Photosynthetic Light Reactions in Chemically Fixed Anacystis nidulans, Chlorella pyrenoidosa, and Porphyridium cruentum

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Abstract. The photochemical activities of various species of unicellular algae (Anacystis nidulans, Chlorella pyrenoidosa, and Porphyridium cruentum) were studied following chemical fixation. Fixation with formaldehyde and glutaraldehyde yielded cells which retained their ability to perform photosystem I and photosystem II reactions. The photochemical efficiencies of some fixed algae are as great as those of unfixed spinach chloroplasts. Fixed algae containing accessory pigments appear to be useful models for further studies of the light reactions of photosynthesis.

In 1966 Park et al. (14) reported that glutaraldehyde fixed Chlorella cells and chloroplasts isolated from glutaraldehyde fixed spinach leaves performed light dependent O₂ production in the presence of suitable electron acceptors. The quantum yields for photosystem II reactions of spinach chloroplasts from fixed leaves were about 25% those of chloroplasts from unfixed leaves. At that time we foresaw that this method might be a useful technique for studying photosystems I and II in algae containing accessory pigments. Such algal cells are generally impermeable to the components of reaction mixtures used to study photosystems I and II. We predicted that aldehyde fixation should remove this permeability barrier and circumvent many of the problems caused by cell breakage in these algae. Cell breakage is usually accompanied by considerable loss of accessory pigment from thylakoid membranes (4, 17).

However in our initial efforts we failed to demonstrate Hill reaction either in glutaraldehyde fixed algae which contained accessory pigments or in spinach chloroplasts which were fixed after isolation. Both these obstacles are now removed. In this paper we report retention of both photosystems I and II in chemically fixed Anacystis nidulans, Chlorella pyrenoidosa and Porphyridium cruentum. In the accompanying paper we show that both photosystem I and II activities can be preserved in spinach thylakoids properly fixed after isolation.

Materials and Methods

Chlorella pyrenoidosa Chick was grown in modified Myers' medium (5) at 20° and Anacystis nidulans (Richt.) Drouet and Dailey was grown in Myers' medium C (11) at 35°. Both cultures were illuminated with 800 to 1000 ft-c (fluorescent light) and aerated with 4% CO₂. Porphyridium cruentum (Agardh) Nageli was grown in artificial seawater (8) at 20°, illuminated with 100 to 200 ft-c (fluorescent light) and aerated with 4% CO₂. Chlorophyll contents of the cultures were estimated according to Arnon (3, 13).

Reagents Used. ¹⁴C-Formaldehyde, specific activity 12 mc/mnole (New England Nuclear), DCMU³ (DuPont), DCPIP (Sigma), paraformaldehyde (Malinckrodt), GA (Fisher Scientific), MA (Matheson), MV (California Chemical), tricine (Calbiochem).

Aldehydes. Formaldehyde was prepared freshly every day from paraformaldehyde and titrated to pH 7.4. Glutaraldehyde was prepared by washing twice for about 20 min with animal bone charcoal and then titrating, if necessary, to pH 7.4 (1, 15).

Fixation. After determination of the absorption at 680 nm, algae were centrifuged at top speed in a clinical centrifuge for 2 to 4 min at room temperature. The precipitate was resuspended in 0.05 M tricine or potassium phosphate (pH 7.4) solution containing aldehyde. Recent experiments have shown that potassium phosphate buffers give better results since aldehydes react with tricine. In the

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³ The following abbreviations are used: DCMU [3-(3,4-dichlorophenyl)-1,1-dithy lurea]; DCPIP (2,6-dichlorophenolindophenol); FA (formaldehyde); GA (glutaraldehyde); MA (methylamine hydrochloride); MV (1,1'-dimethyl-4,4'-dipyridyl dichloride or methyl viologen); tricine [N-tris (hydroxymethyl) methyl glycine].
case of short fixations the algae were immediately centrifuged again; the precipitate was washed once in 0.2 M MA (pH 7.4) to remove unreacted aldehyde, and twice in 0.05 M tricine buffer (pH 7.4). Short fixations lasted about 4 min. The same washing treatment was used after long fixations.

**Fixation With \(^{14}\text{C}-\text{Formaldehyde.** During incubation of the algal suspension with \(^{14}\text{C}-\text{formaldehyde samples were taken at certain times and spread evenly on millipore filters. The filters were washed several times with water. The filters were dissolved in a solution of ethanol, toluene, and p-dioxane, in the ratio 1/1.4/1.6. Four and one-half g of 2,5-di-phenyloxazolyl plus 0.1 g of 1,4-bis-(4-methyl-5-phenyloxazolyl) benzene were used per liter of dissolving solution. The samples were counted with a Packard Tri-Carb (model 3375) liquid scintillation spectrometer.**

**Reaction Mixtures.** A typical photosystem II reaction mixture contained per ml: 0.03 \(\mu\)mole DCPIP, 100 \(\mu\)mole MA (as an uncoupler of photophosphorylation 6.12), and 50 \(\mu\)mole tricine, in a total volume of 2.5 ml at pH 7.4. Chlorophyll concentrations are given in the figure legends. A typical photosystem I reaction (2,9,10) contained per ml: 5 \(\mu\)mole ascorbate, 5 \(\mu\)mole MV, 0.1 \(\mu\)mole DCMU, 0.05 \(\mu\)mole DCPIP and 50 \(\mu\)mole tricine in a total volume of 2.5 ml at pH 7.4. Chlorophyll concentrations are given in the figure legends. The reaction mixture was run in closed cuvettes which were flushed with \(\text{N}_2\) for several min before illumination. Materials with low photosystem I rates due to fixation required up to 10 min illumination before the residual \(\text{O}_2\) in the reaction mixture was used up and measurable MV reduction commenced. Fresh chloroplasts required only 10 to 20 sec illumination before photosystem I commenced. The reactions were performed at room temperature in a Cary model 14 spectrophotometer, modified according to Sauer and Biggins (16). The DCPIP reduction (photosystem II) was measured at 580 nm, the reduction of MV at 395 nm. Actinic light was supplied by a 1000 watt projection lamp (Westinghouse DFT 1000 W) passed through a Bausch and Lomb grating monochromator and red cutoff filters supplied by Corning. For both reactions the assays were illuminated with light of 678 nm, though photosystem II is driven more efficiently by illumination of the accessory pigment. Incident intensities on the various samples were determined with a silicon photodiode calibrated against a standard lamp (16).

**Results and Discussion**

Typical raw data for photosystem II reactions in FA-fixed *Chlorella* cells are shown in Fig. 1. The

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*Fig. 1. Light dependent reduction of DCPIP (photosystem II) by FA fixed *Chlorella* cells. The reaction mixture is described under Methods. Chlorophyll content as *Chlorella* cells was 9.6 \(\mu\)g chlorophyll/ml. Short fixation (see Methods) was performed with 3% (w/v) FA (pH 7.4). The reaction mixture was illuminated with light of 678 nm at voltages given in the figure.*

*Fig. 2. Light dependent reduction of DCPIP (Photosystem II) by *Porphyridium* after various FA additions. The reaction mixture is described under Methods. Chlorophyll content as *Porphyridium* was 8 \(\mu\)g chlorophyll/ml. The reaction mixture was illuminated with light of 550 nm at maximum voltage. Optical density change was measured at 580 nm.*
light intensity was regulated by changing the voltage supplied to the actinic lamp (60-120 volts). Due to the filtering system this procedure produces almost no change in light quality. The intensity dependence of the Hill reaction in the fixed cells is indicated by the changes in slope of the OD trace. The photosystem II activities of all the fixed cells reported in this paper were completely inhibited in the presence of $10^{-4}$ M DCMU.

Fig. 2 shows the fixing effect of FA on Porphyridium cells. Whereas the unfixed control does not show any reduction of DCPIP, presumably because the dye is not able to penetrate into the cell, addition of 0.66% (w/v) FA to the assay initiates dye reduction after a few min. This induction period is shortened by a 10 sec FA-pretreatment on the cells in the dark. A 20 min pretreatment of the algae with 0.66 % FA leads to immediate DCPIP reduction on illumination. The effect of FA on Porphyridium is most easily explained by assuming that FA has changed the permeability of the cell wall and cell membrane so that DCPIP reaches the photosystem II reducing sites.

The fixing effect of FA is dependent both on the aldehyde concentration and on the fixation time. Long fixations (greater than 4 min) often lead to a decrease in photochemical activity. This means that there are optimal FA concentrations and fixing times for each organism which yield optimal activity of the 2 photosystems. These optimum fixation conditions are different for each organism and for the 2 different photosystems I and II. When using the FA-fixation, optimal photosystem II activity for Chlorella is obtained after a short fixation with a 3% FA solution (pH 7.4), whereas Anacystis reaches the highest rate of DCPIP reduction after short fixation with 1.5% FA solution (pH 7.4).

To investigate the kinetics of formaldehyde fixation more thoroughly, the time course of FA uptake by Anacystis was studied using $^{14}$C formaldehyde. Anacystis cells were added to a 1.5% FA solution containing $^{14}$C formaldehyde. At time intervals samples were taken and the $^{14}$C content was determined. Fig. 3 shows the time dependent uptake of $^{14}$C in dpm and the total uptake of FA based on the relative amount of $^{14}$C formaldehyde in the total FA.

![Figure 3](www.plantphysiol.org)

**Fig. 3.** Uptake of $^{14}$C formaldehyde by Anacystis. Reaction mixture contained per ml: 330 A. moles FA, 0.78 $\mu$ mole $^{14}$C-formaldehyde (specific radioactivity 12 mC/m$\mu$), and 30 $\mu$ moles tricine. Chlorophyll content as Anacystis was 0.6, 0.3, and 0.15 $\mu$g chlorophyll/ml for curves A, B, and C respectively. Total volume was 0.6 ml, pH 7.4. At certain time intervals samples of 0.05 ml were taken, spread as thin layer on millipore filters, washed and counted. Radioactivity is presented as dpm.

It is shown that most of the FA uptake, i.e. fixation, occurs during the first 2 to 3 min of incubation. Furthermore the figure shows that the amount of FA taken up is directly related to the amount of cell material present in the assay which was determined here on the basis of micromoles chlorophyll per sample. The ratio of chlorophyll concentrations in the 3 samples A, B, and C are 4:2:1. These ratios are apparent in the quantitative differences among the fixation curves for the 3 samples. The data in Fig. 3 also show that the molar ratio of (chlorophyll
After 30 min incubation it is approximately 1:2.5, independent of sample size and amount of excess FA in the incubation mixture.

Extraction of the fixed algae with petroleum ether (30-60°) followed by extractions with hexane, CHCl₃, chloroform, ether, 80 % (v/v) acetone 70 % ethanol, and 70 % methanol removed less than 10 % of the radioactivity. This indicates that the formaldehyde is bound to a non lipid fraction, probably protein. Further experiments were done to be certain that uptake of FA was not metabolically driven. Fig. 4 compares the uptake of FA by fresh and boiled Anacystis cells. This experiment indicates that aldehyde fixation is not a metabolic process.

In summary these experiments on FA-fixation of Porphyridium, Chlorella, and Anacystis show that:

1) The effect of FA is probably to change the permeability of the cell walls and membranes, enabling dyes and other components of the reaction mixture to penetrate into the cells. This opens the possibility of measuring the properties of photosystems I and II in a number of unicellular organisms which are difficult to study by conventional cell breakage procedures.

2) The effect of FA fixation is a reproducible one, certain aldehyde concentrations and fixation times leading to optimal reduction rates, even with different cultures of the same organism.

3) The main uptake of formaldehyde occurs during the first few min of the reaction concurrent with the permeability changes as seen by the onset of Hill reaction. Measurable FA uptake continues for up to 30 min. 4) The fixation process is not metabolically driven.

These results of FA fixation encouraged us to explore further the photosynthetic electron transport capacity of cells and cell fragments after GA fixation. Successful GA fixation required purifying the commercially obtained compound with animal bone charcoal as described under methods. Though exchange resins were used by Park et al. (14) for purifying GA used to fix intact leaves, we found this treatment did not yield satisfactory GA for experiments on Anacystis and Porphyridium. Optimal photosystem II activities were obtained using the following GA fixation conditions: Porphyridium short fixation with 0.4 % (v/v) GA (pH 7.4), Anacystis short fixation with 1 % GA (pH 7.4), Chlorella short fixation with 0.1 % GA (pH 7.4).

Fig. 5 presents data for optimal fixation of Porphyridium with GA to achieve maximum rates of

![Graph](attachment:image.png)

**Fig. 5. Light dependent reduction of DCPIP (photosystem II) by Porphyridium fixed with various concentrations of GA (9% = v/v). The reaction mixture is described under Methods. The chlorophyll content as Porphyridium was 0.6 μg chlorophyll/ml. Short fixation with 0.1 to 5 % GA at pH 7.4. Reaction mixture was illuminated with 678 nm light. Light intensity is given in millivolts output of the silicon photodiode. A reading of 50 millivolts corresponds to a light intensity of 3.2 nanoeinstens cm² sec⁻¹.**

![Graph](attachment:image.png)

**Fig. 6. DCPIP reduction (photosystem II) by Porphyridium, Chlorella, and Anacystis as a function of absorbed light intensity after fixation under optimal conditions. Reaction mixtures and absorbed intensity measurements described under Methods. AFA-Anacystis, fixed in 1.5 % FA; AGA-Anacystis, fixed in 1 % GA; Chlor.-Chlorella, fixed in 3 % FA; CGA-Chlorella, fixed in 0.1 % GA; PGA-Porphyridium fixed in 0.4 % GA. All fixations are short fixations. Chlorophyll contents of the reaction mixtures for Anacystis, Chlorella, and Porphyridium were 5 μg, 10 μg, and 10 μg respectively.**
The reaction mixture is described under Methods. The chlorophyll content as Anacystis was 20 μg chlorophyll/ml. The reaction mixture was illuminated with 678 nm light.

DCPIP reduction. Suboptimal as well as supraoptimal fixation times and concentrations lead to lower reduction rates than the optimal fixation conditions.

Fig. 6 summarizes the relative efficiencies of various fixed algae compared with unfixed chloroplasts in driving the DCPIP Hill reaction. All algae were fixed under optimal conditions. This figure illustrates 2 points. 1) That the sensitivity of photosystem II of various algae to fixation differs not only with respect to general susceptibility, but also to the chemical nature of the fixative. 2) That under favorable conditions, a fixed unicellular alga such as Chlorella can be comparable in Hill reaction efficiency to unfixed spinach chloroplasts over the intensity range used.

Optimal photosystem I activity in GA-fixed Anacystis was obtained using cells fixed for 30 min in 10% GA. Fig. 7 shows a characteristic photosystem I reaction performed by a suspension of GA-fixed Anacystis. Photosystem I reactions were observed in GA-fixed Chlorella and Porphyridium, but optimal fixation conditions were not investigated. Photosystem I reactions were also observed in FA-fixed Chlorella and Porphyridium.

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Literature Cited