V_{mp} = V_m K_i/(K_i + K_p) - \Delta R_i\, V_e\, K_i/R_e(K_i + K_p) + V_p K_p/(K_i + K_p). \tag{14}$$

Proceeding in the same way as from Eqs. (9) to (12), one obtains:

$$\begin{aligned} &\Delta V + (\Delta R_i\, V_e/R_e)\,[K_i/(K_i + K_p)] \\ &\quad = -K_p V_m/(K_i + K_p) + K_p V_p/(K_i + K_p). \end{aligned} \tag{15}$$

And since the term $(K_i/K_i + K_p)$ is generally not far from 1.0,

$$\Delta V + (\Delta R_i\, V_e/R_e) = -[1/(R_p/R_i + 1)]\,(V_m - V_p). \tag{16}$$

Thus, adding a correction factor $\Delta R_i\, V_e/R_e$ to the measured photoresponse ΔV yields a curve similar to that predicted by Eq. (12). Generally, ΔR_i is negligible in comparison with R_i; this does not mean, however, that $\Delta R_i\, V_e/R_e$ is negligible in comparison with ΔV.

We wish to thank Dr. Charles Edwards of the State University of New York at Albany for the loan of equipment.

This work was supported in part by Grant no. 5 S01 RR 05649-05 from the National Institutes of Health and Grant no. GB 24315 from the National Science Foundation.

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