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ISOLATION AND PROPERTIES OF CHLOROPLAST PARTICLES OF *SCENEDESMUS OBLIQUUS* D_3 WITH HIGH PHOTOCHEMICAL ACTIVITY

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SUMMARY

An improved and standardized procedure for isolation of chloroplast particles from the unicellular green alga, *Scenedesmus obliquus*, D_3, is described. The method is generally applicable to heterotrophically- and autotrophically-grown cells of *Scenedesmus* as well as to *Chlamydomonas reinhardti* and *Chlorella sorokiniana* (7-11-05) cultures. Chloroplast particles with high $NADP^+$ photoreducing capacity are obtained from heterotrophic cultures only when the cell types are random and the culture is in the logarithmic growth phase; maximal rates of 240–260 μmoles $NADP^+$ reduced/h per mg chlorophyll are achieved. Optimal conditions for separation of such chloroplast particles require the use of Tricine buffer (20 mM, pH 7.5), 50 nM EDTA, 10 mM KCl and 0.5 mM dithiothreitol in the breaking medium; for the maintenance of high photochemical activity it is necessary to store particles in a solution consisting of 0.4 M sucrose, 30 mM KCl and 1% bovine serum albumin.

Optimum reaction conditions were developed and the properties of the isolated particles investigated. Maximal activities are obtained when the sucrose concentration is maintained below 0.4 M; the pH optimum with Tricine buffer is between 7.8–8.1; and at least 30 mM Cl^- is required. Red actinic light (wavelength >620 nm) with an intensity of 10^6 ergs/cm^2 per s is required for saturation.

Ferrodoxin and ferredoxin–$NADP^+$ oxidoreductase are lost from the particles during the preparatory procedures and maximum photochemical activity is attained only when they are added back in balanced amounts. Stimulatory effects of added plastocyanin and cytochrome *c*-553 are noted only with particles having an initially low photochemical activity.

INTRODUCTION

Since the original detection of Hill reaction activity by isolated chloroplasts there has been a continuing quest for chloroplast preparation possessing photochemical activities equivalent to those of the intact leaf. The more recent advancement in this type of research has been the attainment of isolated chloroplasts possessing both the soluble substances of the stroma and an intact outer chloroplast membrane[1,2]. With the introduction of mutant strains of unicellular algae for research on the

mechanisms of photosynthesis[3–6], potentially new possibilities have become available to study single events in O_2 evolution, electron transport, ion transport, photophosphorylation, CO_2 fixation, chloroplast ultrastructure, and so on. For our studies on mutants of *Scenedesmus* it became necessary to isolate active chloroplast particles in order to pursue some of the above mentioned tasks in more detail. Previously a variety of breaking methods and isolation media have been tried, but the rates of photochemical activities of the isolated particles were always much lower than those of spinach chloroplasts. Also, the variation in activities obtained in earlier studies[7–17] was extremely high; one suggested reason for this variability is that most unicellular algae have only a single chloroplast and it seemed impossible to rupture the cell without severely damaging the chloroplast. Although this remains a major problem in algal chloroplast studies, we have found that other conditions such as conditions of growth, are very important in determining the resulting activity of the isolated chloroplast fragments.

In this report an optimized method is described for obtaining chloroplast particles from *Scenedesmus* and other unicellular algae with reproducible high rates of $NADP^+$ photoreduction with H_2O as the electron donor. Various factors influencing the activity of these particles are presented.

MATERIALS AND METHODS

Chemicals

All chemicals employed were of reagent grade. Trizma base [tris(hydroxymethyl)aminomethane] was purchased from Sigma Chemical Corp.; Tricine [(*N*-tris(hydroxylmethyl)glycine] from General Biochemicals (Chargrin Falls, Ohio); dithiothreitol and $NADP^+$ from Calbiochem; bovine serum albumin from Sigma (No. A-4378); and EDTA, as the disodium salt, from Matheson, Coleman and Bell.

Plant material

Scenedesmus obliquus strain D_3 was grown either autotrophically, myxotrophically or heterotrophically as previously described[18,19]. *Chlamydomonas reinhardti* was grown autotrophically in modified Bristol's medium and *Chlorella sorokiniana* (7-11-05) was cultured heterotrophically in the modified medium used for *Scenedesmus* culture[19]. Spinach was purchased at the local market. The packed cell volumes of algal cultures were determined as previously described[14].

Preparation of cofactors and analytical methods

Ferredoxin, plastocyanin and cytochrome *c*-553 were prepared from cells of *Scenedesmus* according to the method of Powls *et al.*[16]. These factors were further purified by preparative electrophoresis on polyacrylamide gel (Bishop, N. I. and Wong, J., unpublished). Concentrations of cofactor solutions were determined by employing the following millimolar extinction coefficients: cytochrome *c*-553, $20 \cdot 10^3$ cm^2/mmole at 553 nm[21]; plastocyanin, $9.8 \cdot 10^3$ cm^2/mmole at 597 nm[20]; and ferredoxin, $9.68 \cdot 10^3$ cm^2/mmole at 420 nm[22]. The ferredoxin was free of diaphorase activity.

Ferredoxin–$NADP^+$ oxidoreductase was isolated from *Scenedesmus* as described[16] or from spinach leaves following the method of Shin *et al.*[23]. The concentration of the flavoprotein was assayed by means of its NADPH-dependent dia-

phorase activity[24]. During the assay the NADPH level was kept constant by an excess of glucose 6-phosphate and a sufficient amount of glucose-6-phosphate dehydrogenase (Type VI or VII, Sigma Chem. Co.). The reduction of dichlorophenolindophenol was measured at pH 7.6 and 25 °C by recording the change in absorbance at 610 nm with a Zeiss PMQ II spectrophotometer. One enzyme unit was defined as the amount of protein which gave a $\Delta A_{640\ nm}$ of 1.0 per min under these conditions. Protein concentrations were calculated from the absorbance at 280 and 260 nm using the correction factors of Warburg and Christian[25] and Layne[26].

Isolation of spinach chloroplasts

Spinach leaves were washed, kept overnight at 4 °C in the dark, and then deveined and cut into s mallpieces. 50 g were blended for 20 s at 2 °C and top speed in a Waring blender with 150 ml ice-cold breaking solution containing 0.5 M sucrose, 0.03 M KCl and 0.05 M Tris–HCl buffer, pH 7.8. The brei was strained through 8 layers of cheesecloth and debris was removed by centrifugation for 2 min at $320 \times g$. Chloroplasts were obtained by centrifugation of the supernatant of the last step for 8 min at $750 \times g$. The pellet was gently homogenized with 20 ml ice-cold sucrose–KCl–Tris medium (which contained in addition 1% bovine serum albumin) with a glass rod tipped with cotton; care was taken not to disturb the starch layer. The chloroplasts were sedimented for 8 min at $1450 \times g$, homogenized gently in 2 ml sucrose–KCl–Tris–albumin, and any heavy particles removed by 2 min centrifugation at $270 \times g$. If chloroplast particles were to be used immediately, they were kept in the dark and at 0 °C; otherwise, they were stored at −30 °C.

Measurement of $NADP^+$ photoreduction

The reaction mixtures employed for $NADP^+$ photoreduction are given with the figures and tables for individual experiments. The absorbance at 340 nm was followed with a spectrophotometer modified for illumination of the sample cuvette from the top; the reaction mixture was continuously stirred during the course of the measurement. The temperature in the cuvette chamber was maintained at 28 °C; all samples were preincubated in the dark for 10 min at this temperature. No precautions were taken to prevent contact of the reaction mixture with air. Routinely the change in absorbance at 340 nm was recorded for 2 min in darkness, 2 min in light, and 2 additional minutes in darkness. If the rate of $NADP^+$ photoreduction was measured at more than one light intensity, it was routinely done with the same sample by alternating 2 min dark, 1 min actinic light, 1 min dark periods, starting with the lowest light intensity used. The coefficient used to convert the $\Delta A_{340\ nm}$ into μmoles of $NADP^+$ reduced was 0.2029 (ref. 28).

The light beam of a 150 W Xenon arc lamp (PEK Labs, Sunnyvale, Calif.) was directed through a 1-cm diameter hole in the lid of the cuvette chamber onto the reaction cell. The actinic beam was filtered by a 2-cm water bath and by infrared and red Corning glass filters (No. 2412). Incident-light intensity was measured at the surface of the reaction mixture with a small surface bolometer (Model 65 Radiometer, Yellow Springs Instrument Co.); maximum intensity of the red actinic light was $1.7 \cdot 10^6$ ergs/cm^2 per s. Neutral density filters were used to vary the light intensity. A Corning glass filter (No. 9863) covered the entry port of the photomultiplier housing to protect the phototube from stray actinic light.

RESULTS AND DISCUSSION

Optimum conditions for heterotrophic growth of Scenedesmus

During the heterotrophic growth of *Scenedesmus* factors such as temperature, composition of the growth medium, degree of aeration during growth, the initial size of the inoculum and the stage of development of the cells prior to subculturing are all factors which we have found to influence the resulting activity of isolated chloroplast particles. If these conditions are not observed the designated age of a culture is meaningless in terms of the stage of development of the algal culture. Kok and Datko[7] ascribe the variability of their results to the lack of special attention to the growth conditions.

We found that chloroplast particles with high photochemical activity could be isolated from heterotrophically-grown *Scenedesmus* cells only if the culture was (1) random and (2) in the logarithmic phase of growth; this finding is in agreement with previous ones[7–9,12,15,29]. Cultures of *Scenedesmus* remained in the logarithmic growth phase and were random if subcultured every second day by transferring aseptically an aliquot of the suspension containing 50 μl packed cell volume of cells into 250 ml of fresh medium. A random culture was obtained only after three consecutive transfers of a culture started initially from a slant. Microscopic examination showed that under these conditions most cells divided into 8 autospores and all sizes of cells were present in a culture at any one time. A typical growth curve for *Scenedesmus* is presented in Fig. 1. Additional single values from 11 other cultures are shown in this figure; it is apparent that growth curves of individual cultures, when maintained under the described conditions, are nearly identical.

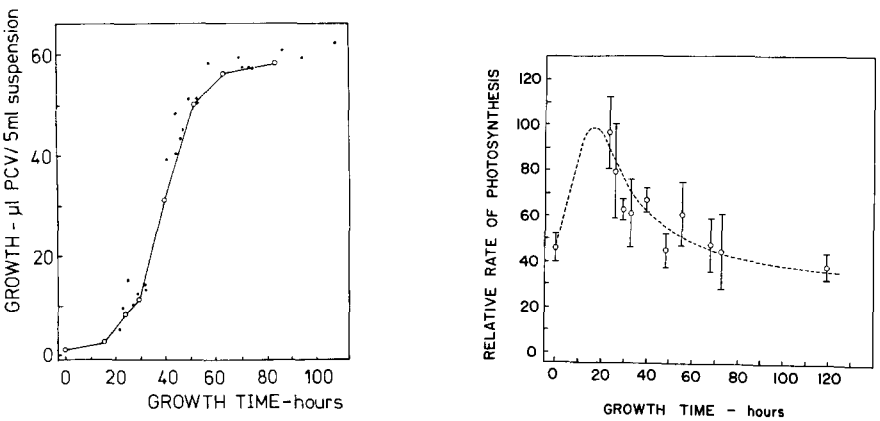


Fig. 1. Typical growth curve of a heterotrophic culture of *Scenedesmus obliquus* D_3. The culture was initiated with an inoculum (50 μl packed cell volume) from a 2-day-old random culture. Growth conditions: temperature, 29 °C; growth medium, NGY (250 ml)[19]. PCV = packed cell volume.

Fig. 2. Photosynthetic capacity (oxygen evolution) of *Scenedesmus obliquus* D_3 following different times of growth after inoculation. Rates determined polarographically with 20 μl packed cell volume per 2 ml. Buffer, 0.1 M Warburg's carbonate–bicarbonate No. 9; temperature, 25 °C; red-light intensity, 10^6 ergs/cm^2 per s. See legend of Fig. 1 and Materials and Methods for additional reaction conditions.

The photosynthetic capacity of an optimized culture of *Scenedesmus* was followed from shortly after the time of inoculation until 5 days of growth (Fig. 2). For this measurement a suspension with a constant packed cell volume was used; according to Senger and Bishop[30], this would allow that the amount of cells and chlorophyll were constant in at least the first 2 days of growth. As is shown in Fig. 2, the photosynthetic capacity of cells of a culture, which had been inoculated with cells from a 2-day-old culture, increased the first day after subculturing but steadily declined thereafter.

Isolation of chloroplast particles from unicellular algae

Chloroplast particles of unicellular algae have been isolated by grinding with sand[29]; by shaking at high frequency with glass beads[7,14]; by freezing and thawing plus rupturing with polyethylene beads in a Waring blender[8]; by sudden drop in pressure in a French press[12]; and by preincubation in hypertonic medium and sonication[17]. Pratt and Bishop (unpublished results) found that shaking algal cell with glass beads in a Vibrogen Zellmühle yielded chloroplast particles with good photochemical activity.

With modification of the method described by Pratt and Bishop[14], optimal activities were achieved if the chloroplast particles were isolated according to the following procedure: an algal culture containing 1–2.5 ml packed cell volume per 250 ml was harvested by centrifugation (5 min at $4000 \times g$) at the late phase of logarithmic growth. The supernatant was discarded and the white top layer of the pellet, which consisted primarily of cell wall residues of divided cells, was rinsed off with distilled water. The pellet was resuspended in 75 ml of breaking solution and washed on the centrifuge (5 min at $3000 \times g$). The supernatant was discarded, any remaining cell residue again rinsed off and the pellet transferred into a 50-ml stainless steel cup of a Vibrogen Zellmühle (Tübingen, Germany) with breaking solution; the total volume of the suspension was adjusted to 19.5 ml. The composition of breaking solution, which was prepared fresh daily, was 20 mM in Tricine–KOH, pH 7.5, 10 mM in KCl, 0.05 mM in EDTA and 0.5 mM in dithiothreitol. Acid-washed technical glass beads of 0.35-mm diameter were added until about 5 mm below the rim of the cup, the mixture stirred and glass beads and/or breaking solution added within 2 mm of the rim. At this point the mixture had a thick consistency, but should still be stirrable. The mixture was precooled for 10 min with ice water and shaken at top speed for 5 min. The glass beads were separated from the broken cells by filtering through a coarse fritted glass funnel into a suction flask and washed 3–4 times with a total of 50 ml of rinsing solution; care was taken to avoid foaming. The rinsing solution contained 20 mM Tricine–KOH buffer, pH 7.5, 10 mM KCl and 0.56 M sucrose to bring the final concentration of sucrose to 0.4 M.

The filtrate was centrifuged for 5 min at $600 \times g$ and the supernatant poured into centrifuge tubes without disturbing the loose light green top layer of the pellet. The supernatant then was centrifuged for 15 min at $27000 \times g$. The pellet from this step showed three layers: pale green, white, and dark green. Only the top layer of the pellet was resuspended in 0.8–1.2 ml of storage solution with a cotton-tipped stirring rod. This solution contained 0.4 M sucrose, 30 mM KCl and 1% (w/v) bovine serum albumin[31,32]. The activity of the chloroplast particles usually was low if this pellet was contractile and sticky. The suspension was centrifuged again (5 min at $620 \times g$)

to remove starch, cell residues and unbroken cells, carefully transferred to another tube, mixed briefly on a Vortex mixer and stored in the dark in an ice bath.

All centrifugations were carried out in a refrigerated Sorvall centrifuge at 0–2 °C; all other steps were performed in an ice bath under dim light. All solutions and glassware, except the glass beads, were maintained in an ice bath.

The elapsed time between breaking of the cells and first possible test was about 1.5 h. For immediate use the suspension was diluted to give a final concentration of 250 μg chlorophyll per ml with a solution containing 0.4 M sucrose, 30 mM KCl, 20 mM Tricine–KOH, pH 7.8, and 1% albumin. Otherwise, the particles were frozen and stored at —30 °C without dilution.

Addition of 10 mM KCl to the breaking medium improved the activity of the isolated chloroplast particles by about 10%; higher KCl levels (30 mM) were often inhibitory. Kok and Datko[7] showed that the addition of EDTA increased the activity of *Scenedesmus* particles if added after grinding. The possible influence of EDTA during breaking of the cells in our system was tested at concentrations from 0.01 to 0.3 mM. 0.05 mM gave the highest stimulation (about 10%); EDTA does not uncouple photophosphorylation at this concentration[33].

The addition of a reducing agent during breakage increased the activity of the chloroplast particles; mercaptoethanol[8,14], glutathione[29], and dithiothreitol were also tested. Dithiothreitol was the most effective of these reagents; maximum stimulation of 60% occurred at 0.5 mM; its effect was examined in a range from 0.2 to 5 mM. In comparison, glutathione and mercaptoethanol caused a maximal stimulation of 35% and 20%, respectively. Ascorbate was not tested.

Varying the pH from 7.0 to 7.5 caused little increase in activity (less than 10%), but 20 mM Tricine–KOH buffer, pH 7.5, yielded 20% higher activity than 20 mM phosphate buffer, pH 7.5. It was also noted that the isolated chloroplast particles had much higher activity (90%) if the sucrose was added immediately after shaking instead of during as was previously noted[14].

Increasing the shaking time caused enhanced yields, but lower activity (15–20%). Decreasing the bead concentration or increasing the air space above the algal–bead suspension gave similar results.

Special care was taken not to include starch granules or residues of broken cells into the final suspension of isolated chloroplast particles since both produce undesirable optical qualities to the algal-chloroplast suspension. If there were unbroken cells in the suspension the activity in $NADP^+$ photoreduction per mg chlorophyll was lowered.

Properties of isolated Scenedesmus chloroplast particles

(a) Initial and steady-state rates of $NADP^+$ photoreduction

The rates of $NADP^+$ photoreduction reported here were obtained from a linear extrapolation of the change in absorbance at 340 nm. Any non-linearity of such tracings usually resulted either from a poor distribution of light through the cuvette, or the establishment of gradients of NADPH in non-stirred assay mixtures. Besides these considerations, it was observed that after some accumulation of NADPH a decrease of absorbance at 340 nm occurred in a following dark period. It was not investigated whether this back reaction was due to the oxidation of NADPH[34] or swelling of the particles[35,36] or other causes. The rate of this back reaction was

dependent upon the concentration of NADPH, ferredoxin, ferredoxin–$NADP^+$ oxidoreductase and cytochrome *c*-553. If the average rate of $NADP^+$ photoreduction was corrected for the dark back-reaction, the steady-state rate, over a 2-min period, was as high as the intial rate (Fig. 3). Somewhat similar results have been obtained with *Euglena* chloroplast particles[13], but at pH 6.5 rather than at pH 8.

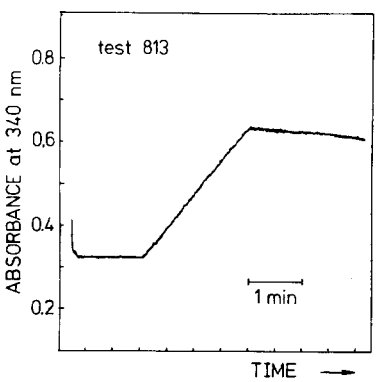
(*b*) *Chlorophyll concentration and light intensity*

The rate of $NADP^+$ photoreduction showed a linear dependency upon the amount of particles[13] in the reaction mixture up to 40 μg chlorophyll per 3 ml. The specific activity decreased with higher density of the reaction mixture, *e.g.* if 86 μg chlorophyll per 3 ml were used instead of 11 μg per 3 ml the rate was 25% less. Consequently, particles sufficient to provide 25 μg chlorophyll per 3 ml were routinely used.

It was found that 10^6 ergs/cm^2 per s actinic red light (at the top surface of the cuvette) often was not saturating for active particles. Extrapolated values of *V* for $NADP^+$ photoreduction could not be achieved by increasing the light intensity because of the resulting photoinactivation (Fig. 6).

(*c*) *Dependency of $NADP^+$ photoreduction on non-proteinaceous substances in the reaction mixture, buffer and pH optimum*

K_2HPO_4–KH_2PO_4, Tris–HCl and Tricine–KOH were tested as buffer in the reaction mixture. At pH 7.5 the chloroplast particles had the same activity in 17 mM Tris–HCl buffer and in 17 mM phosphate buffer; at pH 7.8, however, phosphate buffer was better than Tris–HCl. It might be that some inactivation of Photosystem II (ref. 37) in the Tris–HCl buffer occurred during the preincubation of the particles. Finally, most measurements were done in Tricine–KOH buffer at a pH of 7.8–8.0.



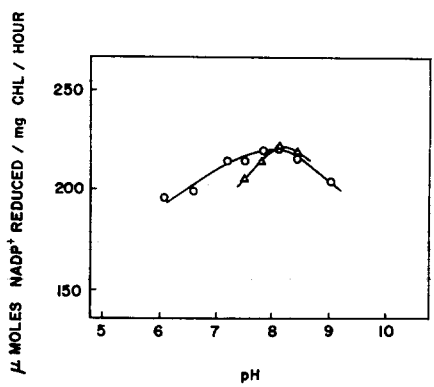


Fig. 3. A typical recorder tracing showing the light-induced reduction of $NADP^+$ by chloroplast particles isolated from a random heterotrophic culture of *Scenedesmus obliquus* D_3. The reaction mixture contained in a total of 3 ml (in μmoles): sucrose, 400; Tricine–KOH buffer (pH 7.8), 50; Cl^-, 57; NH_4^+, 3; $NADP^+$, 1. Also included were 143 μg *Scenedesmus* ferredoxin, 12 units of ferredoxin–$NAPD^+$ oxidoreductase, and 1 mg bovine serum albumin. Chlorophyll concentration, 25 μg.

Fig. 4. Effect of pH on $NADP^+$ photoreduction with chloroplast particles isolated from two separate heterotrophic cultures of *Scenedesmus*. Reaction conditions were the same as indicated in legend of Fig. 3 except for the addition of 107 μmoles Cl^-. Experiments for curves (○—○) and (△—△) contained 100 and 130 μg of ferredoxin, respectively.

Fig. 4 shows the dependency of the rate of $NADP^+$ photoreduction with isolated *Scenedesmus* chloroplast particles on pH.

Sucrose concentration. The particles did not exhibit optimum activity if the sucrose concentration in the reaction mixture was 0.4 M. Higher rates were obtained when the sucrose concentration in the assay medium was decreased (Table I). Under

TABLE I

DEPENDENCY OF $NADP^+$ PHOTOREDUCTION ON SUCROSE CONCENTRATION

The reaction mixture contained in a total volume of 3 ml in μmoles: sucrose as indicated; $MgCl_2$, 6; KCl, 33; phosphate buffer, pH 7.8, 50; 80 μg ferredoxin; $NADP^+$ oxidoreductase (with 20 diaphorase units from *Scenedesmus*); $NADP^+$, 1; and particles equivalent to 30 μg chlorophyll. Temperature, 26 °C, light intensity, 10^6 ergs/cm^2 per s red light. Growth and breakage conditions and Cl^- concentration were not optimal yet.

Sucrose concn (M)	*μmoles $NADP^+$ reduced/mg chlorophyll per h*
0.013	87
0.046	92
0.094	100
0.180	105
0.260	95

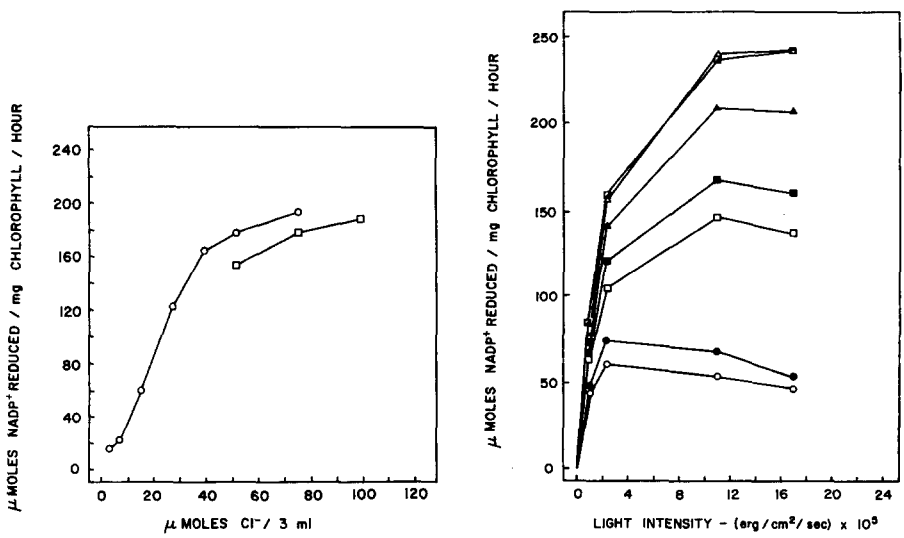


Fig. 5. Cl^- stimulation of the rate of $NADP^+$ photoreduction (H_2O as electron donor) by *Scenedesmus* chloroplast particles. Reaction conditions were identical to those indicated in the legend of Fig. 3. Points (□—□) were obtained with a different chloroplast particle preparation.

Fig. 6. Effect of Cl^- on the light inactivation of $NADP^+$ photoreduction by *Scenedesmus* chloroplast particles. The reaction conditions were the same as indicated in the legend of Fig. 3 except for the use of 138 μg *Scenedesmus* ferredoxin, 500 μmoles sucrose and 50 μmoles Tricine-KOH, pH 8.1. Cl^- concentrations (μmoles/3 ml) were as follows: ○—○, 7; ●—●, 11; □—□, 19; ■—■, 32; △—△, 57; ▲—▲, 107; diagonally filled, squares 157.

these conditions probably the chloroplast particles swell during preincubation. In later experiments, 400 μmoles sucrose were added per 3 ml, unless otherwise indicated; 0.1 ml particles suspension of 0.4 M sucrose contributed another 40 μmoles.

Effects of NH_4Cl, KCl and $MgCl_2$. The photoreduction of $NADP^+$ by *Scenedesmus* chloroplast fragments is completely dependent upon the presence of Cl^- ions in the reaction mixture (Fig. 5). In a series of experiments the protective action of Cl^- against light inactivation was examined. From the curves of Fig. 6 it is apparent that 30 μM Cl^- is sufficient to prevent the photoinactivation observed at the lower concentration. Under the experimental conditions employed for this experiment an approximate 5-fold stimulation of the light-saturated rates of $NADP^+$ was obtained due to the addition of Cl^-. To evaluate if the effect of Cl^- on *Scenedesmus* chloroplast particles is similar to that noted for Hill reaction activity of spinach chloroplasts, the light-intensity dependency of the rate of $NADP^+$ photoreduction was examined according to the method of Lumry and Spikes[38]. Replotting of the data of Fig. 6 by their method revealed that only the intercept of this type of plot is influenced by the addition of Cl^-, *i.e.* only the rate-limiting dark reaction is stimulated; identical effects were obtained with KCl, $MgCl_2$ and $CaCl_2$. The presence of NH_4^+ (3 μmoles/3 ml) in the reaction mixture did not alter the observed effect of Cl^- although an additional 20–30% stimulation was noted. Hind *et al.*[39] found a somewhat similar response of the Hill reaction of spinach chloroplast except that Cl^-, in the presence of NH_4^+, caused an increase also in the rate-limiting light reaction.

(d) Dependency of $NADP^+$ photoreduction on proteinaceous cofactors

Ferredoxin. In the absence of added ferredoxin *Scenedesmus* chloroplast particles show only limited rates of $NADP^+$ photoreduction. Ferredoxin preparations from *Scenedesmus* and spinach were fully interchangeable in restoring $NADP^+$ photoreduction to chloroplast particles obtained from *Scenedesmus*, *Chlamydomonas* and spinach. A double-reciprocal plot of the rate *versus* ferredoxin concentration showed that different ferredoxin preparations gave the same V when tested with the same particles, but the same ferredoxin gave different V if examined with particles from different sources. When $NADP^+$ photoreduction was measured at different

TABLE II

RATE OF $NADP^+$ PHOTOREDUCTION AT DIFFERENT FERREDOXIN CONCENTRATIONS

Reaction mixture and assay conditions as in Fig. 3.

Ferredoxin added (μg)	*μmoles $NADP^+$ reduced/mg chlorophyll per h*	
	Light intensity (ergs/cm² per s): $5 \cdot 10^4$	$1 \cdot 10^6$
0	30	34
15	47	51
29	74	76
50	76	190
95	78	228

TABLE III

RATE OF $NADP^+$ PHOTOREDUCTION AT DIFFERENT FERREDOXIN AND FERREDOXIN–$NADP^+$ OXIDOREDUCTASE CONCENTRATIONS

Chloroplast particles with 25 μg chlorophyll isolated from two separate cultures of autotrophically-grown, synchronized *Scenedesmus* cells (8th h). (For experimental conditions see legend of Fig. 3 and Material and Methods.)

Concn of spinach $NADP^+$ oxidoreductase (diaphorase units)	*Ferredoxin (Scenedesmus) (μg)*	*μmoles $NADP^+$ reduced/mg chlorophyll per h*
0	124	42
12	0	34
12	124	283
12	186	274
		585*
20	95	224
20	143	257
		585*

* Calculated V for ferredoxin $\rightarrow \infty$.

concentrations of ferredoxin and different light intensities, it was seen that the most pronounced effect of ferredoxin was in the light-saturating region (Table II).

Both ferredoxin and the ferredoxin–$NADP^+$ oxidoreductase had to be titrated to obtain maximum rates of $NADP^+$ photoreduction. It is known[40], and was found in many of our experiments, that optimal amounts of ferredoxin were not saturating, *i.e.* an extrapolated V could never be achieved by increasing the ferredoxin concentration (Table III). In its reaction with ferredoxin the flavin enzyme has to compete with O_2 and cyclic electron transport. Therefore a simultaneous increase in the concentration of $NADP^+$ oxidoreductase often gave higher activity.

Ferredoxin–$NADP^+$ oxidoreductase. Addition of ferredoxin–$NADP^+$ oxidoreductase stimulated the rate of $NADP^+$ photoreduction with all particles tested. The activity of isolated chloroplast particles from spinach could be improved up to 60% by addition of 12 diaphorase units. Ferredoxin–$NADP^+$ oxidoreductase usually needed not to be added for $NADP^+$ photoreduction experiments with freshly prepared spinach chloroplasts, and isolated chloroplast particles from *Anacystis nidulans* retain sufficient amounts of the bound reductase[41]. The *Scenedesmus* chloroplast particles, however, showed a high requirement for readdition of the reductase. In a typical experiment stimulation of 360% was obtained by addition of 12 diaphorase units; but for particles with initially high activity, even this amount was not saturating. The lower rates in $NADP^+$ photoreduction with isolated chloroplasts from algae reported so far, seem to be caused, at least in part, by loss of the reductase enzyme during the isolation of the particles. In Fig. 7 three typical test series are shown: the difference in the rate in $NADP^+$ photoreduction with 12 diaphorase units may be due to suboptimal conditions in Curves 1 and 2 rather than to a true difference in the capacity for $NADP^+$ photoreduction of *Scenedesmus* particles obtained from cells

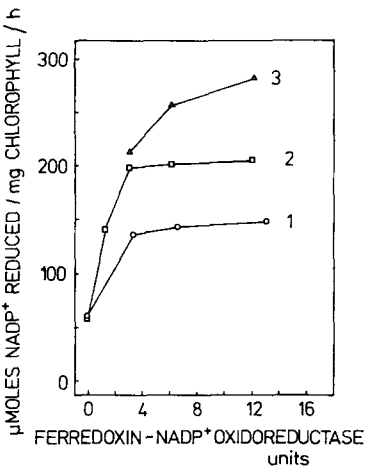


Fig. 7. Concentrations dependency of ferredoxin–$NADP^+$ oxidoreductase on rates of $NADP^+$ photoreduction by *Scenedesmus* chloroplast particles. Reaction conditions were the same as indicated in Fig. 3 with the following exceptions: Curve 1, 58 µg ferredoxin, temperature, 25 °C, chloroplast isolated from non-optimized culture; Curve 2, 124 µg ferredoxin and 100 µmoles Cl^- present, chloroplast particles isolated from non-optimized culture; Curve 3, 124 µg ferredoxin present, chloroplast particles isolated from synchronous autotrophic culture, (8th h). The calculated V, at infinitely high concentrations of the reductase, were 160, 228 and 330, respectively.

TABLE IV

COMPARISON OF STIMULATION IN $NADP^+$ PHOTOREDUCTION BY ADDED SOLUBLE PLASTOCYANIN AND CYTOCHROME *c*-553 IN CHLOROPLAST PARTICLES FROM HETEROTROPHICALLY-GROWN *SCENEDESMUS*

Expt a. In total of 3 ml: 50 mmoles phosphate buffer, pH 7.5; 54 µmoles Cl^-; 400 µmoles sucrose; 87 µg ferredoxin (*Scenedesmus*) $NADP^+$ oxidoreductase with 20 diaphorase units (*Scenedesmus*); temperature, 25 °C; 10^6 ergs/cm² per s red light. Addition of NH_4^+ was without effect. Expt b. 50 µmoles Tricine–KOH buffer, pH 7.8; 107 µmoles Cl^-; 400 µmoles sucrose; 3 µmoles NH_4^+; 60 µg ferredoxin (*Scenedesmus*) and $NADP^+$ oxidoreductase with 12 diaphorase units from spinach; temperature, 28 °C.

Expt	*Plastocyanin (Scenedesmus) (nmoles/3 ml)*	*Cytochrome c-553 (Scenedesmus) (nmoles/3 ml)*	*nmoles $NADP^+$ reduced per mg chlorophyll per h*
a	—	—	36.5
	50	—	57
	—	30	66
	50	30	73
	100	60	92
b	—	—	197
	50	—	219
	—	30	204

grown heterotrophically or autotrophically. In tests for Curve 1 the ferredoxin level was not optimal, and in tests for Curve 2 the age of the culture was 3.5 days.

Like ferredoxin, the ferredoxin–$NADP^+$ oxidoreductase from *Scenedesmus* and spinach were fully interchangeable.

Plastocyanin and cytochrome c-553. In contrast to ferredoxin and ferredoxin–$NADP^+$ oxidoreductase, addition of soluble cytochrome *c*-553 (from *Scenedesmus*) did not stimulate particles with high activity and addition of 50 nmoles external[42] plastocyanin (from *Scenedesmus*) gave only 10–15% stimulation in $NADP^+$ photoreduction. Greater enhancement by both plastocyanin and cytochrome *c*-553 was observed with chloroplast particles with initially low activities (Table IV). Clearly the particles with high photochemical activity have retained their original plastocyanin and cytochrome *c*-553 in amounts sufficient to be saturating.

(*e*) *Storage of the isolated particles*

Any prolonged storage of isolated chloroplast particles at pH 7.8 caused a marked decreased in activity. Also, in non-buffered storage solution (0.4 M in sucrose, 30 mM in KCl and 1% (w/v) in bovine serum albumin) retention at −30 °C did not give consistent and reproducible activities. Addition of glycerol[31] and storage at lower temperature were not tested. Practically constant activity was maintained over a 6-h period, even at pH 8.1 (Tricine–KOH), when the chloroplast particles were stored without freezing at 0 °C in the presence of 30 mM Cl^- and when the particles were preincubated for 10 min and the $NADP^+$ photoreduction was measured in a medium containing 50 mM Cl^-.

In an experiment done at lower Cl^- concentration in the reaction mixture (19 mM) the activity of particles, which were prepared from autotrophically-grown synchronous cells (8th h), appeared to have decreased 85% within 6 h. Even after storage overnight only a slight loss of activity had occurred, when the particles were kept at 0 °C in the dark in sucrose, albumin and 30 mM Cl^-, but without buffer. This loss could be overcome by high Cl^- concentrations in the reaction mixture. Thus Cl^- not only stimulates a rate-limiting dark reaction (compare Figs 5 and 6), and protect against photoinactivation (Fig. 6), but also restores a loss of activity in $NADP^+$ photoreduction during storage for periods up to 30 h.

Comparison of the Hill reaction activity of chloroplast particles from different sources

Data obtained from spinach chloroplasts were included in Table V to show the activity levels observed under our assay conditions. For these particles a sucrose concentration of 13 mM was found to be better than 133 mM. The ferredoxin and the ferredoxin–$NADP^+$ oxidoreductase from *Scenedesmus* and spinach were interchangeable as was previously noted for *Scenedesmus* particles. Our isolation procedure was also applied to heterotrophically-grown *Chlorella* and autotrophically-grown *Chlamydomonas* cultures. As shown in Table V the activity of the *Chlorella* particles was lower than that of either *Chlamydomonas*, *Scenedesmus* or spinach chloroplasts. It should be emphasized that neither the growth conditions nor the assay conditions were optimized for this alga. The described isolation procedure, however, as optimized for *Scenedesmus*, seems to be applicable to studies on chloroplasts of other unicellular algae.

Concluding remarks

The optimized method of isolation and assay offers the possibility to investigate

TABLE V

ACTIVITY IN $NADP^+$ PHOTOREDUCTION OF DIFFERENT CHLOROPLAST PARTICLES

In all tests 1 mM NH_4^+ was present in the reaction mixture. Sucrose concentration was 400 mmoles per 3 ml in all tests except for spinach (40 mmoles per 3 ml). $NADP^+$ oxidoreductase isolated from spinach chloroplasts was used in all experiments except for the first experiment with spinach chloroplasts. *V* determinations for saturating ferredoxin concentrations ($fd \rightarrow \infty$) and light intensity ($erg \rightarrow \infty$) were obtained by graphical extrapolations[38]. Sc, *Scenedesmus*; Sp, spinach.

Organism and growth conditions	*Test No.*	*μmoles $NADP^+$ reduced/mg chlorophyll per h*			*Assay conditions*		
		Measured	*V* $fd \rightarrow \infty$	*V* $erg \rightarrow \infty$	*Cl^- (μmoles/ 3 ml)*	*Ferredoxin (μg)*	*$NADP^+$ oxido-reductase (units)*
Scenedesmus							
Heterotrophic	815	247			57	143 (Sc)	12
	815–18		266		57	var. (Sc)	12
	955	276		395	107	124 (Sc)	13
Myxotrophic*, ***	380	168			57	58 (Sc)	9
Autotrophic	768	154			57	58 (Sc)	19
	771–4		292		57	var. (Sc)	19
Synchronous	794–96	258			57	143 (Sc)	19
(8th h)	809	263		310	57	143 (Sc)	19
	874–79	283		365	57	124 (Sc)	12
	794–6		585		57	var. (Sc)	19
	874–9		585		57	var. (Sc)	12
Synchronous	828	242			57	143 (Sc)	12
(16th h)	828–30		405		57	var. (Sc)	12
	884	356		417	57	124 (Sc)	12
Chlamydomonas							
Autotrophic*, **	640	242			57	300 (Sp)	24
	633–40		336		47	var. (Sp)	19
Chlorella							
Heterotroph*, **, ***	571	112			57	87 (Sc)	12
Spinach	258	270			19	87 (Sc)	9
	747	210			34	143 (Sc)	24
	749–51		340		34	var. (Sc)	24

* Influence of growth conditions not checked and/or growth probably not optimal.

** Optimum conditions for assay mixture not checked, in particular Cl^- requirement and pH optimum.

*** Isolation procedure not optimized.

partial reactions of electron transport and/or photophosphorylation with isolated algal chloroplast particles. Applying artificial electron donor and acceptor systems it may be feasible to investigate the causes for changes in the capacity of Photosystem I and Photosystem II and/or the regulation of non-cyclic and cyclic photophosphorylation.

It should be possible to localize the block(s) in the electron transport chain in various photosynthetic mutant strains of algae and, also, apparent changes in photochemical activity during the synchronous life of various unicellular algae.

NOTE

During the preparation of the manuscript Vol. 23 of *Methods in Enzymology* appeared. No algal chloroplast particles are described with activities in $NADP^+$ photoreduction as high as our particles after optimizing all conditions (see ref. 43). On the other hand reasonable rates in photophosphorylation with algal chloroplast particles have been achieved (see ref. 44). The photophosphorylation capacity of the *Scenedesmus* chloroplast particles have not been examined but because of the observed stimulatory action of NH_4^+ on electron transport, it would seem that these particles are well coupled.

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REFERENCES

1 Cockburn, W., Walker, D. A. and Baldry, C. W. (1968) *Plant Physiol.* 43, 1415–1418
2 Ridley, S. M. and Leech, R. M. (1969) in *Progress in Photosynthesis Research* (Metzner, H., ed.), Vol. I, pp. 229–244, Laupp Jr, Tübingen
3 Levine, R. P. (1963) in *Photosynthetic Mechanisms in Green Plants, Natl. Acad. Sci. – Natl. Res. Council, Publ.* 1145, 158–173
4 Bishop, N. I. (1964) *Record Chem. Progr.* 25, 181–195
5 Russel, G. K. and Lyman, H. (1967) *Plant Physiol.* 43, 1284–1290
6 Wild, A. (1969) in *Progress in Photosynthesis Research* (Metzner, H., ed.), Vol. 2, pp. 871–876, Laupp, Jr., Tübingen
7 Kok, B. and Datko, E. A. (1965) *Plant Physiol.* 40, 1171–1177
8 Kahn, J. S. (1966) *Biochem. Biophys. Res. Commun.* 24, 329–333
9 Levine, R. P. and Gorman, D. S. (1966) *Plant Physiol.* 41, 1293–1300
10 Gorman, D. S. and Levine, R. P. (1967) *Plant Physiol.* 41, 1648–1656
11 Givan, A. L. and Levine, R. P. (1967) *Plant Physiol.* 42, 1264–1268
12 Katoh, S. and San Pietro, A. (1967) *Arch. Biochem. Biophys.* 118, 488–496.
13 Katoh, S. and San Pietro, A. (1968) *Arch. Biochem. Biophys.* 128, 378–386.
14 Pratt, L. H. and Bishop, N. I. (1968) *Biochim. Biophys. Acta* 153, 664–674
15 Cheniae, G. M. and Martin, I. F. (1968) *Biochim. Biophys. Acta* 153, 819–837
16 Powls, R., Wong, J. and Bishop, N. I. (1969) *Biochim. Biophys. Acta* 180, 490–499
17 Boschetti, A. and Grob, E. G. (1969) in *Progress in Photosynthesis Research* (Metzner, H., ed.), Vol. 1, pp. 245–249, Laupp, Jr, Tübingen
18 Bishop, N. I. and Senger, H. (1971) in *Methods in Enzymology* (San Pietro, A., ed.), Vol. 23, Part A, pp. 53–66, Academic Press, New York
19 Kessler, E., Arthur, W. and Brugger, J. E. (1957) *Arch. Biochem. Biophys.* 71, 326–335
20 Katoh, S., Suga, I., Shiratori, I. and Takamiya, A. (1961) *Arch. Biochem. Biophys.* 94, 136–141

21 Gorman, D. S. and Levine, R. P. (1966) *Plant Physiol.* 41, 1643–1647
22 Tagawa, K. and Arnon, D. I. (1968) *Biochim. Biophys. Acta* 153, 602–613
23 Shin, M., Tagawa, K. and Arnon, D. I. (1963) *Biochem. Z.* 338, 84–96
24 Jagendorf, A. T. (1963) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. 6, pp. 430–434, Academic Press, New York.
25 Warburg, O. and Christian, W. (1941) *Biochem. Z.* 310, 384–447.
26 Layne, E. (1957) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. 3, pp. 449–454, Academic Press, New York
27 Holden, M. (1956) in *Chemistry and Biochemistry of Plant Pigments* (Goodwin, T. W., ed.), pp. 461–488, Academic Press, New York
28 Bergmeyer, H. U. (1963) *Methods in Enzymatic Analysis*, pp. 1030–1031, Academic Press, New York
29 Gorman, D. S. and Levine, R. P. (1965) *Proc. Natl. Acad. Sci. U. S.* 54, 1665–1669
30 Senger, H. and Bishop, N. I. (1969) in *Progress in Photosynthesis Research* (Metzner, H., ed.), Vol. 1, pp. 425–434, Laupp. Jr, Tübingen
31 Wassermann, A. R. and Gleischer, S. (1968) *Biochim. Biophys. Acta* 153, 154–169
32 Friedlander, M. and Newmann, J. (1968) *Plant Physiol.* 43, 1249–1254
33 McCarty, R. E. (1966) *Brookhaven Symp. Biol.* 19, 202–214.
34 Lazzarini, R. A. and San Pietro, A. (1962) *Biochim. Biophys. Acta* 62, 417–420
35 Packer, L. and Crofts, Ar. R. (1967) in *Current Topics in Bioenergetics* (Sanadi, D. R., ed.), Vol. 2, pp. 23–64, Academic Press, New York
36 Hildenheger, H. and Menke, W. (1965) *Z. Naturforsch.* 20b, 699–701.
37 Yamashita, T. and Butler, W. L. (1969) in *Progress in Photosynthesis Research* (Metzner, H., ed.), Vol. 3, pp. 1236–1240, Laupp, Jr, Tübingen
38 Lumry, R. and Spikes, J. D. (1957) in *Research in Photosynthesis* (Gaffron, H., ed.), pp. 373–391, Interscience, New York
39 Hind, G., Heath, R. L. and Izawa, S. (1969) in *Progress in Photosynthesis Research* (Metzner, H., ed.), Vol. 2, pp. 1022–1026, Laupp, Jr, Tübingen
40 Neuman, J. and Nelson, N. (1969) in *Progress in Photosynthesis Research* (Metzner, H., ed.), Vol. 3, pp. 1476–1482, Laupp, Jr, Tübingen
41 Bothe, H. and Berzborn, R. J. (1970) *Z. Naturforsch.* 25b, 529–533
42 Hauska, G., McCartry, R. E., Berzborn, R. J. and Racker, E. (1971) *J. Biol. Chem.* 246, 3524–3531
43 Graham, D. and Smillie, R. (1971) *Methods in Enzymology* (San Pietro, A., ed.), Vol. 23, Part A, p. 228, Academic Press, New York
44 Böger, P. (1971) *Methods in Enzymology* (San Pietro, A., ed.), Vol. 23, Part A, p. 242, Academic Press, New York