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GENERATION OF ELECTRIC CURRENT BY CHROMATOPHORES OF *RHODOSPIRILLUM RUBRUM* AND RECONSTITUTION OF ELECTROGENIC FUNCTION IN SUBCHROMATOPHORE PIGMENT-PROTEIN COMPLEXES

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SUMMARY

Lipoprotein complexes, containing (1) bacteriochlorophyll reaction centers, (2) bacteriochlorophyll light-harvesting antenna or (3) both reaction centers and antenna, have been isolated from chromatophores of non-sulphur purple bacteria *Rhodospirillum rubrum* by detergent treatments. The method of reconstituting the proteoliposomes containing these complexes is described. Being associated with planar azolectin membrane, proteoliposomes as well as intact chromatophores were found to generate a light-dependent transmembrane electric potential difference measured by Ag/AgCl electrodes and voltmeter. The direction of the electric field in proteoliposomes can be regulated by the addition of antenna complexes to the reconstitution mixture. The reaction center complex proteoliposomes generate an electric field of a direction opposite to that in chromatophores, whereas proteoliposomes containing reaction center complexes and a sufficient amount of antenna complexes produce a potential difference as in chromatophores. ATP and inorganic pyrophosphate, besides light, were shown to be usable as energy sources for electric generation in chromatophores associated with planar membrane.

INTRODUCTION

Several lines of evidence obtained by some indirect methods suggest transmembrane electrochemical H^+ potential to be generated in chromatophores and intact cells of photosynthetic bacteria, as was originally postulated by Mitchell [1]. These methods are based on the measurement of (1) natural and synthetic ion transport across the membranes of bacteria or isolated chromatophores [2–6], (2) electrochromic spectral shifts of carotenoids [7, 8] and bacteriochlorophyll [9, 10], (3)

Abbreviations: CCCP, 2,4,6-trichlorocarbonylcyanide phenylhydrazone; PP_i, inorganic pyrophosphate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

fluorescence changes of bacteriochlorophyll [11, 12] or exogenous fluorescent dyes such as 8-anilinonaphthalene-1-sulphonate and atebrin [13, 14].

A method of estimating the biological system-generated transmembrane electric potential difference directly with conventional electrometer techniques and macroelectrodes was recently developed in this group [15]. The procedure includes the following stages: (1) incorporation of a purified lipoprotein complex, competent in electric generation, into liposomal membrane, (2) association of proteoliposomes, obtained at stage 1, with planar phospholipid membrane separating two identical electrolyte solutions, and (3) measurement of the electric current and potential difference generated by the incorporated lipoproteins across the planar membrane and induced by the addition of a corresponding energy source.

With this method, generation of photoelectric current by the photosynthetic reaction center complexes of *Rhodospirillum rubrum* was recently demonstrated [16].

Data on the direct measurement of electric generation by native membranes of *R. rubrum* chromatophores and by various types of reconstituted subchromatophore pigment-protein complexes are presented in this paper.

METHODS

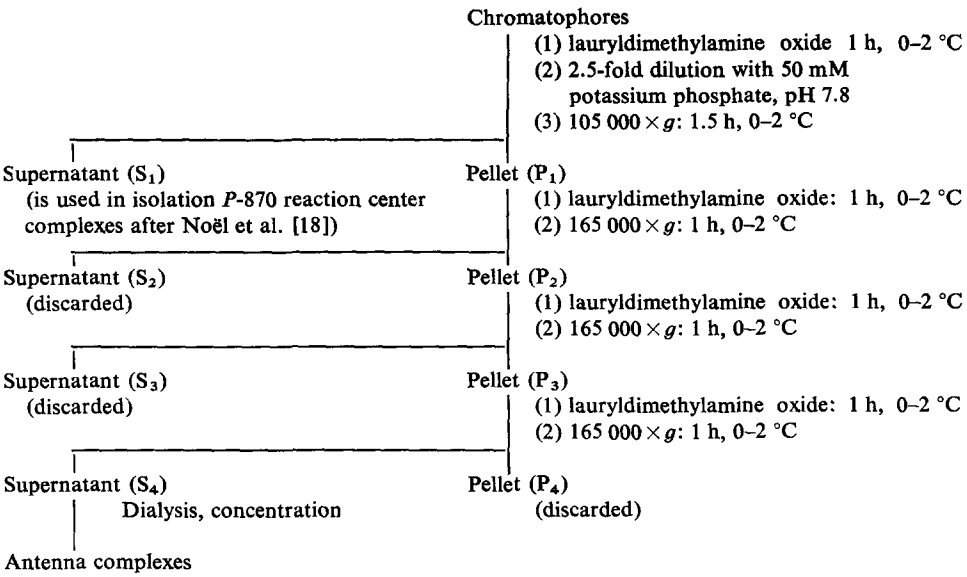
Chromatophores. Non-sulphur purple bacteria *R. rubrum* of wild type were grown semi-anaerobically in the medium described by Cohen-Bazire et al. [17]. The chromatophores were isolated by ultrasonic treatment of the washed bacterial cells [4].

P-870 reaction center complexes were isolated by the method of Noël et al. [18]. The procedure included solubilization of chromatophores with lauryldimethylamine oxide, centrifugation to remove heavy membrane fragments, and fractionation of the supernatant with $(NH_4)_2SO_4$ to purify reaction center complexes. The complexes were then dialyzed and kept at 0 °C in the dark in 50 mM potassium phosphate buffer, pH 7.8.

Antenna complexes. The chromatophores were suspended in 50 mM potassium phosphate and 1 mM sodium ascorbate solution (pH 7.0). The light absorption of the suspension at 880 nm was about 35–40 units of absorbance (here and below, the optical pathlength was 1 cm). The suspension was subjected to successive 4-fold solubilization with 0.25–0.30 % lauryldimethylamine oxide for the pigment-protein complexes of the light-harvesting antenna (antenna complexes) to be isolated. These operations were mainly carried out in the dark (for details, see flow diagram).

Pellets P_1, P_2 and P_3 were suspended in the starting volume of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM sodium ascorbate. Supernatant S_4 containing the pigment-protein complexes of bacteriochlorophyll antenna and carotenoids was dialyzed twice for 24 h against 1.5 l of 50 mM phosphate and 1 mM ascorbate solution (pH 7.8), and concentrated by dialysis against the same buffer containing 10–15 % polyethyleneglycol. The preparation obtained (antenna complexes) was kept at 0 °C in the dark.

Reaction center plus antenna complexes. The chromatophores were suspended in 50 mM potassium phosphate and 150 mM KCl solution (pH 7.8) so as to obtain a final absorbance of 50 units of absorbance at 880 nm. This suspension was used to isolate subchromatophore complexes containing *P*-870 reaction centers, bacterio-



Scheme 1. Isolation of antenna complexes. Flow diagram.

chlorophyll light-harvesting antenna and carotenoids (reaction center plus antenna complexes). The chromatophore suspension was treated with 2 % sodium cholate for 1 h at room temperature with stirring and was centrifuged for 1.5 h at 165 000 × *g* to remove the readily soluble membrane components. The pellet of the resuspended membranes was washed once by the starting volume of potassium phosphate buffer (pH 7.8). The washed pellet was resuspended in a starting volume of the same buffer and treated with 5 % sodium cholate at room temperature with stirring. The suspension was cooled to 0 °C after 30 min incubation, sonicated (20 kcycles) for 3–5 min, incubated for another 30 min at room temperature with stirring and centrifuged for 1.5 h at 165 000 × *g*. The supernatant containing the subchromatophore pigment-protein complexes was kept at 0 °C in the dark.

Proteoliposome reconstitution. Reconstitution of proteoliposomes from a mixture of soya-bean phospholipids (azolectin) and isolated *P*-870 reaction center complexes was carried out as described previously [16].

The proteoliposomes containing reaction center plus antenna complexes were reconstituted by a method similar to that for the isolated *P*-870 reaction center complexes. 50 mg of azolectin were suspended in 1 ml of a mixture containing 100 mM potassium phosphate (pH 7.8), 2 mM $MgSO_4$, 2 % sodium cholate, 0.2 mM CoQ_6 and reaction center plus antenna complexes ($A_{880\,nm} = 0.5$). The mixture was sonicated eight times for 30 s with 30-s intervals at 0 °C and dialyzed for 18 h at 3 °C in the dark against 50 mM potassium phosphate (pH 7.8) and 0.5 mM dithiothreitol. Proteoliposomes were sedimented at 165 000 × *g* for 45 min, suspended in 10 mM Tris · HCl (pH 7.5) and 0.25 M sucrose and kept at 0 °C in the dark.

A similar procedure was used to reconstitute proteoliposomes from a mixture of isolated *P*-870 reaction center complexes, antenna complexes, 0.03 % lauryldimethylamine oxide, 2 % sodium cholate and azolectin. The quantitative ratio of

the reaction center complexes to antenna complexes is denoted in figure captions.

Measurements. Phenyldicarbaundecaborane concentration changes in the chromatophore or proteoliposome suspensions were monitored with phospholipid membrane techniques [19, 20]. Association of chromatophores or proteoliposomes with planar azolectin membrane and the measurement of transmembrane electric potential differences were carried out as described by Drachev et al. [15]. The cuvette used in the experiments consisted of two compartments, separated by a Teflon partition with an 0.8 mm aperture closed by a thick (coloured) planar membrane formed from the mixture of azolectin and decane. The compartments contained solutions of an identical composition. Asymmetry of the system was attained by adding chromatophores or proteoliposomes to one of the compartments. Association of chromatophores (proteoliposomes) with the planar membrane was initiated by Mg^{2+} or Ca^{2+}. These cations (like any additions other than chromatophores and proteoliposomes) were added to both compartments to retain identity of composition of the solutions separated by the planar membrane. The electric potential difference across the planar membrane was measured with Ag/AgCl electrodes connected to a vibrating capacitor electrometer and a KSP-4 recorder. Actinic light of saturating intensity ($\lambda > 700$ nm) was obtained from a tungsten lamp.

The absorption spectra were measured with a Unicam SP. 800B and a Hitachi EPS-3 spectrophotometers. The concentration of *P*-870 in the preparations was determined from the light-induced absorption changes at 870 nm, using a molar extinction coefficient of 12 600 $M^{-1} \cdot cm^{-1}$ for *P*-870 [21]. The action spectrum of the 920 nm fluorescence excitation of bacteriochlorophyll was measured with an Aminco-Bowman spectrofluorimeter.

Electron microscopy of the subchromatophore pigment-protein complexes, proteoliposomes and chromatophores was carried out in an HU-11B electron microscope. For negative staining, samples were placed on collodion-carbon-coated copper grids and stained with 2 % phosphotungstic acid (pH 7.2) for 30 s. Excess fluid was removed with filter paper and the grid was air-dried for at least 30 min before the electron microscope analysis.

RESULTS

Absorption spectra of subchromatophore complexes

The absorption spectra of *R. rubrum* chromatophores and subchromatophore complexes are shown in Fig. 1. One can see that the spectra of the chromatophores (curve 1) and the reaction center plus antenna complexes (curve 2) differ only slightly. As for isolated antenna complexes (curve 4), they demonstrate a lower carotenoid content, a higher absorbance in the spectral region of 250–300 nm, the disappearance of the absorption band at 800 nm and the presence of a shoulder in the region of 770 nm. The spectral differences at 250–300 nm are probably due to different pigment-to-protein ratios in the systems studied. The absorption spectrum of the *P*-870 reaction center complexes (curve 3) is similar to that described previously [18].

The bacteriochlorophyll fluorescence spectra of chromatophores, the antenna complexes and the reaction center plus antenna complexes were found to be indistinguishable (not shown in the figures). The fluorescence band maximum of preparations was in the region of 905–910 nm. The relative quantum yield of the fluorescence of

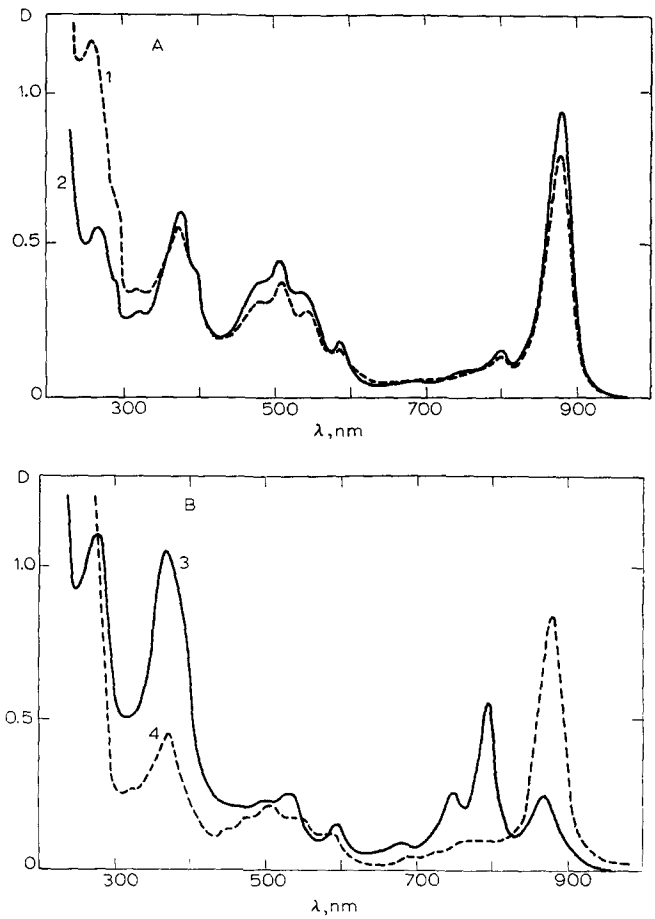


Fig. 1. A. Absorption spectra of *R. rubrum* chromatophores (1), reaction center plus antenna complexes (2), B. reaction center complexes (3) and antenna complexes (4). Incubation mixture: 50 mM potassium phosphate (pH 7.8). Additions: 2 % sodium cholate in 2, 0.03 % lauryldimethylamine oxide in 3 and 4.

antenna complexes was 4–6-fold higher than that of chromatophores.

The ratio of bacteriochlorophyll reaction centers to the total bacteriochlorophyll in chromatophores, as well as in isolated reaction center plus antenna complexes, was 1 to 45–55. The reaction center concentration in the isolated antenna complexes was negligible (no more than one per 1000–1200 bacteriochlorophylls).

The isolated reaction center complexes and the antenna complexes were devoid of cytochromes, according to the data of the differential spectrophotometry (light minus dark and oxidation by ferricyanide minus reduction by dithionite). The content of cytochrome *c* in the reaction center plus antenna complexes treated by acetone was 20–30-fold lower than that of *P*-870.

Electron microscopy study

The *P*-870 reaction center complexes were rod-like structures which seem to be

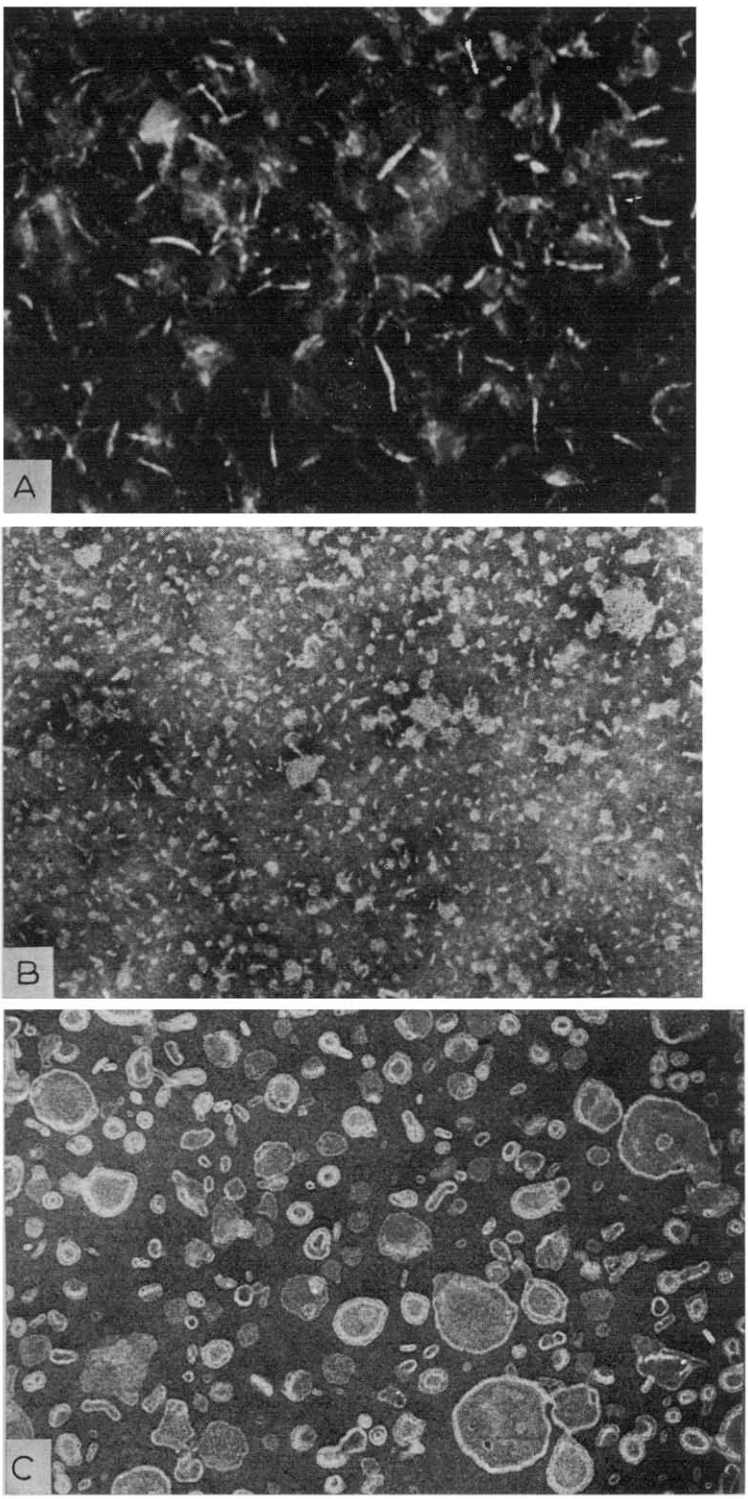


Fig. 2. For legend see opposite page.

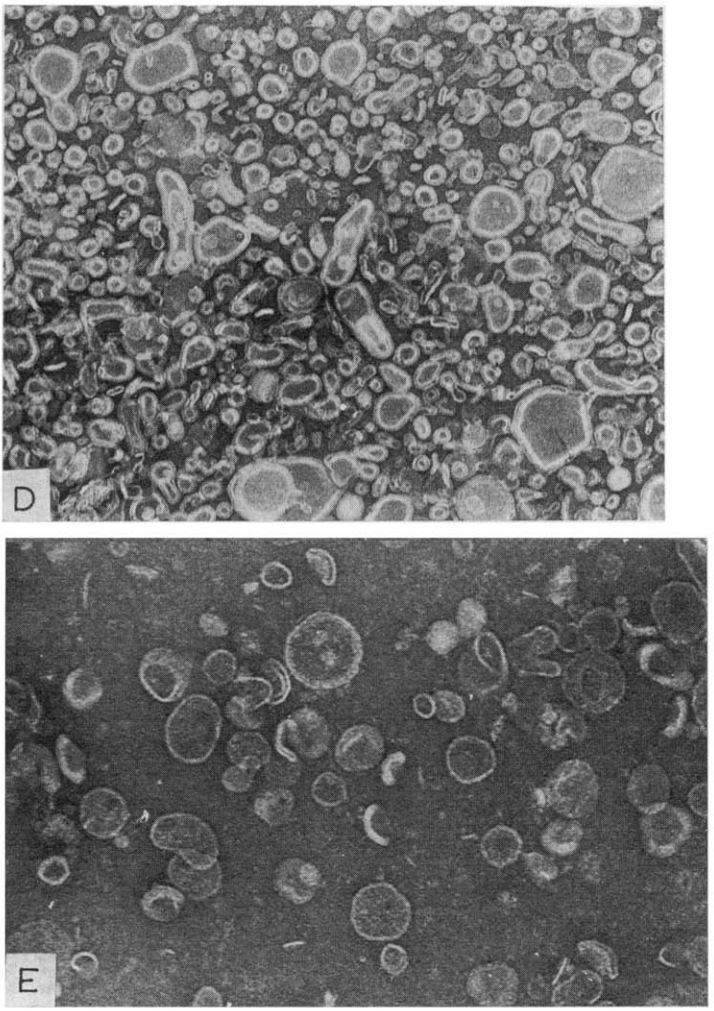


Fig. 2. Electron micrographs of isolated subchromatophore complexes, proteoliposomes and chromatophores of *R. rubrum*. (A) *P*-870 reaction center complexes (magnification × 150 000). (B) Antenna complexes (× 103 000). (C) Proteoliposomes containing reaction center complexes (× 79 000). (D) Proteoliposomes containing reaction center plus antenna complexes (× 79 000). (E) Chromatophores (× 79 000). Incubation mixture for A and B: 50 mM potassium phosphate (pH 7.8), for C, D and E: 50 mM Tris · HCl buffer (pH 7.8) and 250 mM sucrose.

composed of a number of 60–80 Å subunits (Fig. 2A). Subunit associations were observed only in the absence of lauryldimethylamine oxide. Similar data were obtained by Feher [22] with *P*-870 reaction center complexes of *Rhodopseudomonas sphaeroides* R-26.

The antenna complexes (Fig. 2B) formed rod-like structures (80–100 Å across) tending to aggregate into particles of different shapes.

Membrane vesicles are not found in the preparations of any of the subchromatophore pigment-protein complexes studied.

Figs. 2C, 2D and 2E demonstrate the electron micrographs of the proteoliposomes containing the reaction center complexes, proteoliposomes containing the reaction center plus antenna complexes and the isolated chromatophores of *R. rubrum*, respectively. In all cases, vesicles 500–1200 Å in diameter are observed. Multimembrane structures were formed when a protein component was not added to the reconstitution mixture.

Generation of transmembrane electric potential difference by chromatophores

As has been revealed in previous studies carried out in this group with synthetic penetrating anion probe [4–6], a transmembrane electric field is generated in energized *R. rubrum* chromatophores ("plus" inside). This conclusion has now been confirmed by direct voltmeter measurement.

Chromatophores were added to one of the two compartments separated by a Teflon partition with an aperture closed by the planar azolectin membrane. To induce association of the planar and chromatophore membranes, the negative surface charges of phospholipids were neutralized by Mg^{2+} (or Ca^{2+}). The results of a typical experiment are shown in Fig. 3. It is seen that illumination of the chromatophore-treated planar membrane results in generation of a small electric potential difference between the two compartments (positive on the chromatophore-free side of the planar membrane). The photoelectric response greatly increases on addition of 1,4-naphthoquinone plus TMPD (Fig. 3A). Naphthoquinone without TMPD was ineffective, whereas TMPD without naphthoquinone stimulated a photoresponse, but only slightly (see Fig. 6). Addition of ascorbate after 1,4-naphthoquinone and TMPD caused a further increase in the light-induced effect, which reached 130 mV in this sample. The highest value of membrane potential observed in such a system was 215 mV at $1 \cdot 10^{-11}$ A current. The electric response was inhibited by *o*-phenanthroline (Fig. 3A).

A study of the naphthoquinone effect showed that it can be reproduced by vitamin K-3. Naphthoquinone (or vitamin K-3) addition proved unnecessary when the mixture for planar membrane formation was supplemented with CoQ_6.

As for TMPD, it could be substituted by phenazine methosulphate, another dye, which shunts some steps of the chromatophore cyclic redox chain. In the latter case the photoeffect was greatly enhanced by ascorbate (Fig. 3B).

The phenyldicarbaundecaborane experiments on the chromatophore suspension revealed that naphthoquinone does not influence the light-dependent electric potential across the chromatophore membrane, whereas TMPD (or phenazine methosulphate) somewhat increases it (Fig. 4).

The action spectrum of photoelectric response in the system "chromatophores-planar membrane" is shown in Fig. 5. One can see that the maxima in the action spectrum coincide with those of bacteriochlorophyll absorption in chromatophores. The light absorbed by carotenoids was less effective in electric generation than that absorbed by bacteriochlorophyll. Similar relationships were obtained for the excitation of the 920 nm bacteriochlorophyll fluorescence.

When non-specific light scattering was taken into account, the carotenoids were found to be several times less effective in both the fluorescence excitation and electric generation as compared with bacteriochlorophyll. Similar relationships between carotenoid- and bacteriochlorophyll-mediated fluorescence excitation effectivities in

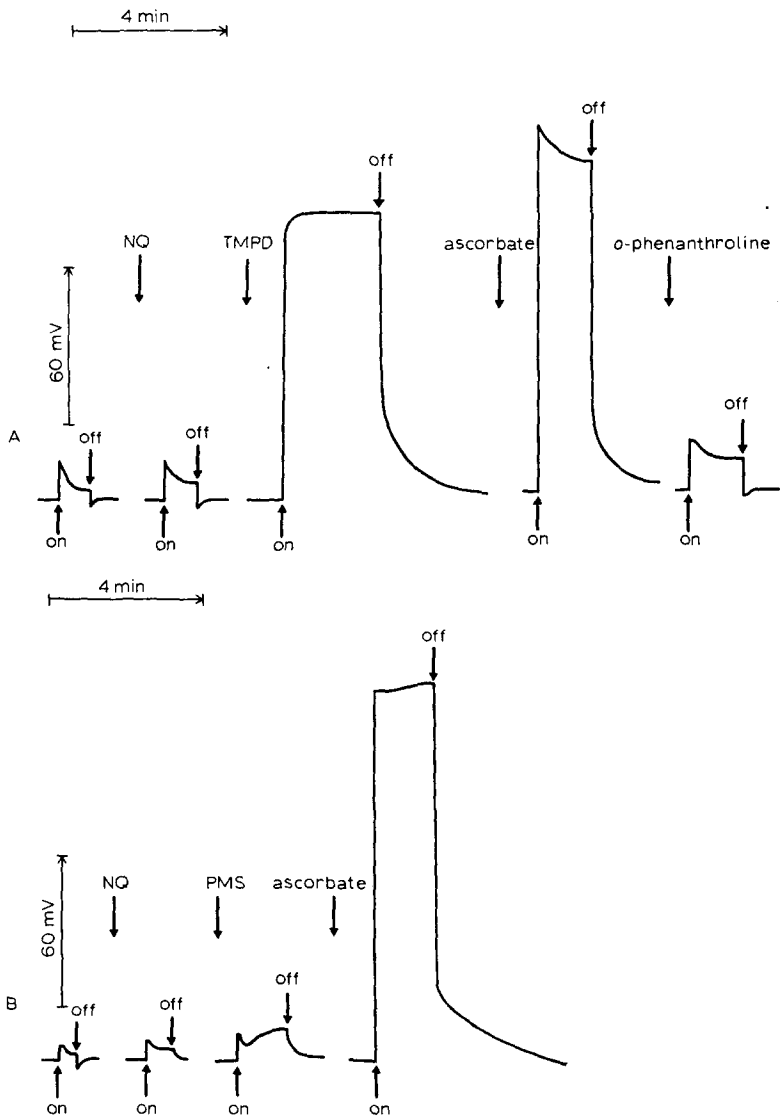


Fig. 3. Photoelectric generation by *R. rubrum* chromatophores associated with the planar azolectin membrane. Incubation mixture: 50 mM Tris · HCl buffer (pH 7.5), 10 mM $MgSO_4$ and chromatophores ($A_{880\,nm} = 1.9$; here and below, the length of the optical pathway is 1 cm). Additions (here and below, to both compartments of the experimental cell): 0.1 mM 1,4-naphthoquinone (NQ), 0.5 mM TMPD, 5 mM sodium ascorbate, 3 mM *o*-phenanthroline, 0.01 mM phenazine methosulphate (PMS). Positive charging of the chromatophore-free compartment is shown as an electric potential increase.

R. rubrum were previously described by Duysens [29].

The electric potential difference across the planar membrane was shown to be generated not only by light, but also by the ATP or inorganic pyrophosphate (PP_i) hydrolysis.

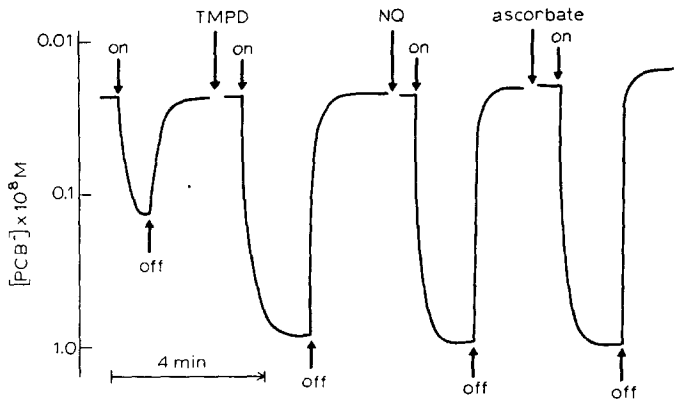


Fig. 4. Light-induced uptake of phenyldicarbaundecaborane (PCB) anions by *R. rubrum* chromatophores. Incubation mixture: 250 mM sucrose, 50 mM Tris · HCl buffer (pH 7.5) and chromatophores ($A_{880\,nm} = 3.1$). Additions: 0.2 mM TMPD, 0.1 mM 1,4-naphthoquinone (NQ), 5 mM sodium ascorbate.

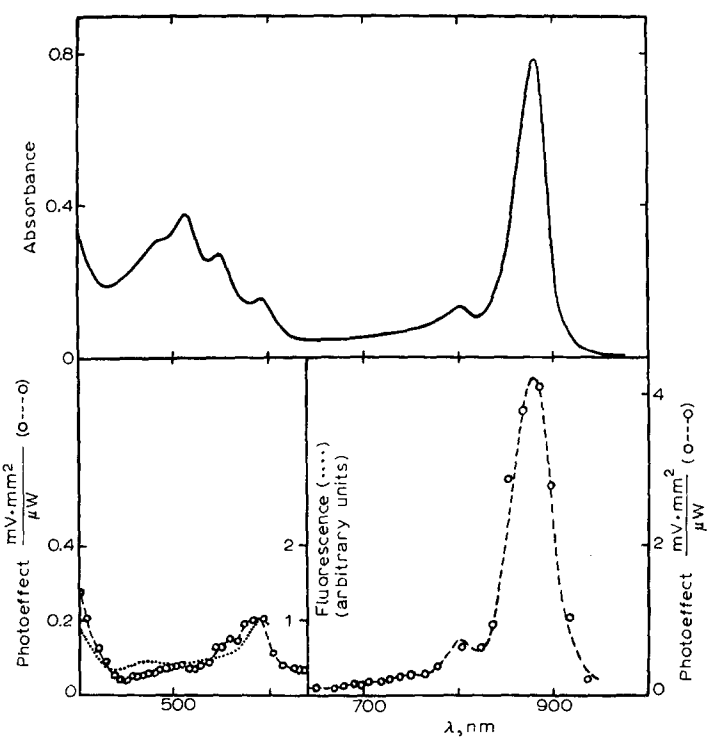


Fig. 5. The absorption spectrum of *R. rubrum* chromatophores (solid line) and the action spectra of the photoelectric response in the system chromatophores-planar membrane (broken line) and of 920 nm fluorescence excitation in chromatophores (dotted line). Incubation mixture: 50 mM Tris · HCl buffer (pH 7.5), 10 mM $MgSO_4$, chromatophores ($A_{880\,nm} = 1.7$), 0.5 mM TMPD, 0.1 mM 1,4-naphthoquinone (NQ), 5 mM sodium ascorbate.

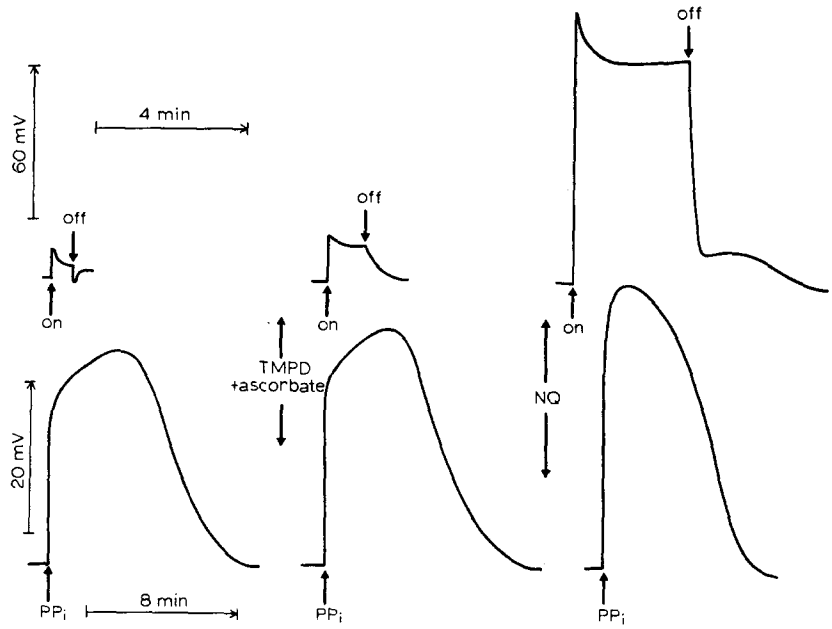


Fig. 6. Light- or PP_i-induced electric responses in the system chromatophores-planar membrane. Incubation mixture: 50 mM Tris · HCl buffer (pH 7.5), 10 mM $MgSO_4$ and chromatophores ($A_{880\,nm} = 1.4$). Additions: 0.5 mM TMPD, 5 mM sodium ascorbate, 0.1 mM 1,4-naphthoquinone (NQ), 0.1 mM PP_i.

Fig. 6 shows that the addition of a small amount of PP_i in the dark causes the formation of an electric potential difference across the planar membrane. The effect disappeared within several min, apparently due to PP_i exhaustion. Repeated additions of PP_i gave rise to fresh electric response cycles. The direction of the electric field (positive in the chromatophore-free compartment) generated by pyrophosphatase coincides with that induced by light. TMPD, ascorbate and 1,4-naphthoquinone, which cause a strong increase in the light-induced effect, have only a very slight influence on the PP_i-dependent electric generation. The magnitude of the PP_i-induced responses was lower than that of the photoeffect in optimal conditions.

Shunting of the planar membrane by an external electric resistance (Fig. 7A) was shown to cause a decrease in the light-induced effect and a characteristic change in its form (differentiation). The protonophorous uncoupler CCCP ($1 \cdot 10^{-6}$ M) strongly lowered the light-induced response, but without differentiation. The PP_i-induced response was also decreased by CCCP. The electrogenic function of pyrophosphatase was also found to be inhibited by F^- and La^{3+}, whereas oligomycin proved ineffective. Unlike CCCP, which is an uncoupler increasing the proton conduction of both the planar and chromatophore membranes, ionophorous antibiotic gramicidin has a very slight effect on the electric resistance of the planar membrane, but inhibits the photoelectric responses apparently due to an increase in the ion permeability of chromatophore membranes (Fig. 7B).

Of some interest are the experiments performed with the use of the thick planar membrane made of a mixture of chromatophores, azolectin and decane. The electric

responses in this system are a superposition of the electrogenic effects of the chromatophores associated with the right and left sides of the planar membrane. The direction and the form of the photoeffect vary from membrane to membrane most probably being determined by the ratio of the chromatophore amounts associated with the right and left sides of the membrane. The results of two experiments with such a system are shown in Figs. 7C and 7D. One can see that responses to switching the light on and off are rather small and biphasic due to the operation of two sets

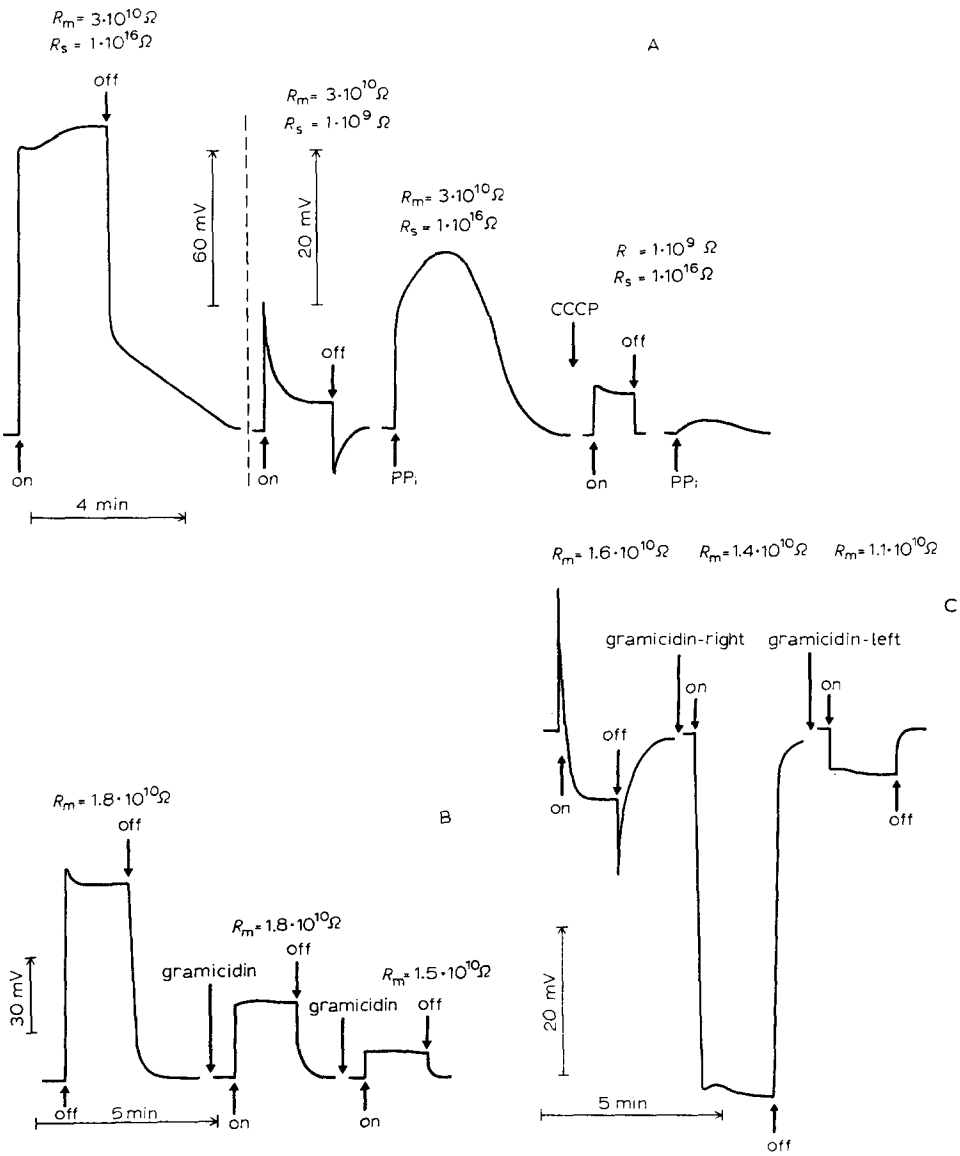


Fig. 7. For legend see opposite page.

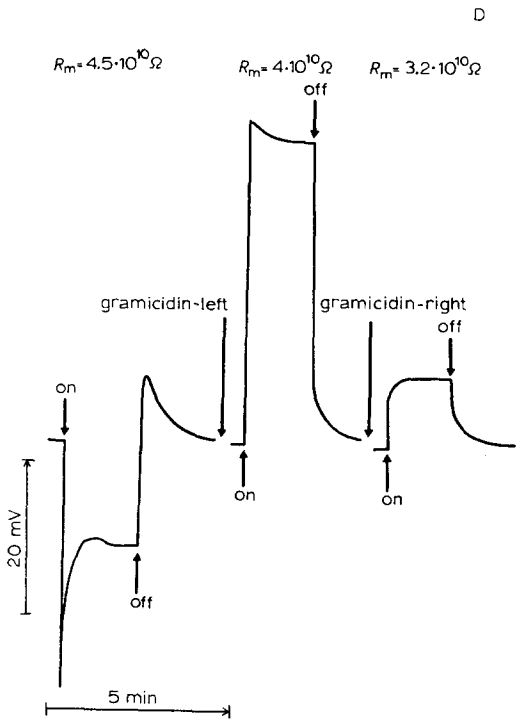


Fig. 7. (A) Shunting effects of an external electric resistance and uncoupler CCCP on the light- and PP_i-induced electric responses in the system chromatophores-planar membrane. Incubation mixture: 50 mM Tris · HCl buffer (pH 7.5), 10 mM $MgSO_4$, chromatophores ($A_{880\,nm} = 1.6$,) 0.02 mM phenazine methosulphate, 5 mM sodium ascorbate, 0.1 mM 1,4-naphthoquinone. Additions: 0.1 mM PP_i, $1 \cdot 10^{-6}$ M CCCP. R_m, electric resistance of the planar azolectin membrane; R_s, shunting electric resistance (see Figs. 12a and 12b). (B) Effect of gramicidin on the light-induced responses in the system chromatophores-planar membrane. Incubation mixture: 50 mM Tris · HCl buffer (pH 7.5), 45 mM KCl, 7.5 mM $MgSO_4$, 0.5 mM TMPD, 3 mM sodium ascorbate, 0.1 mM 1,4-naphthoquinone, chromatophores ($A_{880\,nm} = 1.5$). Additions: $1.5 \cdot 10^{-7}$ M gramicidin to the chromatophore-containing compartment. (C and D) Effect of gramicidin on the light-induced responses of planar membrane made of a mixture of chromatophores, azolectin and decane. Incubation mixture: 50 mM Tris · HCl buffer (pH 7.5), 0.5 mM TMPD, 3 mM sodium ascorbate, 0.1 mM 1,4-naphthoquinone (NQ). Additions: $1.5 \cdot 10^{-7}$ M gramicidin. R_s value in B, C and D is $1 \cdot 10^{16}$ Ω. Positive charging of the left compartment is shown as an electric potential increase.

of photobatteries producing electric potential gradients of opposite directions. Gramicidin added to the right (Fig. 7C) or the left (Fig. 7D) compartment, increasing the asymmetry of the system, causes an increase in the electric responses of the chromatophores associated with the opposite side of the planar membrane. The photoelectric responses are inhibited on addition of gramicidin to both compartments.

Data on the electrogenic action of ATPase in the chromatophores associated with the planar membrane are shown in Fig. 8. It is shown that ATP, like PP_i, causes the formation of electric potential difference across the planar membrane. Subsequent switching on of the light considerably enhanced the membrane potential. The magnitude of the photoeffect observed in the presence of ATP was lower than in its absence, so that steady levels of membrane potential in light with and without ATP proves

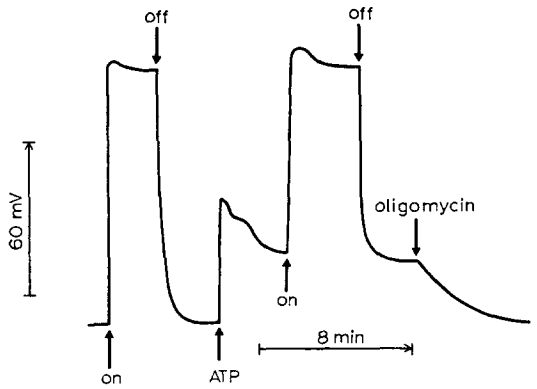


Fig. 8. Light- and ATP-induced electric responses in the system chromatophores-planar membrane. Incubation mixture: 50 mM Tris · HCl buffer (pH 7.5), 10 mM $MgSO_4$, chromatophores ($A_{880\,nm}$ = 1.5), 0.5 mM TMPD, 5 mM sodium ascorbate, 0.1 mM 1,4-naphthoquinone (NQ). Additions: 5 mM ATP, 20 μg/ml oligomycin.

to be the same. Oligomycin inhibited the electrogenic function of ATPase.

A special study was carried out to demonstrate that the events described above are due to the chromatophore material which is incorporated in the artificial membrane. Two pieces of evidence suggest that this is the case.

(1) No light-induced electric potential difference was formed across the planar membrane when Mg^{2+} (or Ca^{2+}) inducing the association of membrane vesicles with the planar membrane [15] were omitted. Control experiments showed that the formation of electric potential difference across the chromatophore membrane does not require Mg^{2+} (Ca^{2+}) addition, as judged from the light-dependent phenyldicarbaundecaborane responses which were found to be the same both in the presence and in the absence of divalent cations. Besides, in the planar membrane experiments the binding of Mg^{2+} (Ca^{2+}) by excess EDTA added when the association process is over, does not diminish the photoeffect. It was also found that Mg^{2+} is not necessary to the photoeffect produced by the planar membrane made of a mixture of chromatophores, azolectin and decane.

(2) Both light-induced and PP_i (or ATP)-induced electric responses can be demonstrated after substitution of chromatophore-free solution for mixture containing chromatophores, if the Mg^{2+}(Ca^{2+})-dependent association was completed. The most convenient system for this experiment proved to be a Teflon filter impregnated with azolectin. It has recently been found in this group [23] that such a filter can be used instead of the planar membrane to study biological generators of electric current. The Teflon filter with 50 μm pores covered a 1 cm aperture in the Teflon partition separating the two solutions. Chromatophores were added to one of the compartments and after 1 h incubation in the presence of Mg^{2+} the solution with chromatophores was removed and replaced by chromatophore-free solution. Experiments showed that such a phospholipid-impregnated, chromatophore-treated filter demonstrates electric responses similar to those in the chromatophore-planar membrane system.

Reconstitution of the electrogenic function in subchromatophore pigment-protein complexes

It has previously been demonstrated that the transmembrane electric potential difference is generated in illuminated proteoliposomes reconstituted from a mixture of azolectin and isolated *R. rubrum* photosynthetic reaction center complexes containing no light-harvesting antenna [16]. The direction of the electric field in the

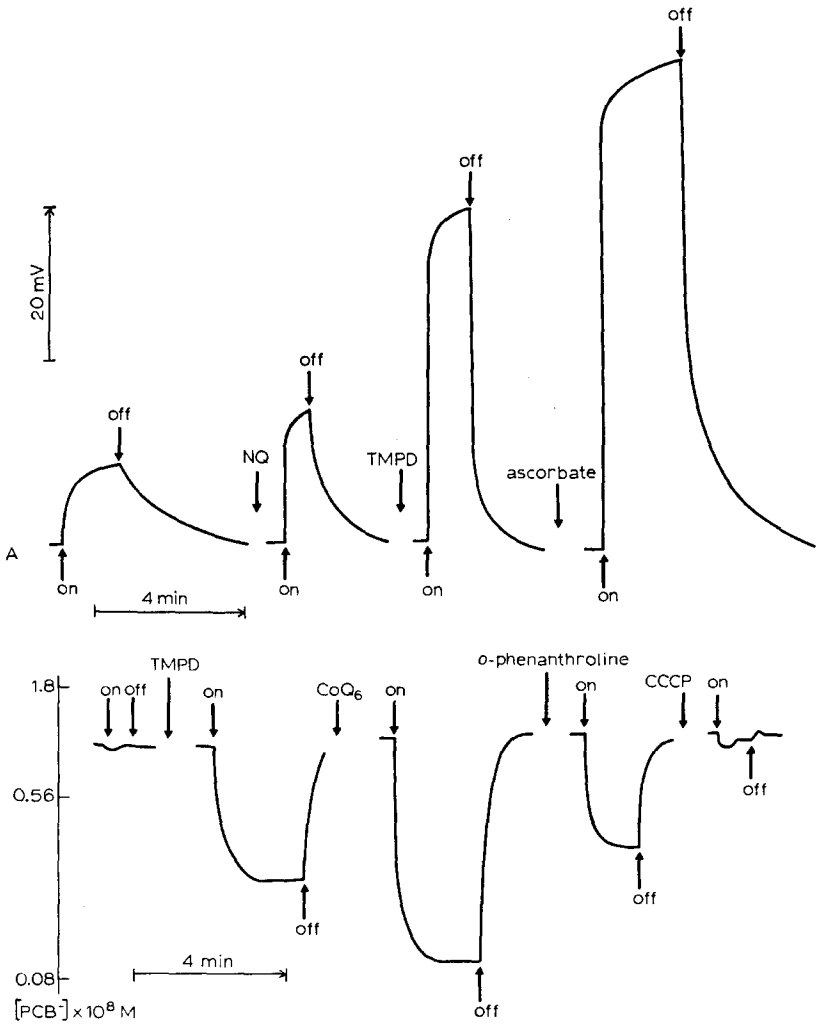


Fig. 9. (A) Light-induced electric responses of the proteoliposomes containing the reaction center plus antenna complexes in the system with planar azolectin membrane. Incubation mixture: 50 mM Tris · HCl buffer (pH 7.8), 30 mM $CaCl_2$ and proteoliposomes reconstituted with CoQ_6 ($A_{880\,nm}$ = 0.25). Additions: 0.1 mM 1,4-naphthoquinone (NQ), 0.5 mM TMPD, 5 mM sodium ascorbate. (B) Light-induced uptake of phenyldicarbaundecaborane (PCB^-) anions by proteoliposomes containing reaction center plus antenna complexes. Incubation mixture: 250 mM sucrose, 50 mM Tris · HCl buffer (pH 7.5), 5 mM $MgSO_4$ and proteoliposomes reconstituted with CoQ_6 ($A_{880\,nm}$ = 0.5). Additions: 0.5 mM TMPD, 0.1 mM CoQ_6, 2 mM *o*-phenanthroline, $4 \cdot 10^{-6}$ M CCCP.

proteoliposomes ("minus" inside) was found to be opposite to that in the chromatophores ("plus" inside). In this study, proteoliposomes containing both reaction center complexes and antenna have been investigated.

Fig. 9 shows photoelectric generation in proteoliposomes reconstituted from the reaction center plus antenna complexes in the presence of CoQ_6. Being associated with the planar membrane, the proteoliposomes, like chromatophores, generate electric potential difference which is positive in the proteoliposome-free compartment (Fig. 9A). Photoelectric response increases on addition of 1,4-naphthoquinone, TMPD and ascorbate.

Fig. 9B illustrates the result of an experiment in which generation of membrane potential was detected with the phenyldicarbaundecaborane probe. Switching on the light causes an uptake of phenyldicarbaundecaborane anions by the proteoliposomes containing the reaction center plus antenna complexes in the presence of TMPD. The direction of the light-dependent translocation of phenyldicarbaundecaborane anions (the uptake) indicates the generation of electric potential difference across the proteoliposomal membrane with "plus" inside the vesicles. The process was reversible in the dark, could be stimulated by CoQ_6, suppressed by a non-beam iron inhibitor, *o*-phenanthroline, and a protonophorous uncoupler, CCCP.

Fig. 10A shows the photoelectric generation by proteoliposomes containing reaction center complexes. It is demonstrated that the photoelectric response measured in the system "proteoliposomes-planar membrane" increases significantly on addition of TMPD (proteoliposomes were reconstituted with CoQ_6). The direction of the

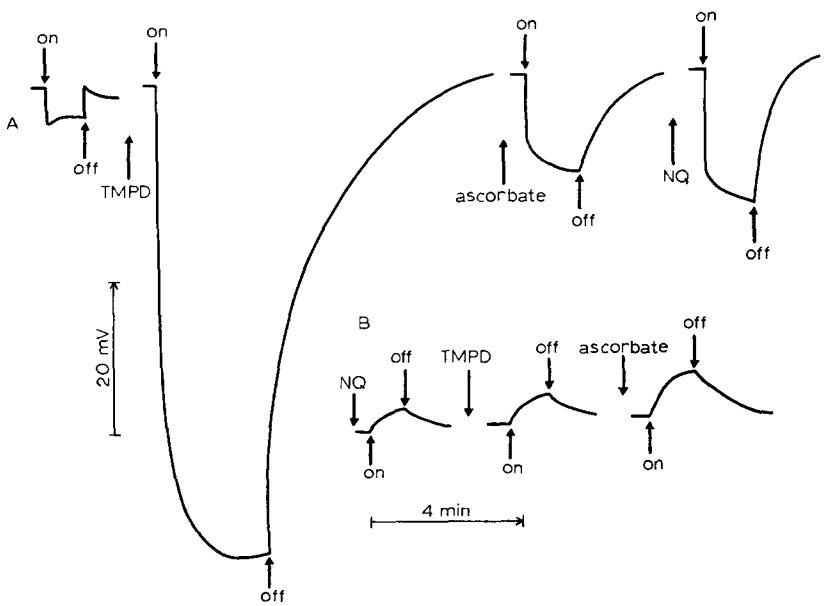


Fig. 10. Light-induced electric responses of proteoliposomes containing isolated reaction center complexes (A) or isolated antenna complexes (B) in the system with planar azolectin membrane. Incubation mixture: 50 mM Tris · HCl buffer (pH 7.5,) 20 mM $MgSO_4$ in A or 30 mM $CaCl_2$ in B, proteoliposomes reconstituted with CoQ_6. $A_{870\,nm} = 0.05$ in A and $A_{880\,nm} = 3.4$ in B. Additions: 0.5 mM TMPD, 5 mM sodium ascorbate, 0.1 mM 1,4-naphthoquinone (NQ).

electric field ("minus" in the proteoliposome-free compartment) is opposite to that revealed in the systems with the proteoliposomes containing the reaction center plus antenna complexes. Ascorbate inhibits the photoeffect (cf. the stimulating action of ascorbate in experiments with chromatophores and the reaction center plus antenna proteoliposomes). Subsequent addition of 1,4-naphthoquinone influences the photoeffect only slightly. Illumination induces the extrusion of phenyldicarbaundecaborane anions from the proteoliposomes containing the reaction center complexes (not shown), which indicates the negative charging of the proteoliposome interior.

As noted previously [16], the photoelectric generation in the proteoliposomes containing the reaction center complexes is inhibited by *o*-phenanthroline and CCCP.

The proteoliposomes reconstituted from phospholipids and isolated complexes of the antenna (light-harvesting bacteriochlorophyll plus carotenoids) are capable of only negligible photoelectric generation not exceeding 5–6 mV (Fig. 10B). The process required 1,4-naphthoquinone, being stimulated by TMPD and ascorbate. It was probably due to contamination of the bacteriochlorophyll reaction center.

In the last series of experiments, proteoliposomes were reconstituted from a mixture containing the reaction center complexes and antenna complexes added in different proportions. As is shown in Fig. 11A, proteoliposomes with a reaction center-to-antenna ratio equal to 1 : 13 showed photoelectric responses of the same direction as those without antenna ("minus" in the proteoliposome-free compartment). The effect was stimulated by TMPD and 1,4-naphthoquinone and inhibited by ascorbate. The magnitude of light-induced membrane potential was lower than in proteoliposomes containing the reaction center complexes only (cf. Fig. 10A).

When the reaction center-to-antenna ratio decreases down to 1 : 65 (Fig. 11B), the direction of the electric vector in the presence of TMPD is found to be the same as in Fig. 11A. Subsequent addition of 1,4-naphthoquinone causes a change in the form of the light-dependent response. The initial positive charging of the proteoliposome-free compartment was followed by a negative one. The disappearance of electric potential in the dark was also biphasic. The complicated character of the response observed indicates that the two types of photoelectric generators operate in opposite directions: one charges the membrane like the reaction center, and the other, like the reaction center plus antenna complexes. Ascorbate, which inhibits the first type of generators, was shown to modify the photoelectric response which became similar to that of the reaction center plus antenna complexes (Fig. 11B).

Fig. 11C shows the photoelectric responses in proteoliposomes reconstituted in conditions of a very low reaction center-to-antenna ratio (1 : 325). One can see that the small negative photoelectric response observed after TMPD treatment becomes the opposite on subsequent addition of 1,4-naphthoquinone. The photoeffect is significantly stimulated by ascorbate. The magnitude of the positive photoelectric response was much higher in Fig. 11C than in Fig. 11B.

Thus, the direction and magnitude of the electric potential difference generated in the system "proteoliposomes-planar membrane" can be regulated by changing the reconstitution mixture composition. The reconstitution of the reaction center complexes in the presence of the antenna complexes proves favourable for obtaining a system generating a membrane potential of the same direction as in chromatophores.

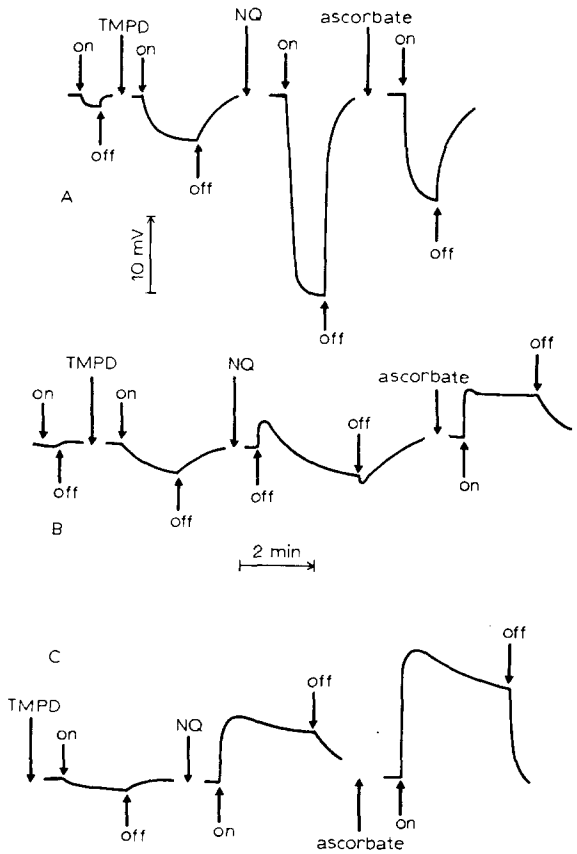


Fig. 11. Light-induced electric responses of proteoliposomes containing isolated reaction center complexes and isolated antenna complexes, in the system with planar azolectin membrane. (A) $A_{870\,nm} = 0.05$, $A_{870\,nm} : A_{880\,nm} = 1 : 13$. (B) $A_{870\,nm} = 0.04$, $A_{870\,nm} : A_{880\,nm} = 1 : 65$. (C) $A_{870\,nm} = 0.01$, $A_{870\,nm} : A_{880\,nm} = 1 : 325$. Incubation mixture: 50 mM Tris · HCl buffer (pH 7.5), 30 mM $CaCl_2$ and proteoliposomes. Additions: 0.5 mM TMPD, 0.1 mM 1,4-naphthoquinone (NQ) and 5 mM sodium ascorbate.

DISCUSSION

Isolation of the pigment-protein complexes

The pigment-protein complexes used in the above experiments were isolated from *R. rubrum* chromatophores by detergent treatments. Predominant solubilization of the *P*-870 reaction center complexes takes place during the initial stages of the treatment of chromatophores by a detergent, lauryldimethylamine oxide. Repeated detergent treatments sharply lowers the *P*-870 content in the chromatophores which after this prove to be a good source for the preparation of the pigment-protein complexes of light-harvesting antenna.

Pigment-protein complexes isolated from chromatophores with the use of cholate contain both reaction centers and antenna. The spectral characteristics of

these complexes are similar to that of chromatophores, as well as that of the complexes isolated after the combined treatment of *R. rubrum* chromatophores with urea and Triton X-100 at alkaline pH [24].

Measurements of electric generation by chromatophores or proteoliposomes associated with the planar membrane

The method of polyvalent cation-induced association of reconstituted lipoprotein vesicles with the planar membrane, which were used in this group to measure electric generation by bacteriorhodopsin, cytochrome oxidase, H^+-ATPase and the bacteriochlorophyll reaction center complexes [15, 16], has now been applied to a native membranous system, namely *R. rubrum* chromatophores. With the help of this approach, electric potential generation by the chromatophore photosynthetic redox chain, H^+-ATPase and inorganic pyrophosphatase has been measured with a voltmeter.

A scheme illustrating the electric events in a vesicle attached to a planar phospholipid membrane is given in Fig. 12A. According to the scheme, either the switching on of a chromatophore electric generator by illumination, or ATP (or PP_i) additions causes the appearance of a charge gradient across the chromatophore membrane ("plus" inside the chromatophore), and of the electric currents I_1, I_2 and I_3 through the planar and chromatophore membranes.

It is suggested that gramicidin, added to the proteoliposome-containing compartment, specifically decreases the resistance of the thin proteoliposomal

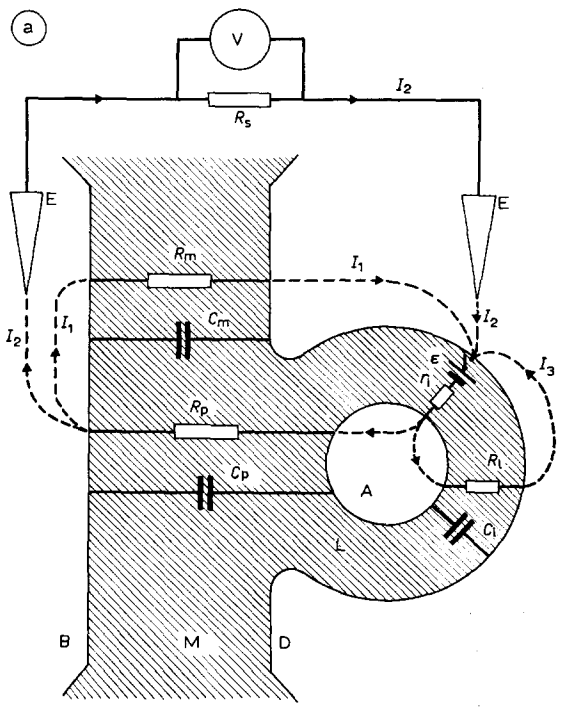


Fig. 12. For legend see following page.

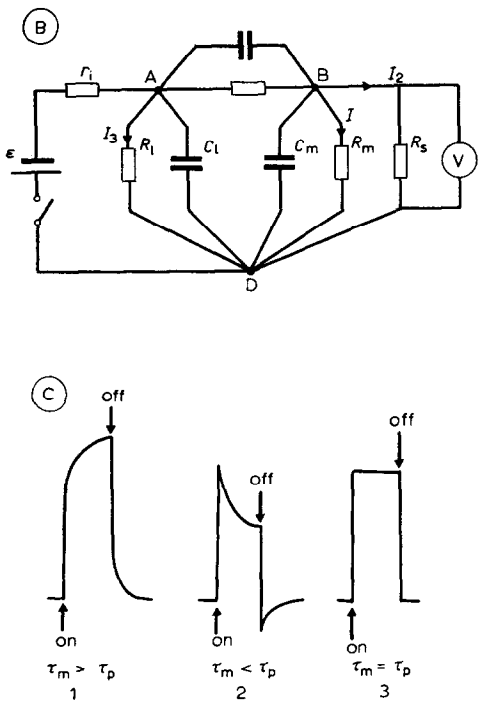


Fig. 12. Schemes of electric potential measurement in the system chromatophores (proteoliposomes)-planar membrane: (a) supposed scheme of electric currents in the chromatophore-planar membrane system; (b) equivalent electric scheme; (c) the forms of the electric responses at different $\tau_t : \tau_m$ ratios. Designations: M, planar phospholipid membrane; L, membrane of the vesicle (chromatophore or proteoliposome); A, internal volume of the vesicle; B and D, compartments of the cuvette with electrolyte solutions; E, Ag/AgCl electrode; V, voltmeter; R_s, electric resistance, shunting the planar membrane; R_m and C_m, electric resistance and capacity of planar membrane, respectively; R_l and C_l, electric resistance and capacity of visicle membrane, respectively; R_t and C_t, transitional electric resistance and capacity corresponding to a fusion region of the planar and vesicle membranes; ε and r_i, electromotive force and the internal resistance of the molecular electric generator; I_1, I_2 and I_3, electric currents; $\tau_t = R_t \cdot C_t$, $\tau_m = R_m \cdot R_s \cdot C_m / R_m + R_s$.

membrane (R_l) but not of the thick planar membrane (R_m) measured in the light. As a result, the photoeffect is inhibited due to an increase in I_3. On the other hand, CCCP decreases both R_l and R_m since the membrane thickness is not critical for its action. In this case, the photoeffect inhibition parallels lowering of the planar membrane resistance.

Fig. 12b shows an equivalent scheme of an electric chain. Fig. 12c illustrates the electric responses calculated by using the equivalent scheme at different ratios between the time constants τ_t and τ_m (for designations, see the Fig. 12 caption). The electric response observed in experiments at high R_s values usually corresponds in form to the $\tau_t < \tau_m$ type. A decrease in the R_s magnitude and, hence, in τ_m gives rise to electric responses of $\tau_t > \tau_m$ type (see, e.g. Fig. 7, the second light response). Undoubtedly, representation of the system "chromatophores (proteoliposomes)-planar membrane" by a linear electric chain is too simple to be absolutely equivalent to actual conditions. In particular, the scheme sets aside the possibility of substituting

ΔpH for electric potential during the functioning of this system. With high electric currents, i.e. when R_s and/or R_m are low, this substitution can result in an electric response of the type shown in Fig. 12c, 2.

It is noteworthy that the formation of ΔpH, shunting the planar or chromatophore membrane and other probable factors complicating the exact measurement of the electromotive force of the biological generator in the system used, can only result in the true electromotive force being underestimated. Therefore, the values obtained in the above experiments should be appreciated as the lower limit of the true electromotive force. The degree of such underestimation may be roughly evaluated in the following way. For the photoelectric response of chromatophores, the time constant of the membrane potential generation (τ_p) is calculated as

$$\tau_p = r_i \cdot C_l + \left(\frac{C_m \cdot C_t}{C_m + C_t}\right)$$

Assuming that the value of $C_l + (C_m \cdot C_t / C_m + C_t)$ is of the same order of magnitude as the C_m value of the membrane in the dark, one can calculate $r_i = \tau_p / C_m$. The τ_p value, determined by means of oscillographic measurement of the initial rate of the photoelectric response, was found to be < 0.1 s. The measured values of C_m were usually about $0.5 \cdot 10^{-9}$ F. Taking these data into account, we obtain $r_i < 2 \cdot 10^8\ \Omega$. This value is at least two orders lower than R_m which was usually equal to $2 \cdot 10^{10}$–$5 \times 10^{10}\ \Omega$. The electric potential difference measured by voltmeter V (Fig. 12A) is determined by the ratio between R_t and $R_m \cdot R_s / R_m + R_s$. The electric resistances of the "chromatophore-planar membrane" system, determined from the current voltage relationship in the light and in the dark, differ by a factor from 1.5 to 3. Consequently, the difference between the R_t and R_m values should not be too large. Assuming $R_s \gg R_m$ and $R_l \gg r_i$, we come to the conclusion that the measured voltage should be from 3 to 1.5 times lower than the real electromotive force of the photosynthetic redox chain generator in chromatophores.

Mechanism of TMPD and 1,4-naphthoquinone activation of photoelectric responses

It was found in the above experiment that the photoelectric responses of both chromatophores and proteoliposomes can be very sharply increased by adding TMPD (or phenazine methosulphate) and 1,4-naphthoquinone (or vitamin K-3). All these compounds have no effect on the ATP- and PP_i-dependent electric responses, this fact indicating that the phenomenon considered is connected with the operation of the chain of light-induced electron transport, i.e. photoelectric generator per se, rather than with the other electric parameters of the system studied. The observations that 1,4-naphthoquinone and vitamin K-3 are not necessary when (1) CoQ_6 is included in the mixture for planar membrane formation and (2) the phenyldicarbaundecaborane responses of the chromatophore suspension were measured, allowed the effect of these quinone derivates to be accounted for by the removal of planar membrane-induced inhibition of the chromatophore redox chain at the CoQ level. The simplest explanation of this inhibition consists in the assumption that endogenous CoQ is extracted from chromatophores by decane, a constituent of the planar membrane-forming solution.

As for the TMPD and phenazine methosulphate effect, it may be due to the shunting of the cytochrome part of the redox chain. It is noteworthy that TMPD

and phenazine methosulphate increase the light-induced phenyldicarbaundecaborane uptake by chromatophores and desensibilize this process to antimycin, the agent inhibiting the chain at the cytochrome *b* level. This action may be explained by the fact that the electromotive force of the bacteriochlorophyll reaction center generator is higher than that of the second generator of the photosynthetic redox chain localized in the cytochrome system (most probably in the region of cytochrome *b*). In shunting the cytochrome *b* segment of the redox chain, we allow the bacteriochlorophyll generator to produce its maximal voltage.

In the chromatophore-planar membrane system, there is one more probable reason for the increase in the photoeffect when the cytochrome *b* region is shunted. It is known that the antimycin-sensitive step of the mitochondrial respiration chain is inhibited by a wide range of uncharged hydrophobic compounds of very different structure [25, 26]. Decane as a hydrophobic agent may inhibit cytochrome *b* of the chromatophore redox chain, and TMPD (or phenazine methosulphate) may abolish this inhibition. In this way, one can explain why TMPD (phenazine methosulphate) stimulation of the photoresponse is greater in the planar membrane experiments than in those with phenyldicarbaundecaborane.

Probable points of action of 1,4-naphthoquinone, vitamin K-3, TMPD and phenazine methosulphate are illustrated in Fig. 13. A tentative scheme of the cyclic photosynthetic redox chain of the chromatophores given in Fig. 13 was assumed in

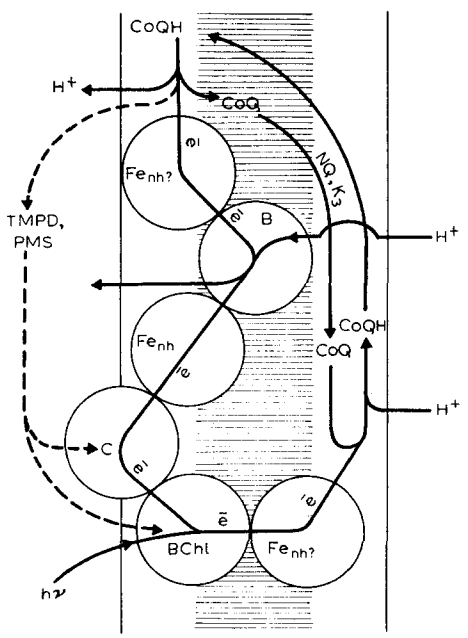


Fig. 13. Scheme of the cyclic photosynthetic redox chain in *R. rubrum* chromatophores. BChl, bacteriochlorophyll of the reaction center; Fe_{nh}, non-haem iron; B and C, cytochromes. It is proposed that 1,4-naphthoquinone (NQ) or vitamin K-3 can replace CoQ in the natural pathway of the hydrogen atom transport through the hydrophobic barrier of the chromatophore membrane. As for TMPD and phenazine methosulphate (PMS), they are suggested to organize artificial shunt of the reducing equivalents transfer with no cytochrome *b* involved.

this group [27] on the basis of the analogy between mitochondrial and chromatophore electric generators. The scheme implies that CoQ functions as a transmembrane H atom carrier, "bacteriochlorophyll primary acceptor" system, as a transmembrane electron carrier, and cytochrome *b*, as a redox proton pump carrying electrons along the membrane in a fashion coupled with H^+ transport across the membrane.

Regulation of proteoliposome reconstitution

A study on proteoliposome reconstitution revealed that some proteins can be spontaneously oriented in the reconstituted membrane, mainly in one of the possible positions. This was the case with the bacteriorhodopsin [15, 28] and *R. rubrum* reaction center complexes [16]. The data of this paper demonstrate that, in the latter system, we can regulate the process of reconstitution.

It was found that orientation of the photoelectric generator in the proteoliposomal membrane prove opposite if the reaction center complexes are reconstituted in the presence or in the absence of antenna complexes (Fig. 11). It may be of importance that the reaction center plus antenna complexes yield a system generating an electric field of the same direction as in the native chromatophore membrane. The effect was found to depend on the ratio between the isolated reaction center complexes and the antenna complexes in the mixture used for proteoliposome reconstitution.

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