

Chloroplast Integrity and Biochemical Function^{1, 2}

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The experiments to be presented here are directly concerned with the importance of chloroplast integrity and function in photoreduction and photophosphorylation. In an earlier communication (10), we were able to show both by biochemical criteria involving CO₂ fixation as well as direct electron microscope observations that the isolation of chloroplasts in 0.35 M NaCl is noxious to the structure and function of the chloroplast. Not only is the outer membrane destroyed, but stromal ground substance is lost and the grana are disrupted and swollen. Less than 20 % of the grana remain in an intact configuration. Concomitant with this structural degradation is the loss of ability to incorporate C¹⁴O₂ into carbohydrates and other products of photosynthesis. When chloroplasts are isolated avoiding the use of salts and extensive dilution, a preparation is obtained in which the outer membrane is ruptured and ground substance lost, but the grana appear to be intact. It is this semi-intact preparation that has been utilized as starting material in the experiments presented here.

The photoreduction of NADP⁺ is now known to proceed through the mediation of a so-called System 1 (13,19) in which electrons are taken from the level of chlorophyll to reduce ferredoxin and thence by way of a flavoprotein enzyme to the pyridine nucleotides (21). The recent work of Becker et al. (7) has been concerned with the consecutive degradation by sonication and osmotic shock of spinach chloroplasts and with their ability to carry out the photoreduction of ferricyanide. They were able to obtain fractions, centrifuging between 15,000 and 20,000 × *g*, which were extremely active, of even higher specific activity than the quantasomes isolated by Park and Pon (17,18). For optimal activity Park and Pon were required to add back supernatant fraction to their particles.

The classical experiments of Vishniac and Ochoa (25) which first demonstrated the ability of chloroplast preparations to photoreduce pyridine nucleotide led to the discovery by San Pietro and Lang (20) that both the chloroplasts or chloroplast fragments and soluble protein were necessary to carry out the overall photoreduction of pyridine nucleotide. It is important to emphasize the very labile interrelationship that exists between the soluble fraction and the chloroplast fragments. Recently Gressel and

Avron (11) have prepared chloroplasts from swiss chard by a salt fractionation procedure, and have used both saponification and enzymatic techniques to disrupt them. Their measurements of photoreduction as a function of disintegration used ferricyanide and 2,6-dichlorophenolindophenol (DPIP) reduction. They did not measure pyridine nucleotide reduction.

The first reports of photophosphorylation in a chloroplast preparation by Arnon and his collaborators (2) used an isolation technique which we now recognize yields only broken chloroplasts and disrupted grana. In these preparations, photophosphorylation was stimulated by the addition of various dyes which served as electron carriers in the cyclic phosphorylation pathway. Subsequently, the investigations of Park and Pon (18) disclosed that rather vigorous disruption of these chloroplast preparations led to the formation of the quantasome unit able to carry out very low levels of photophosphorylation. Gressel and Avron (11), using a variety of degradative techniques, were able to show that the incorporation of inorganic phosphate in the light was extremely sensitive to both pancreatic lipase and phospholipase as well as sonication. We emphasize again the fact that their starting material was already a partially degraded chloroplast prepared in salt.

We have now examined the photoreduction and photophosphorylation of our semi-intact chloroplasts prepared in the absence of salt and dilution. We will demonstrate that these chloroplasts have approximately 10 times the endogenous photophosphorylating activity of chloroplasts reported heretofore in the literature (5,9). They are also quite active in their ability to carry out pyridine nucleotide reduction, achieving maximal rates when supplemented with exogenous photosynthetic pyridine nucleotide reductase (PPNR). The ability of these preparations to maintain such high endogenous rates is directly dependent on their structural integrity.

Materials and Methods

Preparation of Chloroplasts. The method of preparation is essentially that described in our earlier communication (10). One hundred g of spinach (*Spinacea oleracea*) were harvested from the Earhart Greenhouse at the California Institute of Technology. Petioles and midribs were removed, the leaves washed several times in distilled water, placed in a polyethylene bag and chilled to 0° prior to grinding. Twenty ml of a buffer containing Tris, 0.1 M, pH 7.5; glutathione (GSH), 10⁻⁴ M; ethylene diamine tetraacetate (EDTA), 10⁻⁴ M; and MgCl₂, 10⁻³ M were added to each 100 g of spinach to be ground. Grinding in the absence of sand was carried

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out in a cold room using mortar and pestle prechilled to 0°. The extract was filtered through 8 layers of cheesecloth previously moistened in buffer. This filtrate was then centrifuged at $300 \times g$ in a Sorvell refrigerated centrifuge to remove cell debris and whole cells. The supernatant remaining was centrifuged at $1500 \times g$ for 7 minutes. The pellet so obtained was used as the chloroplast preparation in the experiments to follow. In some experiments, when ascorbate was added to the grinding medium, its final concentration was 0.05 M.

The chloroplast pellet was suspended in 20 ml of 0.05 M Tris, pH 7.5, containing 2×10^{-3} M $MgCl_2$, 5×10^{-3} M EDTA, and 5×10^{-5} M GSH. In the ascorbate experiments the concentration of that compound was 2×10^{-2} M in the suspending buffer. It should be noted that there was considerable variability in the biochemical activity of these preparations which seems to be a function of the growing conditions of the plants.

Sonic disruption of the chloroplast fraction was carried out using a Heat Systems sonicator equipped with a rosette cell. The sonicator was operated at 20 kc and 110 w. The rosette cell was cooled using a salt-ice bath at -5° . At no time did the temperature in the cell rise above 4° during sonication of the chloroplast preparation. The times of sonication are those indicated on the graph.

Chlorophyll concentrations were measured as described by Arnon (1). Suitable dilutions were made so that the final concentration of the chlorophyll in the preparation was 250 μg per 2.5 ml for experiments of photophosphorylation and 100 μg per 2.5 ml in photoreduction experiments.

Measurement of Photoreduction of NADP. The method employed is essentially that described by San Pietro (20, 21). A solution containing NADP⁺, 2 μ moles; P_i, 10 μ moles; and ADP, 1 μ mole was added to 2.5 ml of the buffered chloroplast suspension containing 100 μg of chlorophyll. The final volume was 3.0 ml. In those experiments where ascorbate was tested, it was added in the amount of 50 μ moles per 3.0 ml. When PPNR was assayed in the system, 60 μg of this material were added. The reactions were carried out in 30 ml screw top glass vials. For the dark experiments, the bottles were carefully covered with aluminum foil and black tape. The reaction was maintained at pH 7.4, the temperature 20° in a Dubnoff shaking incubator. The bottles were 2 cm below the surface of the water bath in order to absorb some of the heat. Light was provided by 2 Sylvania Sun-Guns Model 11. The measured illumination (Weston meter 756) falling upon the vials was 4000 ft-c. The concentration of NADPH was determined directly with a Gilford modification of a Beckman DU spectrophotometer using light of 340 m μ . The dark control vial was used to zero the instrument. All values are then relative to the dark control.

Measurement of Photophosphorylation. The concentration of chlorophyll in the chloroplast prepara-

tion used for photophosphorylation was adjusted to 250 μg per 2.5 ml of buffer solution. To this chloroplast suspension was added 10 μ moles of potassium phosphate, pH 7.0, containing approximately 3 μc of P-32, as well as 1 μ mole of adenosine diphosphate (ADP). The final volume was adjusted to 3.0 ml. Various electron carriers and cofactors were added at the following concentrations when tested: phenazene methosulfate (PMS), 0.1 μ mole; 2,6-dichlorophenolindolphenol (DPIP), 0.1 μ mole; PPNR, 60 μg ; NADP⁺, 2 μ moles; and $K_3Fe(CN)_6$, 5.0 μ moles. The reactions were carried out under the light and temperature conditions described for photoreduction. The reactions were stopped with 0.5 ml of 20% trichloroacetic acid (TCA). Precipitate was removed by centrifugation and the supernatant decanted and saved. The precipitate was washed twice, the first time with 1.5 ml of 10% TCA, the second with 1.0 ml of ion-free water, and all 3 supernatants combined. The final volume of the supernatants was approximately 6.0 ml.

The determination of phosphate incorporated in the organic fraction was carried out in the same fashion as described by Avron (4). A 0.3-ml aliquot of the combined supernatants and 1.2 ml of acetone were placed in an 8×180 mm test tube and chilled in ice. 1.2 ml of ion-free water was added which had previously been saturated at 0° with a 1:1 mixture of isobutanol-benzene. Following this, 7.0 ml of 1:1 isobutanol benzene mixture previously equilibrated with water was added. To this chilled 2 phase system was added 0.8 ml of a 4.0 M sulfuric acid solution containing 5% by weight ammonium molybdate. The mixture was allowed to stand for 5 minutes. The extraction of the inorganic phosphomolybdate was carried out on a Vortex mixer for 15 to 20 seconds at high speed. The organic phase was removed by suction and discarded. Another 7.0 ml of butanol-benzene mixture was added as well as 20 μl of 10^{-2} M potassium phosphate. The latter was added to assure the carrier removal of radioactive inorganic phosphate. The mixture was allowed to stand again for 5 minutes and was shaken and extracted as above. Suitable aliquots of the aqueous phase, 100 μl each, were removed and the radioactivity of the P-32 determined by the liquid scintillation methods as described in our earlier communication (10). For all conditions, both a zero time control as well as a dark control were run to be certain of respiratory phosphate incorporation as well as any artifacts that might be encountered.

Results

The Effect of Exogenous Electron Carriers and Sonication on NADPH Formation. The ability of several electron carriers, particularly DPIP, ascorbate, and PPNR, to enhance endogenous level of pyridine nucleotide reduction is shown in figure 1. The addition of PPNR to the chloroplast preparation caused a 3-fold increase in the rate of reduction.

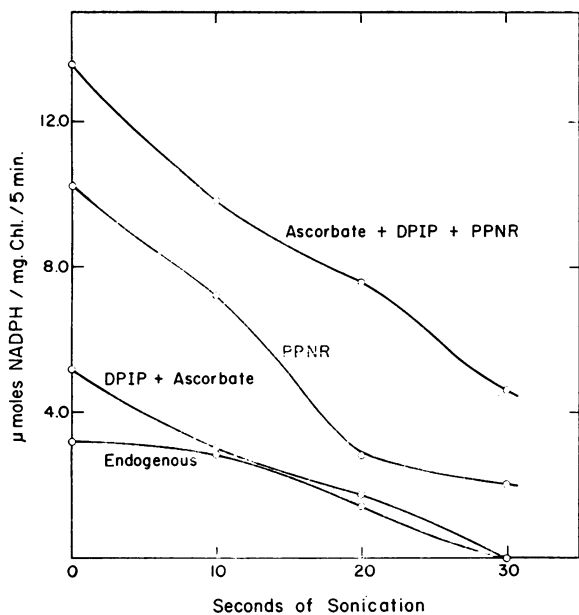


FIG. 1. The effect of sonication on photoreduction. The reaction contained chloroplasts, 100 μg chlorophyll; Tris, 125 μmoles , pH 7.5; MgCl_2 , 7.5 μmoles ; GSH, 0.1 μmole ; EDTA, 0.1 μmole ; ADP, 1.0 μmole ; P_i , 10 μmoles ; and NADP, 2.0 μmoles in a final volume of 3.0 ml. The respective concentrations of cofactors added are: ascorbate, 50 μmoles ; DPIP, 0.1 μmole ; and PPNR, 60 μg . The duration of illumination was 5 minutes at 20° and 4000 ft-c.

This was to be expected and has been seen before by many other investigators (20, 21). The addition of DPIP and ascorbate to provide an additional source of electrons to the system (22) did, indeed, enhance the rate somewhat. In the presence of both the electron donating system and PPNR, optimal rates were obtained. The disruption of the chloroplasts by sonication caused a rapid inhibition in the rate of reduction and led ultimately to a complete loss of activity at 30 seconds. DPIP and ascorbate did little to protect the chloroplast. However, when exogenous PPNR was added, the rates were maintained at an appreciable level. The addition of ascorbate, DPIP and PPNR restored activity to the disrupted chloroplasts from the sonic treatment.

The results of our first experiments in which ascorbate was added and shown to be an effective donor of electrons to the photoreduction system led us to examine the preparation of chloroplasts in the presence of ascorbic acid throughout the isolation procedure. The results from such experiments are presented in figure 2. There was a striking increase in the rate of pyridine nucleotide reduction when PPNR was added to these ascorbate prepared chloroplasts. The rate was about twice that observed in experiments of figure 1. Furthermore there was pronounced protection of photoreduction from sonic disruption in these chloroplasts.

The Kinetics of Phosphate Esterification. The rate of phosphate esterified per mg chlorophyll as a function of time of incubation in the light is presented in figure 3. Within 15 minutes of exposure to light, the chloroplasts lost all ability to esterify inorganic phosphate. We therefore chose as our assay time in the experiments to follow, a 5-minute period of illumination. A possible explanation for reaching an asymptote in the time course of phosphate esterifica-

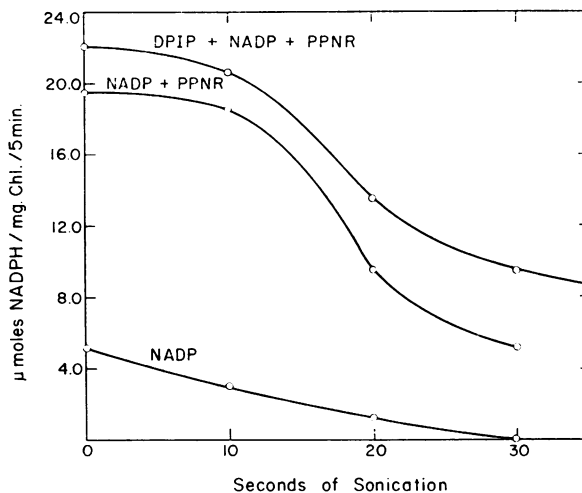


FIG. 2. The effect of sonication on the photoreduction of chloroplasts prepared in the presence of ascorbate. The reaction mixture, pH 7.5, contained chloroplasts, 100 μg chlorophyll; Tris, 125 μmoles ; ascorbate, 50 μmoles ; GSH, 0.1 μmole ; EDTA, 0.1 μmole ; MgCl_2 , 7.5 μmoles ; NADP⁺, 2.0 μmoles ; ADP, 1.0 μmole ; and P_i , 10 μmoles in a final volume of 3.0 ml. The respective concentrations of cofactors, when added were DPIP, 0.1 μmole , PPNR, 60 μg , and the duration of illumination was 5 minutes at 20° and 4000 ft-c.

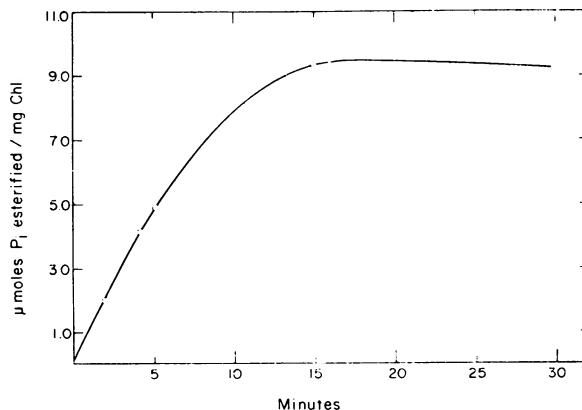


FIG. 3. Time course of photophosphorylation. The reaction mixture, pH 7.5, contained chloroplasts, 250 μg chlorophyll; Tris, 125 μmoles ; EDTA, 0.1 μmole ; GSH, 0.1 μmole ; MgCl_2 , 7.5 μmoles ; ADP, 1.0 μmole , and P_i , 10 μmoles (containing 3 μC P^{32}) in a total volume of 3.0 ml. The reaction was run at 20° and 4000 ft-c.

tion would be the depletion of ADP acceptor to permit the reaction to continue. We therefore carried out a time course analysis for a series of initial ADP concentrations which is shown in figure 4. There is an endogenous phosphate acceptor present. At relatively low levels of ADP, 0.5 μ mole, the reaction is rapid and attains essentially maximal rate. When the ADP is raised to 5.0 μ moles, there appears to be substrate inhibition.

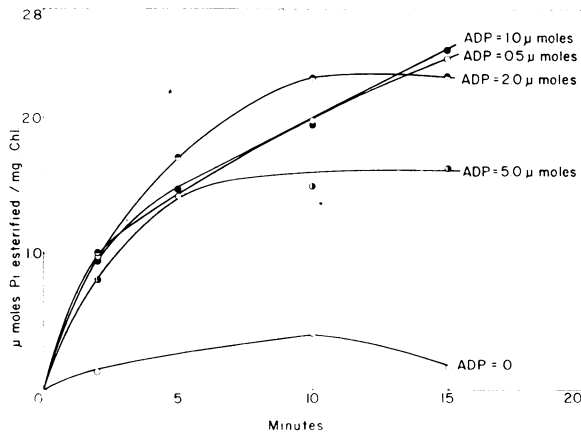


FIG. 4. Time course of photophosphorylation as a function of initial ADP concentration. Conditions were the same as those used for experiments presented in figure 3.

As an alternate technique for providing ADP acceptor, an exogenous hexokinase trap was tested in the system by adding 15 μ g per vial of purified yeast hexokinase (Sigma Chemical Company) and 10^{-3} M glucose. The reaction was carried out using the optimal concentration of ADP, 1.0 μ mole per vial. These results are presented in figure 5 along with the time course of phosphate esterification in the presence of other co-factors to stimulate electron transport and phosphorylation. It is clear that the addition of the hexokinase and glucose did not alter the kinetics of phosphate esterification. Furthermore, the capacity of the endogenous phosphorylating system is far greater than that supplied exogenously. With PMS added to stimulate cyclic phosphorylation there is an 8-fold increase in the absolute amount of phosphate esterified in 10 minutes. The general shape of the time course for all reactions studied is similar in the sense that by 10 minutes the system ceases function. With ferricyanide and PMS there is a marked inhibition observed after about 15 or 20 minutes, possibly caused by hydrolysis of ATP or phosphate esters.

We have isolated the 80% ethanol soluble fraction from chloroplasts which had been allowed to photophosphorylate in the presence of P-32. This extract was subjected to high voltage electrophoresis. The major fraction of radioactivity was associated with the sugar phosphates and diphosphates. Radioactive ATP was also identified.

Effect of Various Co-factors on Photophosphorylation. The initial rates of photophosphorylation for a variety of conditions is shown in table I. Of particular importance was the very high rate of photophosphorylation observed in the absence of any added co-factors other than inorganic phosphate and the ADP accep-

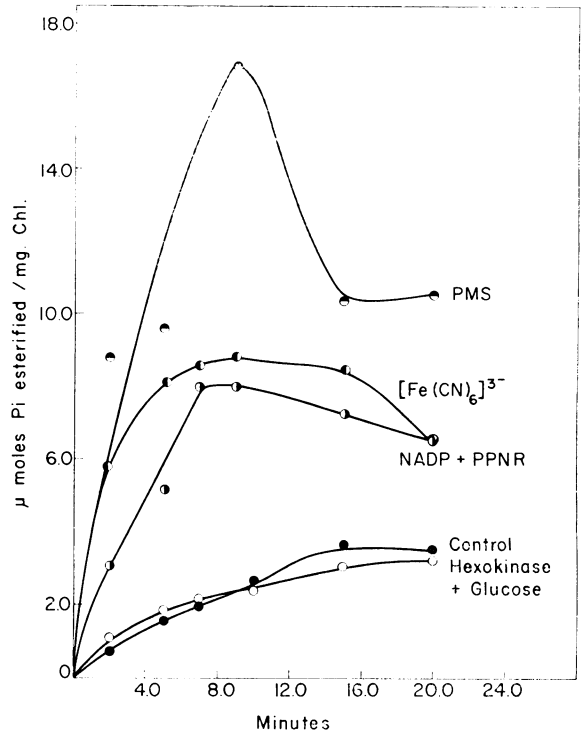


FIG. 5. Time course of photophosphorylation in the presence of exogenous hexokinase plus glucose and cofactors to stimulate electron transport. Conditions for control are those indicated for figure 3. Concentrations of indicated constituents were: hexokinase, 15 μ g; glucose, 10^{-3} M; NADP⁺, 8.0 μ moles; PPNR, 60 μ g; [Fe(CN)₆]³⁻, 8 μ moles; PMS, 0.1 μ mole.

Table I. Rates of Photophosphorylation with Various Cofactors

The reaction mixture contained chloroplasts, 250 μ g chlorophyll; Tris, 125 μ moles; EDTA, 0.1 μ mole; GSH, 0.1 μ mole; MgCl₂, 7.5 μ moles; ADP, 1.0 μ mole; Pi, 10 μ moles (containing 3 μ c P³²). When cofactors were added PMS was 0.1 μ moles, NADP, 2.0 μ moles, ascorbate, 50 μ moles, DPIP, 0.1 μ mole, K₃Fe(CN)₆, 5.0 μ moles, and PPNR, 60 μ g. Reactions were carried out in a final volume of 3.0 ml for 5 minutes at 20° and 4000 ft-c.

Conditions	μ moles P _i esterified mg chl hr
ADP + P _i	62
ADP + P _i + Asc	110
ADP + P _i + TPN + PPNR	178
ADP + P _i + TPN	95
ADP + P _i + FMN	230
ADP + P _i + PMS	346
ADP + P _i + K ₃ Fe(CN) ₆	174

tor. This rate was about 6 to 10 times that reported for other chloroplast preparations (5, 9). When ascorbate was added as a potential donor of electrons, a 2-fold increase of phosphorylation was seen. The flow of electrons could also be stimulated by the addition of PPNR and NADP^+ as had been shown above, and a concomitant increase in phosphorylation was noted. That this effect was not due to the pyridine nucleotide alone was clear from the observations made in the absence of PPNR where the rate was only slightly enhanced.

We then determined the stoichiometry and concentration dependence of phosphate esterification as a function of NADP^+ . This is shown in figure 6, where the net increase in phosphate, i.e. the amount esterified with NADP^+ minus the control, is plotted. Between 0 and 2.0 μmoles of NADP^+ per assay vial, there is a linear relationship of phosphate esterified with NADP^+ . Calculation of the $\text{P}/2\text{e}$ is 1 in this region. The coupling between electron transport and phosphorylation appears extremely tight. At concentrations higher than 2 μmoles of NADP^+ , there is no increase in phosphate esterification. The addition of FMN, an electron carrier which stimulates pseudocyclic phosphorylation (23), and of PMS, an electron carrier which is known to act in the cyclic pathway

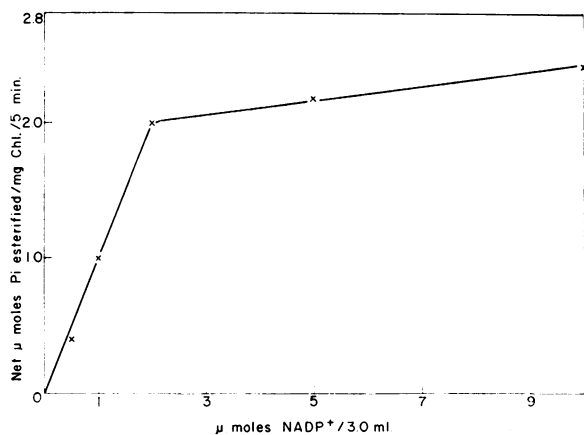


FIG. 6. Net photophosphorylation as a function of NADP^+ concentration. Values are reported as the difference between observed phosphate esterified in the presence of NADP^+ minus that in its absence. Conditions were those described in figure 3 except for NADP^+ concentration.

(3, 23), caused very large increases in phosphorylation. Ferricyanide is also a potent stimulator of phosphate esterification (6). If we plot the net phosphate esterified, i.e. the total phosphate esterified in the presence of ferricyanide minus the endogenous rate in its absence, as a function of ferricyanide concentration, we obtain the data of figure 7. It is seen that there is no net increase in phosphate esterified up to 0.5 μmole of ferricyanide in the assay mixture. This is caused by the reaction of ferricyanide with reducing agents such as GSH and other factors within the chloroplast produced during the illumina-

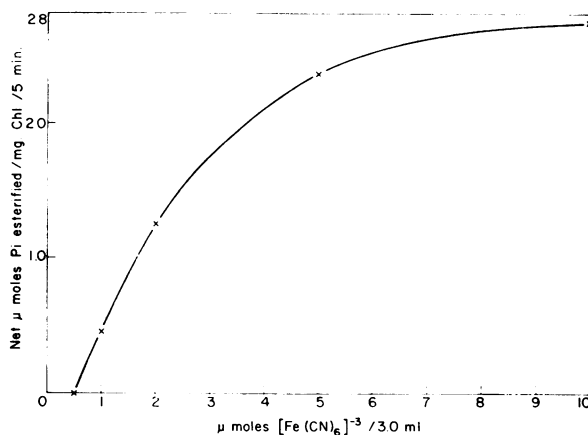


FIG. 7. Net photophosphorylation as a function of $[\text{Fe}(\text{CN})_6]^{3-}$. Values reported are phosphate esterified in the presence of $[\text{Fe}(\text{CN})_6]^{3-}$ minus that observed in the absence of this anion. Conditions were those described in figure 3 except for added $[\text{Fe}(\text{CN})_6]^{3-}$.

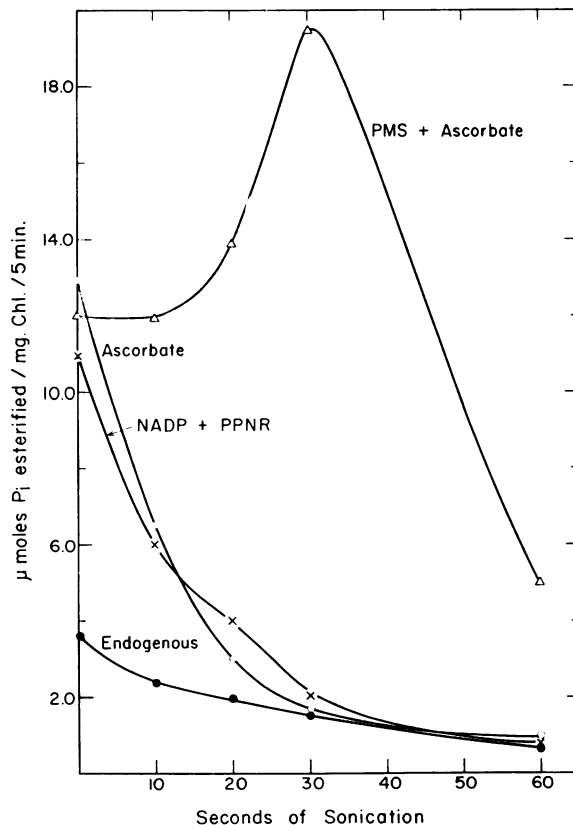


FIG. 8. Effect of sonication on photophosphorylation. Reaction mixtures contained Tris, 125 μmoles ; pH 7.5; EDTA, 0.1 μmole ; GSH, 0.1 μmole ; MgCl_2 , 7.5 μmoles ; ADP, 1.0 μmole ; P_i , 10 μmoles containing $\sim 3 \mu\text{C}$ P^{32} . When cofactors were added, ascorbate was 50 μmoles ; NADP^+ , 2.0 μmoles ; PMS, 0.1 μmole ; PPNR, 60 μg . The reactions were run in a final volume of 3 ml for 5 minutes at 20° and 4000 ft-c.

tion. However, between 2.5 and 5 μ moles of ferricyanide, the P/2e ratio is 1. This is further indication that these semi-intact systems are very tightly coupled.

Effect of Disruption of Chloroplasts on Rates of Photophosphorylation. Experiments presented above for photoreduction showed that sonication destroyed the integrated ability of the chloroplast to carry out NADPH synthesis. Similar results were obtained when we studied photophosphorylation. When chloroplasts were sonicated for periods as long as 60 seconds, it is seen, figure 8, that the endogenous rate of photophosphorylation fell slightly more than 50%. The addition of ascorbate and NADP⁺ plus PPNR stimulated photophosphorylation by the semi-intact chloroplast. However, this enhancement was rapidly lost upon sonication and after 30 seconds fell to the level of the endogenous rate. When PMS and ascorbate were added, quite a different effect was observed. After 20 seconds of sonication, there was a significant stimulation of photophosphorylation of the

cyclic variety which reached a maximum at 30 seconds and then quickly dropped. Even at 60 seconds, however, these disrupted chloroplasts were quite active in their ability to carry out photophosphorylation in the presence of PMS and ascorbate.

An objection could be made to the severity of sonication in the disintegration procedure. We therefore carried out an additional series of experiments where we allowed the chloroplasts, as initially prepared, to stand at 20° for increasing periods prior to assaying for photophosphorylation. The results of such mild disruption are presented in figure 9. Although the time scale was extended, the results were in basic agreement with those observed using sonication.

Discussion

Integrity of chloroplast structure and biochemical function should be considered from 2 points of view: the photoreduction of pyridine nucleotide and photophosphorylation. It is quite clear from the results presented here, and as have been described by many investigators (12, 20, 21), that a major fraction of the PPNR system is lost from the chloroplast where it most probably is located in situ. The ability of exogenous PPNR to increase the endogenous rate of photoreduction by a factor of at least 3 is evidence for this. There also appears to be a limited flow of electrons through the chlorophyll system responsible for the photoreduction of NADPH. The addition of ascorbate to a chloroplast preparation as a potential electron donor in the pathway of photoreduction causes a slight increase in the NADPH formed. If the chloroplasts are prepared in the presence of ascorbate, throughout the course of their isolation, the rate is enhanced as has been demonstrated by Whatley et al. (24). But now the addition of PPNR causes approximately a 5-fold increase in rate. We believe that these experiments point to an involvement of ascorbate at at least 2 levels. It is possible that this compound serves to prevent destruction of important intermediates in electron transport as well as to serve as a direct electron donor to the system. Ascorbate also protects the chloroplast from sonic degradation, possibly by protecting against noxious peroxides that are formed during the process of sonication (24).

The integrity of the chloroplast is also directly linked to its ability to photophosphorylate. This is most strikingly shown by the endogenous rates of phosphate esterification obtained with our preparation. As indicated above, it is approximately 10 times that observed for other techniques of preparation utilizing high salt, extensive dilution, or sucrose. That these high rates are truly a function of chloroplast integrity can be seen by comparison of their structure as revealed in the electron microscope (10). The observation that the thylakoids are swollen or ruptured and that lamellar structures have lost the dimensions that they subtend in the intact leaf is evidence to support causal involvement of structure and

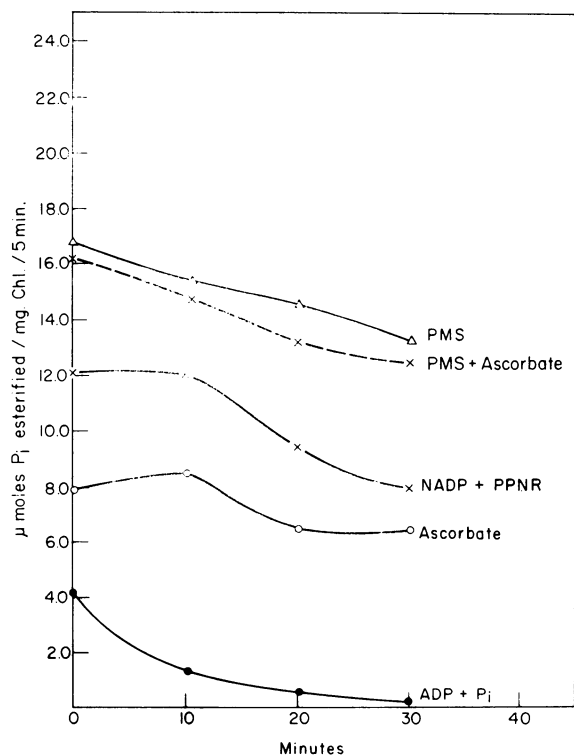


FIG. 9. Effect of aging chloroplasts by standing in buffer on photophosphorylation. The chloroplasts were suspended in 0.05 M Tris, pH 7.5, and allowed to stand at room temperature for the indicated times before the reaction. The reaction mixture contained Tris, 125 μ moles; EDTA, 0.1 μ mole; GSH, 0.1 μ mole; $MgCl_2$, 7.5 μ moles; ADP, 1.0 μ mole; and P_i , 10 μ moles containing 3 μ c P^{32} . When cofactors were added, PMS was 0.1 μ mole; NADP⁺, 2.0 μ moles; ascorbate, 50 μ moles; and PPNR, 60 μ g. Reactions were carried out in a final volume of 3.0 ml for 5 minutes at 20° and 4000 ft-c.

photophosphorylating activity. Chloroplasts isolated by our technique and allowed to stand at room temperature also rapidly lose the high initial endogenous rates. Even the maintenance of these preparations in the cold for periods of time up to 1 or 2 hours causes loss of 50 to 60 % of the original activity measured. The chloroplast appears to be a most sensitive subcellular organelle with which to work.

The presence of a very effective hexokinase-like trapping mechanism for high energy phosphate generated during photophosphorylation in this preparation may be an important consideration in the efficiency of chloroplast function. In the presence of 1.0 μ mole of ADP acceptor, the system is functioning near optimal levels. The addition of increased amounts of ADP or an exogenous hexokinase glucose trap is completely ineffective in stimulating photophosphorylation. The principal question raised by the experiments on the kinetics of photophosphorylation is that of what factor(s) may be involved in causing the photophosphorylation system to cease functioning. In some measure, PPNR must be implicated, but certainly it is not the critical system involved. The addition of this compound does not prolong the effective time of photophosphorylation in the preparation.

We can gain some insight into the biochemical effects of structural derangement. The addition of various compounds which are involved in electron transport immediately enhances the rate of photophosphorylation. Thus, it is seen that ascorbate, when added to chloroplasts prepared in the absence of these compounds, causes a 60 % increase in phosphate esterified. Further, preparation of chloroplasts with ascorbate present throughout their isolation causes a 2-fold increase in the rate of photophosphorylation. When NADP⁺ and PPNR are added, compounds which are known to be operative in the electron transport system, the rate is increased to more than twice the original endogenous level. That electron flow is important is evidenced by the fact that NADP⁺ alone is not as effective as it is in the presence of PPNR. When PMS is added to stimulate cyclic phosphorylation (3, 23), the rates are 5 to 6 times higher than the endogenous.

The effect of sonication on photophosphorylation reveals a rather rapid loss of activity as a function of time of sonication. Whereas the rate of photoreduction was completely destroyed after 30 seconds of sonication, the rate of phosphorylation remained about 50 % that of the semi-intact preparation. Further sonication for as long as 60 seconds did not abolish activity. There was a basal level of phosphorylation, approximately 30 % that of the original endogenous value. This indicates that electrons are still capable of flowing through the phosphorylation site, but are not able to reduce NADP⁺ because of rapid loss of PPNR activity. Even in the presence of ascorbate, NADP⁺ and PPNR, there was a rapid initial loss of phosphorylating ability, reaching the same 30 % level at 60 seconds of sonication. The

most striking effect of sonication is observed in the presence of ascorbate and PMS. The disruption of the structural integrity of the chloroplast leads to an enhanced ability of the PMS to interact with the cyclic phosphorylating system. The 50 % increase of rate observed in the presence of these 2 electron carriers is lost after 30 seconds of sonication. However, it is obvious that there is still a great enhancement of phosphorylating activity via the cyclic route.

These experiments also suggest the involvement of 2 sites for photophosphorylation: one on the cyclic pathway, the other on the noncyclic. The fact that we do not completely abolish phosphorylation even after long sonication would suggest a stable as well as an unstable phosphorylating site. The unique activation by PMS of the cyclic pathway which is not observed for electron carriers in the noncyclic pathway is also suggestive of 2 sites. Evidence from Baltscheffsky (8) and from Avron (6) supports such a conclusion. Their experiments on the effects of different inhibitors such as valinomycin were the first evidence that this was a likely possibility.

We believe that the experiments presented demonstrate the close relationship of structure and function of chloroplasts, not only in fixation of CO₂ and the pathways by which this comes about both in light and dark, but also in the processes of photoreduction and photophosphorylation. All attempts that we have made to isolate intact chloroplasts have been unsuccessful. There have been some reports (14, 15) that claim isolation of chloroplasts with their outer membrane intact and carrying their complete complement of enzymes. However no biochemical evidence for their integrity exists. Certainly the experiments of measuring swelling of isolated chloroplasts as a function of light (16) were a measure of changes in the volume of the grana or lamellar structures and not that of an intact chloroplast acting as an osmometer or contractile organelle. It is hoped that one or more techniques will be developed for isolation of truly intact chloroplasts. With such a preparation, we will then be able to learn more of the intricate relationships of enzyme distribution and substrate flux across subcellular membrane barriers.

Summary

Semi-intact chloroplasts have been isolated from spinach leaves which contain grana and lamellar structures in the same conformation as they appear in the intact leaf. However, their outer membrane and stromal ground substance has been lost. The abilities of these semi-intact chloroplasts to carry out the photoreduction of NADP⁺ and the photophosphorylation of ADP has been tested. The relationship of integrity of the grana structure to the chloroplasts' ability to carry out these 2 important intermediate reactions of photosynthesis was studied using sonic disruption as well as a more gentle aging procedure.

Photoreduction of NADP⁺ can be most markedly stimulated and protected from the effects of sonica-

tion by the addition of photosynthetic pyridine nucleotide reductase (PPNR) to the chloroplast. It appears that this enzyme is quickly lost from the chloroplast during its preparation and must be added back for optimal activity. The addition of exogenous sources of electrons also enhances photoreduction. Upon disruption by sonication, the ability to reduce NADP^+ is rapidly lost.

The rate at which phosphate can be esterified in the light in the absence of any added electron carriers or enzymes is 6 to 10 times greater in this semi-intact chloroplast than has been observed for other isolated chloroplast preparations under similar assay conditions. This rate can be stimulated by the addition of various cofactors involved in cyclic and non-cyclic photophosphorylation. Although disruption of the chloroplasts by sonication or aging causes a loss of phosphorylating ability, there is a residual low activity. Phenazinemetosulfate, a stimulator of the cyclic pathway, is unique in that after short periods of sonication its activity is enhanced. Evidence is presented to indicate that 2 independent sites for photophosphorylation may be operative.

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