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Is Nicotinamide Adenine Dinucleotide Phosphate an Obligatory Intermediate in Photosynthesis?

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ABSTRACT

The site of action of the inhibitors disalicylidenepropanediamine and pyrophosphate was more closely defined as acting on ferredoxin. Three inhibitors which act on the electron transport path between ferredoxin and NADP: disalicylidenepropanediamine, pyrophosphate, and phosphoadenosinediphosphate ribose, had no effect on photosynthesis in cell free preparations of *Dunaliela parva* at concentrations which completely inhibited the enzymic activity on which each inhibitor acts. The addition of disalicylidenepropanediamine to dark-grown *Euglena gracilis* cells prevented the light-induced formation of NADP-dependent glyceraldehyde-3-phosphate dehydrogenase, but not of photosynthesis, chlorophyll synthesis, or NAD-dependent glyceraldehyde-3-phosphate dehydrogenase.

The above results are interpreted as indicating that, at least under some conditions, a reduced product of photosystem I preceding ferredoxin in the electron transport path can serve as the reductant of CO_2 in photosynthesis.

In a previous communication (8) we described the photochemical activities of the halophilic alga *Dunaliela parva*. One of the unusual features of this alga was the inability of cell-free preparations to perform any ferredoxin-dependent reactions. This was true even for preparations which retained the ability to fix CO_2.

This communication will describe experiments designed to test, in view of the above observations, the requirement for the photoproduction of NADPH in photosynthesis. Two types of experiments will be described. In the first, the effect of inhibitors whose site of action was reported between photosystem I and NADP will be compared on NADP photoreduction and CO_2 fixation in cell-free preparations of *D. parva*. In the second, the light-dependent production of the NADP-dependent glyceraldehyde-3-phosphate dehydrogenase in dark-grown *Euglena gracilis*, which was previously shown to be closely correlated with the development of photosynthetic capability, will be shown to be inhibited under conditions which do not inhibit the development of photosynthesis.

MATERIALS AND METHODS

Dunaliella parva cells were grown as previously described (7). *Euglena gracilis* cells were grown in the dark and in the light in Hutner's medium (20). Chloroplasts were isolated from lettuce leaves as previously described (3).

D. parva "chloroplasts" were obtained by suspending the cells in Walker's medium (13). Chloroplast fragments were obtained by suspension of the cells in a reaction mixture containing a final concentration of 25 mM NaCl (8).

Ferredoxin, DCIP, and cytochrome *c* reductions were measured in an Aminco-Chance dual-wavelength spectrophotometer. The reference wavelength used was 540 nm, with a measuring wavelength of 497 nm for ferredoxin (4), 575 nm for DCIP, and 550 nm for cytochrome *c*.

NADP and NAD reductions or NADPH and NADH oxidations were measured by following the change of fluorescence of NADPH or NADH in an Eppendorf fluorimeter attached to a recorder and modified for illumination of the cuvette. The fluorescence exciting light was passed through the Eppendorf 313 + 366 nm filter. The photomultiplier was protected by a Corning filter 4-96 and a Strand Electric Co. cinemoid filter No. 62.

O_2 evolution was measured with a Yellow Springs Instrument Clark type oxygen electrode. Illumination in the above reactions was provided by a 500 w projector and filtered through a Baird-Atomic sharp cut-off interference filter peaking at 640 nm. Light intensity was about 4×10^4 ergs cm^{-2} sec^{-1}.

The incorporation of $^{14}CO_2$ was carried out at 22 C with 55,000 lux of white light. The reaction mixture contained: 0.33 M sorbitol, 50 mM Tricine pH 7.6, 1 mM $MgCl_2$, 1 mM MnCl, 5 mM $NaHCO_3$ containing 1 μc ^{14}C per sample and algae containing about 90 μg of chlorophyll in a total volume of 3 ml. The reaction was stopped by adding 0.5 ml 30% trichloroacetic acid. Aliquots were removed and dried on planchets. The ^{14}C content of the acid stable compounds was then determined with a Nuclear Chicago gas-flow counter.

Preparation of extracts from *Euglena* to determine NADP- or NAD-dependent glyceraldehyde-3-P dehydrogenase was essentially as described by Hudock and Fuller (19). *Euglena* cells were grown in the dark for several days. The culture was diluted 2-fold in a regular heterotrophic medium plus or minus 0.1 mM DSPD[1] and transferred to light. Samples were taken at daily intervals and assayed as previously described (19).

NADP- and NAD-dependent glyceraldehyde-3-P dehydrogenases were assayed by the decrease of fluorescence of NADPH and NADH as described by Müller (26). P-ADPR was prepared as previously described (9). Chlorophyll was assayed following the method of Arnon (1).

RESULTS

Effect of Inhibitors. DSPD was previously shown to inhibit the photoreduction of NADP and other ferredoxin-dependent reactions (30). Since no inhibition of nitrite reduction was ob-

[1] Abbreviations: DSPD: disalicylidenepropanediamine; sulfo-DSPD: disulfodisalicylidenepropanediamine; P-ADPR: phosphoadenosinediphosphateribose.

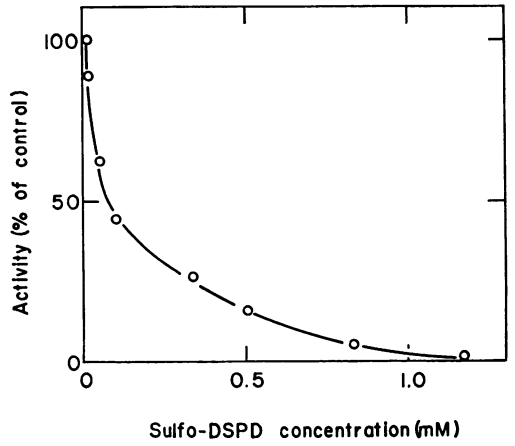

FIG. 1. Effect of sulfo-DSPD on the photoreduction of ferredoxin. Reaction mixture contained in a total volume of 3 ml: 25 mM Tricine buffer, pH 7.8; 25 mM NaCl; 4.5 μM ferredoxin, and lettuce chloroplasts containing 53 μg of chlorophyll. Other details are as described in "Materials and Methods." Activity is defined as the extent of ferredoxin reduction in steady state. 100% refers to a ΔA of 2.5×10^{-3} at 497 minus 540 nm.

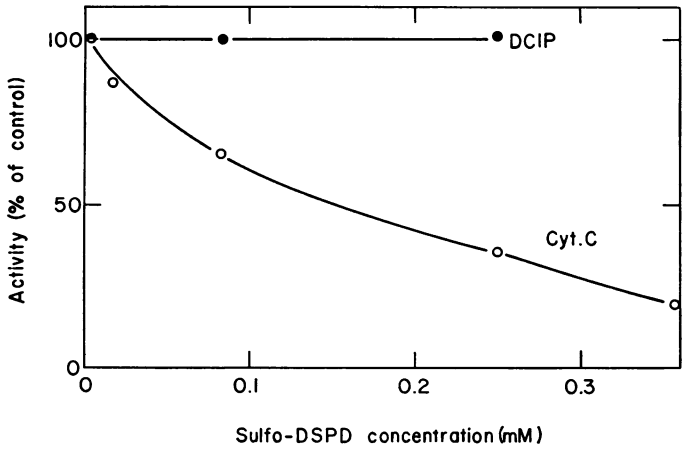

FIG. 2. Effect of sulfo-DSPD on electron transport from NADPH to cytochrome *c* and from NADPH to DCIP. Reaction mixture contained in a total volume of 3.0 ml: 25 mM Tricine buffer, pH 7.8, 25 mM NaCl; 40 μM NADPH; partially purified flavoprotein; and either 20 μM DCIP or 3.5 μM cytochrome *c* and 3 μM ferredoxin. Other details are as described in "Materials and Methods." An activity of 100% refers to 8 nmoles DCIP reduced min^{-1} or 10 nmoles cytochrome *c* reduced min^{-1}.

served, its site of action was concluded to be between photosystem I and ferredoxin. Figure 1 illustrates the inhibition of the photoreduction of ferredoxin by isolated lettuce chloroplasts. This reaction was also sensitive to the inhibitor, with 50% inhibition observed around 0.1 mM. Since no inhibition was observed of the photoreduction of low potential dyes like Diquat, these observations confirm the proposed site of action between photosystem I and ferredoxin. However, the following observations suggest that the site of action is ferredoxin itself, rather than a preceding position, as previously suggested (30). Thus the enzymic reduction of cytochrome *c* by NADPH (25), which requires the participation of the flavoprotein ferredoxin-NADP-reductase and ferredoxin, was fully sensitive to DSPD, while that of DCIP, which requires only the flavoprotein, was totally insensitive (Fig. 2).

At the concentrations of DSPD required for 50% inhibition of these ferredoxin catalyzed reactions, no effect could be observed on photosynthetic O_2 evolution of *D. parva* cells (Fig. 3). This is in agreement with previously reported effects of this inhibitor in other algae (18).

Pyrophosphate was previously shown to inhibit all ferredoxin-dependent reactions, except ferredoxin reduction (14, 15), and was therefore suggested to inhibit the interaction of reduced ferredoxin with the flavoprotein. As can be seen in Figure 4, pyrophosphate inhibited both NADP reduction and ferredoxin reduction to a similar extent. It is therefore concluded that pyrophosphate also interacts directly with ferredoxin. The failure of previous investigators to observe the inhibition of ferredoxin reduction may have been due to an absorption artifact due to the ascorbate + DCIP which they employed as the electron donor couple (14).

Pyrophosphate was previously shown to stimulate rather than inhibit CO_2 fixation in chloroplast preparations (12, 22), and this apparent contradiction has already been pointed out (15). Pyrophosphate did not affect O_2 evolution in a chloroplast preparation of *D. parva*.

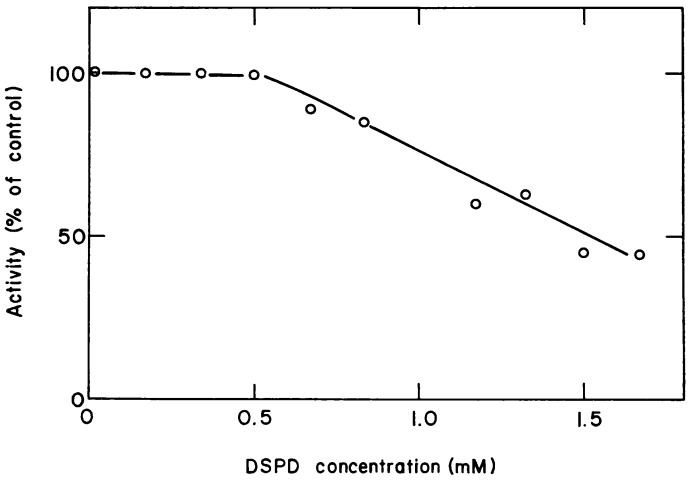

FIG. 3. Effect of DSPD on O_2 evolution in *D. parva*. Reaction mixture contained in a total volume of 2.5 ml: 1.5 M NaCl, 25 mM Tricine pH 7.6, *D. parva* cells containing 54 μg of chlorophyll. Other details are as described in "Materials and Methods."

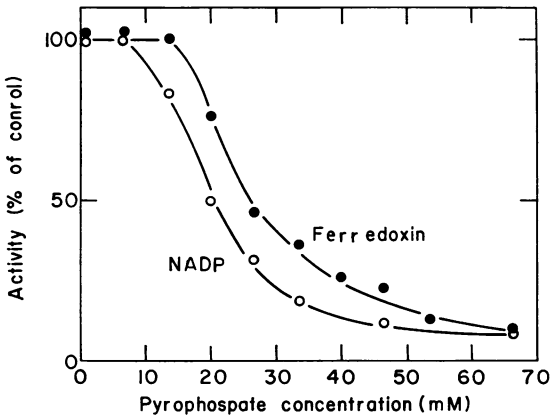

FIG. 4. Effect of pyrophosphate on the photoreduction of ferredoxin and NADP. Reaction mixture contained in a total volume of 3.0 ml: 25 mM Tricine buffer, pH 7.8, 25 mM NaCl, lettuce chloroplasts containing 42 μg of chlorophyll, and either 4.5 μM ferredoxin or ferredoxin and 0.1 mM NADP. Other details are as described in "Materials and Methods." An activity of 100% refers to a change of ΔA of 2×10^{-3} at 497 − 540 nm, or 120 μmoles NADP reduced mg chl^{-1} hr^{-1}.

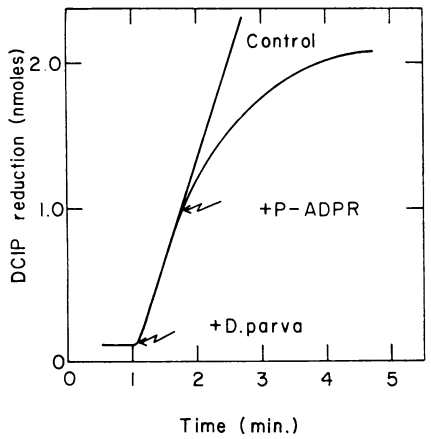

FIG. 5. Effect of P-ADPR on DCIP reduction in chloroplasts of *D. parva*. Reaction mixture contained in a total volume of 3.0 ml: 50 mM HEPES buffer, pH 7.6, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.33 M sorbitol, 1 mM isocitric acid, 150 μg of isocitrate dehydrogenase (Sigma), 12 μM DCIP, 3.3 μM NADP, and *D. parva* cells containing 11.4 μg of chlorophyll. Where indicated, 0.13 mM P-ADPR was added. Other details are as described in "Materials and Methods."

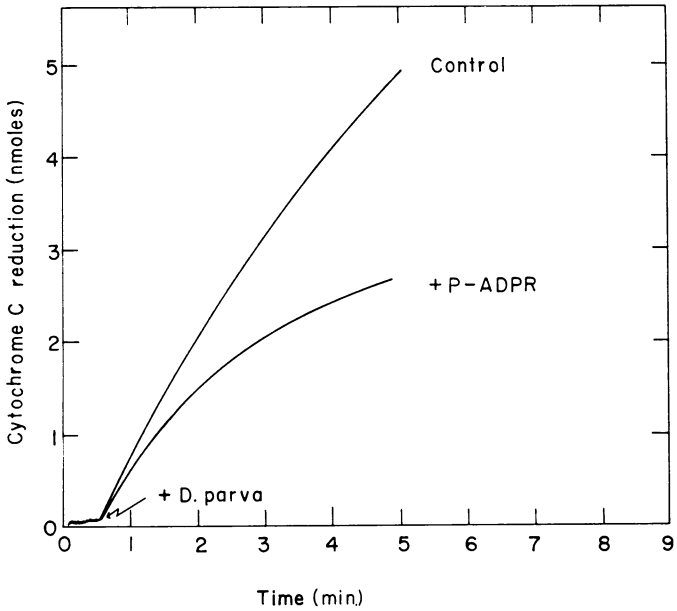

FIG. 6. Effect of P-ADPR on cytochrome *c* reduction in chloroplasts of *D. parva*. Reaction mixture contained in a total volume of 3.0 ml: 50 mM HEPES, pH 7.6, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.33 M sorbitol, 0.5 mM isocitric acid, 11 μM cytochrome *c*, 150 μg isocitrate dehydrogenase (Sigma), 3 μM ferredoxin, 0.5 μM NADP, and *D. parva* cells containing 8.8 μg of chlorophyll. Where indicated, 0.65 mM P-ADPR were added. Other details are as described in "Materials and Methods."

P-ADPR was previously shown to inhibit specifically the flavoprotein ferredoxin-NADP-reductase (9). As can be seen in Figure 5, the flavoprotein activity of *D. parva* chloroplasts was indeed inhibited by P-ADPR. A similar inhibition was obtained in the reduction of cytochrome *c* by the *D. parva* chloroplast flavoprotein supplemented by Swiss chard ferredoxin (Fig. 6).

At concentrations which inhibited the chloroplast flavoprotein reactions, P-ADPR had no effect on the chloroplasts ability to fix CO_2 and evolve O_2.

Effect of DSPD on the Development of Photosynthetic and NADP-Glyceraldehyde-3-P Dehydrogenase Activities. It was shown that the activity of NADP-dependent glyceraldehyde-3-P dehydrogenase appeared in parallel with photosynthetic capability and chlorophyll formation, on transfer of dark-grown *Euglena* cells to light (11, 19). If, as our results indicate, NADPH formation may not be a necessary prerequisite for photosynthesis, it may be possible to inhibit the formation of this NADPH-dependent enzyme without inhibiting photosynthesis. Thus, one would assume that the enzyme is induced via the production of NADPH, as a side product of the developing photosynthetic apparatus. It would be expected, therefore, that the addition of any of the above inhibitors, which will block the formation of NADPH, will in turn prevent the induction of the NADP-dependent glyceraldehyde-3-P dehydrogenase, without inhibiting photosynthesis.

Only DSPD, of the inhibitors used in this study, penetrates whole cells *in vivo*, and therefore the experiment was conducted utilizing DSPD as the specific inhibitor. Figure 7 illustrates that in the presence of an appropriate concentration of DSPD, only a small inhibition was observed in chlorophyll formation per cell and photosynthetic capability per cell. No inhibition was observed of photosynthetic ability per mg chlorophyll formed or of NAD-dependent glyceraldehyde-3-phosphate dehydrogenase. However, the activity of NADP-dependent glyceraldehyde-3-phosphate dehydrogenase was severely inhibited. Thus, under these conditions the cells were able to photosynthesize at a rate of 390 μatoms oxygen evolved per mg chlorophyll per hr while their NADP-dependent glyceraldehyde-3-phosphate dehydrogenase activity was 120 μmoles NADPH oxidized per mg chlorophyll per hr.

DISCUSSION

Our previous study (8) has indicated that cell-free preparations of the obligate autotrophic halophilic alga *Dunalliela parva* were unable to perform any photoinduced reaction which involved ferredoxin, despite the presence of both ferredoxin and the flavoprotein ferredoxin-NADP-reductase in the alga. Since *D. parva* preparations could photosynthesize even in cell-free chloroplast preparations, this raised the question of whether the path ferredoxin → ferredoxin-NADP-reductase → NADP was indeed an absolute requirement for photosynthesis as has been generally assumed (2).

In the results presented it was shown that inhibitors of this path, two acting on ferredoxin and one on the flavoprotein, failed to inhibit photosynthesis at concentrations which fully inhibited the activity of the proteins in question. One may speculate that, at least under these conditions, an earlier reduced product of photosystem I served as the reductant of CO_2. The existence of such a reduced product of a sufficiently low redox potential has been amply demonstrated (10, 24, 32), and similar suggestions have appeared (5, 6, 15, 23, 29).

It was shown that, on addition of DSPD to whole algae, photosynthesis is not inhibited, but the formation of glycolate is favored over that of sugars (17, 18). This again may be the result of the accumulation of the early reduced product of photosystem I, which has been postulated to be a necessary intermediate during the production of glycolate (21, 28). We have shown here that the addition of DSPD to dark-grown whole *Euglena* cells does not prevent the development of photosynthetic capability in the light, but does inhibit severely the formation of the NADP-dependent glyceraldehyde-3-P dehydrogenase. The latter enzyme is considered essential for the utilization of NADPH as a source of reducing power in photosynthesis (6).

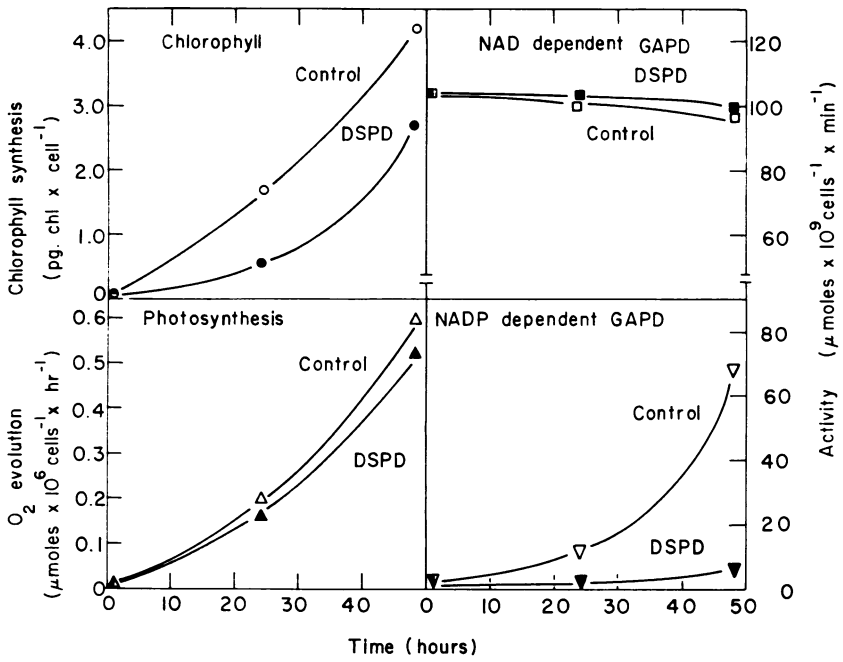

FIG. 7. Effect of DSPD on the formation of chlorophyll, NAD- and NADP-dependent glyceraldehyde-3-phosphate-dehydrogenases, and photosynthetic capability during illumination of dark-grown *Euglena gracilis*. The assay medium for the enzymes contained: 50 mM tris-Cl, pH 7.6, 8 mM $MgSO_4$, 1.2 mM ATP, 3 mM dithiothreitol, 0.16 mM NADPH or NADH, 4.5 mM phosphoglyceric acid, a saturating amount of phosphoglycerate kinase, and the extract from the *Euglena* cells. Other details are as described in "Materials and Methods."

D. parva may be a special natural case of an alga which is incapable of utilizing NADPH in photosynthetic CO_2 fixation. This may have developed from the recently described sensitivity of the ferredoxin-flavoprotein complex to the high salt conditions which are essential for the growth of these algae (14–16, 27). It may also be related to the observations that the major product of CO_2 fixation in *D. parva* is glycerol (31), and that the activity of the NADP-dependent glyceraldehyde-3-phosphate dehydrogenase per unit chlorophyll in these alga is only one-fifth of that of *E. gracilis*, whereas the photosynthetic activity on a chlorophyll basis is at least equal to that of *E. gracilis*.

A side product of these investigations was a better definition of the site of action of pyrophosphate and DSPD. Both of these inhibitors seem to interact directly with ferredoxin. The former may just be a special case of salt inhibition (14–16, 27), and the latter a more specific kind of interaction which should prove useful in further studies of this part of the electron transport path.

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