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SULPHYDRYL GROUPS IN PHOTOSYNTHETIC ENERGY CONSERVATION

I. LIGHT-DEPENDENT INHIBITION OF PHOTOPHOSPHORYLATION BY THE SULPHYDRYL REAGENT 2-2'DITHIO BIS-(5-NITROPYRIDINE)

CARLOS S. ANDREO and RUBEN H. VALLEJOS

Departamento de Bioquímica, Facultad de Ciencias Bioquímicas, Universidad Nacional de Rosario, Suipacha 531, Rosario (Argentina)

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SUMMARY

1. The sulphydryl reagent 2-2'dithio bis-(5-nitropyridine) (DTNP) inhibited photophosphorylation when the chloroplasts were preincubated with the reagent in the light. A maximum inhibition of about 50 % was obtained in the presence of pyocyanine and $MgCl_2$ at 0.3 μmol DTNP per mg chlorophyll and was completed in about 40 s of preillumination.

2. Dithioerythritol, ADP plus P_i (or arsenate) and uncouplers prevented the inhibition when present during the preillumination while phloridzin, Dio-9 and discarine B were ineffective. Low concentrations of ADP or ATP afforded partial protection but other nucleotides had no effect.

3. DTNP inhibited the coupled electron transport rate to the basal level and had no effect on the uncoupled electron transport. The stimulation of proton uptake and inhibition of electron transport by ATP was prevented by DTNP.

4. The trypsin-activated but not the light- and dithioerythritol-triggered ATPase was inhibited by light preincubation of chloroplasts with DTNP.

5. Reversal of DTNP inhibition of photophosphorylation was obtained by a second preillumination in the presence of thiol groups.

6. More DTNP reacted with chloroplasts in the light than in the dark. Two mol of thione were formed in the light per mol of DTNP disappeared.

7. The results suggested that DTNP inhibition is related to the oxidation by DTNP of chloroplast vicinal dithiols probably exposed by a light-induced conformational change.

INTRODUCTION

The photosynthetic generation of ATP in spinach chloroplasts requires the

Abbreviations: DTNP, 2-2'dithio bis-(5-nitropyridine); FCCP, carbonyl cyanide trifluoromethoxyphenylhydrazone; TES, *N*-tris (hydroxymethyl)methyl-2-aminoethane sulfonic acid.

membrane-bound coupling factor 1 [1, 2]. This protein has a high molecular weight (325 000), has been purified to homogeneity [3] and has a latent ATPase activity [2, 3] that can be unmasked by sulphydryl groups plus light [4], by trypsin treatment or by heat [2]. There are five different subunits in coupling factor 1 [4] designed α, β, γ, δ, and ε, and the probable composition of coupling factor 1 is α_3, β_3, γ, δ, ε [5]. The properties of several ATPases from other sources, involved in the synthesis of ATP, are remarkably similar to those of coupling factor 1 [6, 7].

There is increasing evidence that the conformation of membrane-bound coupling factor 1 changes when it is energized by light, acid-base transition or ATPase activity as revealed by tritium incorporation from ^3H_2O to coupling factor 1 [8, 9]. These observations were reinforced by the demonstration of light-dependent inhibitions of photophosphorylation in spinach chloroplasts by inorganic sulfate [10], *N*-ethylmaleimide [11] and permanganate [12]. The delayed light emission from chloroplasts [13, 14], the interactions of adenine nucleotides with coupling factor 1 [11, 15, 16] and a synergistic uncoupling by combinations of photophosphorylation inhibitors and FCCP [17] have also been related to conformational changes of coupling factor 1.

The irreversible, light-dependent inhibition of photophosphorylation and ATPase activity by *N*-ethylmaleimide suggest that light induces a conformational change in the membrane-bound coupling factor 1, that exposes the group(s) reacting with *N*-ethylmaleimide [11, 18]. McCarty and Fagan have shown [18], with radioactive-labeled reagent, a light-dependent incorporation of *N*-ethylmaleimide into only one of the five coupling factor 1 subunits. Although the nature of the reacting group(s) is not known, it is suspected to be a SH group [11, 18]. In order to elucidate this point, a reagent more specific for thiol groups than *N*-ethylmaleimide was needed. DTNP is a reagent that reacts specifically with sulphydryl groups of several compounds [19].

In the present paper we show that low DTNP concentrations inhibit photophosphorylation only when spinach chloroplasts are preilluminated in its presence. The effect is prevented by thiol compounds but it is not reversed by them, unless the thiol compounds are present during a second preillumination of the inhibited chloroplasts.

RESULTS

When spinach chloroplasts were preincubated in the light with 50 μM DTNP followed by 0.5 mM dithioerythritol after illumination the cyclic photophosphorylation measured subsequently was inhibited by about 50 % (Table I). Dark pretreatment or addition of DTNP up to 1 mM to the reaction mixture for photophosphorylation had no effect. If dithioerythritol was added before chloroplasts in the preilluminated experiment it prevented the inhibition (Table I). This result was expected since sulphydryl compounds readily react with DTNP forming mixed disulphides [19]. The concentration of DTNP for maximal inhibition of photophosphorylation depended on the chloroplast concentration. Fig. 1 shows that the maximum effect was obtained with 0.3 μmol of DTNP per mg of chlorophyll when DTNP concentration varied between 5 and 100 μM at fixed concentration of chloroplast (50 μg chloro-

TABLE I

LIGHT-DEPENDENT INHIBITION OF PHOTOPHOSPHORYLATION BY DTNP

Experimental conditions were as described in the text. Pretreatment was carried out in the dark or light for 1 min as stated. DTNP was 0.5 μmol/mg chlorophyll and dithioerythritol, 0.5 mM. Dithioerythritol was added in the dark after the preincubation except when stated otherwise.

Conditions of pretreatment	Cyclic photophosphorylation (μmol ATP/mg chlorophyll/h)
Dark	173
Dark+DTNP	163
Dark+DTNP, dithioerythritol added before chloroplasts	176
Light	164
Light+DTNP	75
Light+DTNP, dithioerythritol added before chloroplasts	151

phyll). The same result was obtained with varying concentrations of chloroplast (25–500 μg chlorophyll) at a fixed DTNP concentration (30 μM).

The preillumination of chloroplasts required pyocyanine and $MgCl_2$ for optimal inhibition by DTNP since when either of them were omitted from the preincubation medium the synthesis of ATP decreased only by 13 % and 28 % respectively.

Fig. 2 shows the effect of the time of preillumination on the development of photophosphorylation inhibition by DTNP. The time for half maximum effect was 6.6 s and in 40 s the effect was complete. This result is very similar to the kinetics of

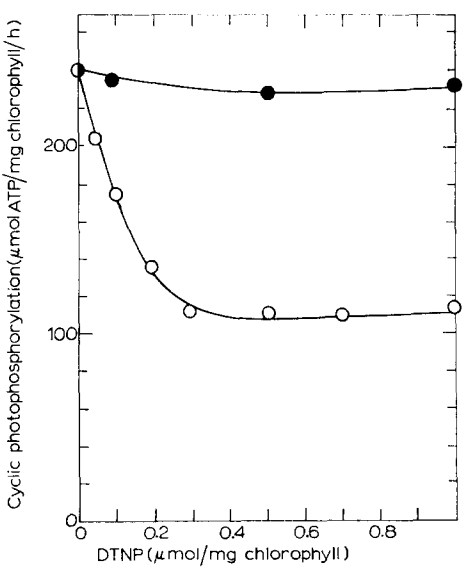


Fig. 1. Effect of DTNP on cyclic photophosphorylation in dark- or light-pretreated chloroplasts. Dark (●—●) or light (○—○) pretreatment of chloroplasts (50 μg chlorophyll) with increasing concentrations of DTNP and the subsequent determination of photophosphorylation were as described in the text and in the legend to Table I.

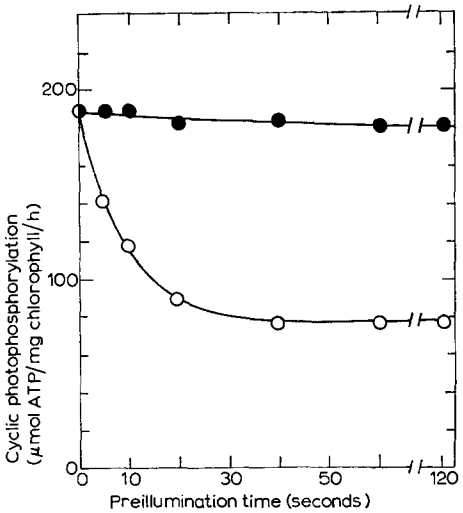


Fig. 2. Effect of preillumination time on the inhibition of cyclic photophosphorylation by DTNP. Experimental conditions were as described in the text and Fig. 1 except that the time of preillumination in the absence (●—●) or in the presence (○—○) of DTNP (0.5 μmol/mg chlorophyll) was as stated.

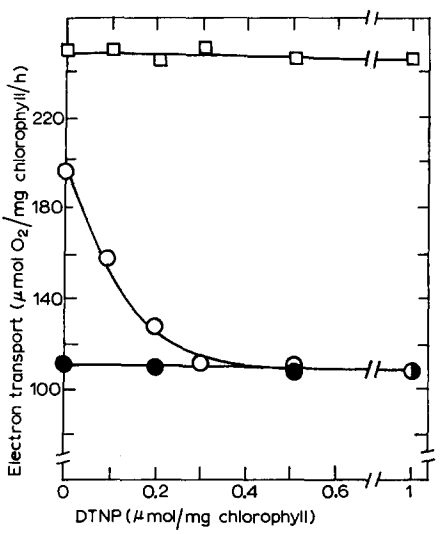


Fig. 3 Effect of light preincubation of chloroplasts with DTNP on electron transport. Electron transport from water to methylviologen was measured as oxygen uptake with a Teflon-covered Clark electrode (Gilson Oxygraph) as previously described [17]. Chloroplasts (50 μg of chlorophyll) were pretreated as described in the text except that the incubation medium contained 50 μM methylviologen and 0.5 mM NaN_3 instead of pyocyanine. After pretreatment, the rate of oxygen uptake was determined in the following conditions: in the absence of ADP and P_i(●—●); in the presence of 2 mM ADP and 2 mM P_i (○—○) and in the presence of 2 mM ADP, 2 mM P_i and 5 mM NH_4Cl (□—□). Preincubation of chloroplasts with DTNP in the dark did not affect the reactions tested.

TABLE II

PROTECTION OF PHOTOPHOSPHORYLATION FROM DTNP INHIBITION BY ADENINE NUCLEOTIDES, UNCOUPLERS AND INHIBITORS

Experimental conditions were as described in the text and in the legend to Table I. The numerals in parenthesis are per cent of inhibition.

Additions to the preillumination stage	Cyclic photophosphorylation (μmol ATP/mg chlorophyll/h)	
	DTNP (μmol/mg chlorophyll) (0)	(0.5)
None	313	139 (56)
2 mM ADP; 2 mM P_i	300	290 (3)
2 mM ADP; 2 mM Arsenate	314	292 (7)
2 mM P_i	304	148 (51)
2 mM Arsenate	321	187 (42)
2 mM ADP	327	260 (20)
2 mM ATP	313	229 (27)
5 mM NH_4Cl	313	318 (0)
2 μM FCCP	323	327 (0)
Dio-9 (5 μg/ml)	303	156 (48)
1 mM Phloridzin	240	110 (54)
200 μM Discarine B	303	140 (54)

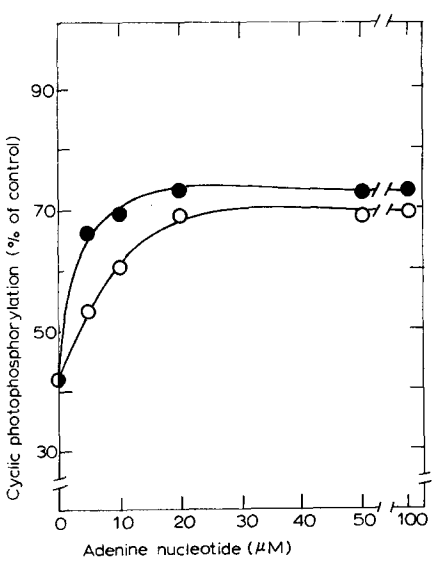


Fig. 4. Effect of ADP and ATP on the inhibition of cyclic photophosphorylation by DTNP. Experimental conditions were as described in Fig. 1 except that the stated concentrations of ADP (● — ●) or ATP (○ — ○) were present with DTNP (0.5 μmol/mg chlorophyll) in the preillumination stage.

other light-dependent effects such as tritium labelling of coupling factor 1 and the inhibition of photophosphorylation by permanganate, sulfate or *N*-ethylmaleimide [8–12].

Light-dependent inhibition by DTNP was also observed on non-cyclic photophosphorylation. The activity associated with methylviologen reduction was diminished from 96 to 50 μmol ATP/mg chlorophyll/h by DTNP (0.5 μmol/mg chlorophyll).

Fig. 3 shows that the basal and the uncoupled electron transport from water to methylviologen were not affected by DTNP while the coupled activity (i.e. in the presence of ADP and P_i) was decreased to the basal level. It is surprising that the concentration of DTNP that achieved this effect inhibited photophosphorylation only partially (cf. Fig. 1). Coupled electron transport was also more inhibited than photophosphorylation by permanganate [12]. The inhibition of electron transport by DTNP is similar to that of energy transfer inhibitors.

The reason why inhibition of ATP synthesis by DTNP required pretreatment with light is evident from the data of Table II: the presence of ADP and P_i in the preillumination step completely prevented the inhibition by DTNP. Arsenate can replace phosphate. However, 2 mM phosphate or arsenate did not protect photophosphorylation by themselves. On the other hand, 2 mM ADP or ATP alone afforded partial protection while 2 mM UTP, CTP, GTP, GDP, IMP or AMP had no effect. Phosphorylating conditions also prevent the light-dependent incorporation of tritium to coupling factor 1 [9] and the inhibitory effects of sulphate, permanganate and *N*-ethylmaleimide [10–12] and are required for the synergistic uncoupling by combinations of FCCP and inhibitors [17].

Table II also shows that uncouplers like NH_4Cl or FCCP completely prevented the inhibitory effect of DTNP while the energy transfer inhibitors [20–22] Dio-9, phloridzin and discarine B were not effective.

The concentration of ADP or ATP that gave partial protection of photophosphorylation against DTNP may be much lower than that shown in Table II. Fig 4 shows that indeed only 20 μM nucleotide were necessary and half-maximal effect was

TABLE III

EFFECT OF ATP AND DTNP ON PHOTOSYNTHETIC ELECTRON TRANSPORT AND PROTON UPTAKE BY CHLOROPLASTS

Experimental conditions for determination of basal electron transport from water to methylviologen were as described in legend to Fig. 3 and for proton uptake were as described [17]. Chloroplasts (50 μg chlorophyll) suspended in 100 mM KCl were pretreated in dark or light for 1 min without or with DTNP (0.6 μmol/mg chlorophyll) in a medium (2 ml) of 100 mM KCl, 5 mM $MgCl_2$ and 50 μM pyocyanine. After pretreatment, 0.5 mM dithioerythritol was added and proton uptake was determined at an initial pH of 8. When added, ATP was 60 μM. Numerals in parenthesis indicate inhibition (−) or stimulation (+) per cent.

Additions	Electron transport (μmol O_2/mg chlorophyll/h)	Proton uptake (nmol H^+/mg chlorophyll)
None	122	106
DTNP pretreatment	118	112
ATP	69 (−43)	159 (+50)
ATP after DTNP pretreatment	93 (−21)	134 (+20)

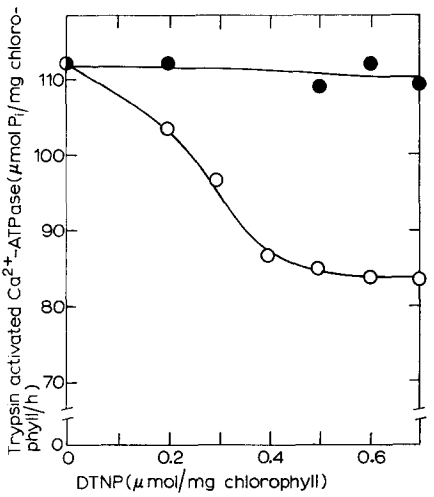


Fig. 5. Effect of dark- or light-pretreatment of chloroplasts with DTNP on trypsin-activated Ca^{2+}-ATPase. Chloroplasts were pretreated in the dark (●—●) or light (○—○) with increasing concentrations of DTNP; the activity of Ca^{2+}-ATPase was determined as described in the text.

TABLE IV

REVERSAL OF DTNP INHIBITION OF PHOTOPHOSPHORYLATION BY A SECOND PREILLUMINATION IN THE PRESENCE OF THIOL GROUPS

Experimental conditions were as described in the text except that the first preillumination of chloroplasts with or without DTNP was followed by a second 2-min stage under the conditions stated. The thiol compounds tested had no effect in the dark (shown for dithioerythritol). Numerals in parenthesis indicate inhibition per cent.

Conditions for second pretreatment of chloroplasts	Cyclic photophosphorylation (μmol ATP/mg chlorophyll/h)	
	DTNP (μmol/mg chlorophyll) (0)	(0.5)
None	220	124 (44)
Light	208	110 (47)
Dark	216	110 (49)
Dark, plus 0.5 mM dithioerythritol	208	110 (47)
Light, plus 0.5 mM dithioerythritol	235	187 (20)
Light, plus 0.5 mM dithioerythritol, 2 mM ADP and 2 mM P_i	204	106 (48)
Light, plus 2 mM dithioerythritol	220	196 (11)
Light, plus 2 mM β-mercaptoethanol	192	177 (8)
Light, plus 2 mM 3-mercapto-1,2-propanediol	204	178 (13)

attained with 2μM ADP or 5 μM ATP. Low concentrations of ADP and ATP have been shown to protect photophosphorylation from *N*-ethylmaleimide [11, 23].

Low concentration of ATP (or ADP) have been shown to inhibit basal photosynthetic electron transport and to enhance proton uptake by chloroplasts [15, 16, 24]. Table III shows that DTNP had effect neither on proton uptake nor on basal electron transport. The proton uptake at an initial pH of 6.5 was not affected either (not shown). However, DTNP pretreatment of the chloroplasts partially prevented the ATP-induced inhibition of electron transport and stimulation of proton uptake.

Chloroplasts have a latent ATPase activity that may be triggered by illumination in the presence of thiol agents or by treatment with trypsin [2]. DTNP has no effect on the light- and dithioerythritol-triggered ATPase activity of chloroplasts (not shown). However, the trypsin-activated Ca^{2+}-ATPase was diminished by light (but not dark) DTNP pretreatment of chloroplasts (Fig. 5).

In all the experiments described up to now, 0.5 mM dithioerythritol was added in the dark after the preillumination step. This dithioerythritol addition did not affect the inhibition by DTNP. However, if light was turned on a second time in

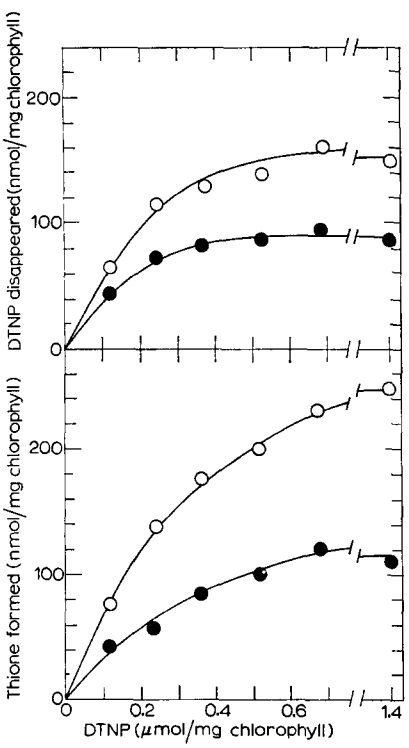


Fig. 6. Determination of DTNP reacting in the dark or in the light with chloroplasts and of the thione formed. Chloroplasts (50 μg of chlorophyll) were incubated with the stated concentrations of DTNP in the dark (●—●) or in the light (○—○) for 1 min in the experimental conditions described in the text except that the final volume was 1.5 ml. Then, the test tubes were centrifuged in the dark for 4 min in an Eppendorf microcentrifuge. The concentration of the DTNP disappeared and of the thione formed were calculated from the absorption of the supernatant solutions at 314 and 386 nm respectively. Appropriate controls with chloroplasts supernatant alone were substracted.

the presence of dithioerythritol the inhibition of photophosphorylation by DTNP was partially reversed (Table IV). Higher concentrations of dithioerythritol (2 mM) or of other thiol compounds such as β-mercaptoethanol or 3-mercapto-1,2-propanediol present during a second preillumination induced nearly complete reversion of the DTNP inhibition. ADP and P_i blocked this reversal of DTNP effect (Table IV). These results also explained the lack of effect of DTNP on the dithioerythritol-triggered ATPase.

DTNP has a maximum absorption peak in the ultraviolet at 314 nm that, after reaction with thiol groups, shifts toward longer wavelengths [19]. The thione formed has the absorption maximum at 386 nm. The molar absorption of both substances is known [19]. Therefore, it was possible and desirable to study the reaction of DTNP with the sulphydryl groups of chloroplasts both in the light and in the dark.

Fig. 6A shows the disappearance of DTNP from the supernatant solution after dark or light incubation with chloroplasts at different concentrations of DTNP. More DTNP disappeared in the light than in the dark experiments at all concentrations tested.

Similar results were obtained when the concentration of thione formed was determined spectrophotometrically in the same experimental conditions, i.e. more thione was formed in the light than in the dark incubations of chloroplasts with DTNP (Fig. 6B).

It is noteworthy that although more DTNP reacts with chloroplasts in the dark than during illumination, DTNP had no effect in the dark on the reactions tested.

When the light minus dark supernatant concentrations of DTNP and thione were calculated it was observed that at all the concentrations of DTNP tested 2 mol of thione were formed per mol of DTNP that disappeared (Table V). On the other hand, the relationship of DTNP disappeared to thione formed in the dark experiments was one. When ADP plus P_i were present in the light experiments the concentrations of DTNP disappeared and of thione formed were reduced to the dark level (experiment not shown).

TABLE V

AMOUNT OF DTNP REACTING WITH CHLOROPLASTS AND OF THIONE FORMED DURING ILLUMINATION

Experimental conditions were as described in the legend to Fig. 6.

Initial DTNP concentration (μmol/mg chlorophyll)	DTNP (Light minus dark supernatant concentration in nmol/mg chlorophyll)	Thione
0.12	19	34
0.24	41	84
0.36	45	92
0.52	50	96
0.68	66	116
1.40	68	140

DISCUSSION

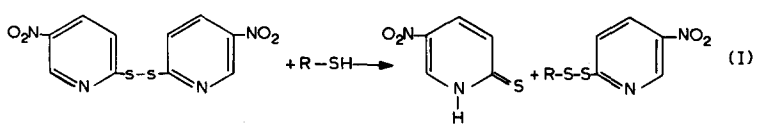
The results presented above show that DTNP, a reagent specific for thiol groups, diminished in a light-dependent process the synthesis of ATP by chloroplasts, the coupled electron transport and the trypsin-activated ATPase activity.

These effects of DTNP have the following common aspects with the light-dependent inhibition of photophosphorylation by sulfate [10], *N*-ethylmaleimide [11] and permanganate [12]: (a) all have similar kinetics, (b) the effect levels off at about 50 %, (c) ADP and P_i (or arsenate) and uncouplers prevent the inhibition and (d) the ATPase activity of coupling factor 1 is affected.

The light-dependency of DTNP inhibition can be explained admitting that DTNP reacts with sulphydryl groups exposed by a conformational change of coupling factor 1 induced by light. Such a conformation change has been shown by Ryrie and Jagendorf [8, 9]. The kinetics of coupling factor 1 titration observed by them [9] was very similar to that of DTNP inhibition and both effects were prevented by uncouplers.

Localization of at least some of the reacting sulphydryl groups on coupling factor 1 may be deduced from the inhibition of the trypsin-activated Ca^{2+}-ATPase (Fig. 5) and from the protection afforded by low concentrations of ADP and ATP (Fig. 4) but not by other nucleotides including GDP (see ref. 23). The light stimulated incorporation of *N*-ethylmaleimide into coupling factor 1 [18] supports the possibility that sulphydryl groups are exposed by light in chloroplast. Further evidence was obtained by us from quantitative determinations of the DTNP reacting with chloroplasts and of the thione formed.

DTNP may react with a monothiol giving a mixed disulfide as shown in reaction I or with a vicinal dithiol according to reaction II:



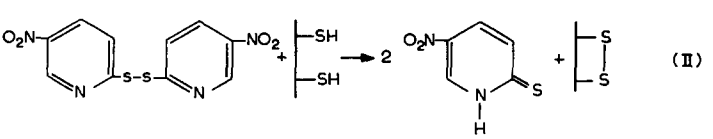


Fig. 7

In the latter case, i.e. when a dithiol is oxidized to a disulfide, 2 mol of thione are formed per mol of DTNP reacting instead of one as in reaction I. Such was indeed the stoichiometry found for the reaction of DTNP with chloroplasts during illumination (Table V). The same stoichiometry was found for the reaction of 5,5'dithiobis-(2-nitrobenzoate) with α-amylase [25]. This result was explained assuming that two SH groups were oxidized by the reagent. These results suggest that DTNP reacts with vicinal dithiols in illuminated chloroplasts. Therefore the light-dependent inhi-

bition by DTNP of ATP synthesis and hydrolysis and of coupled electron transport in chloroplasts may be related to the oxidation of vicinal dithiols to the corresponding disulfides. Localization of the dithiols involved are presently under study.

The reversal of DTNP inhibition by a second preillumination in the presence of SH groups suggests that the disulfide is protected from reduction by thiols in the dark but that light exposes it again. This effect of light is similar to the tritium discharge from coupling factor 1 that occurred when labeled chloroplasts were reilluminated [9].

We found a light-dependent inhibition of photophosphorylation by 5,5'-dithio-bis-(2-nitrobenzoate) similar to that of DTNP although the latter was about seven times more effective (unpublished results).

Results obtained with a sulphydryl reagent specific for vicinal dithiols such as *o*-iodosobenzoic acid (Vallejos and Andreo, manuscript in preparation) support our conclusions.

EXPERIMENTAL

Chloroplasts were isolated from spinach leaves (*Spinacea oleracea* L) as previously described [26] and suspended in 250 mM sucrose, 20 mM TES/NaOH buffer (pH 7.8) and 5 mM $MgCl_2$ unless otherwise stated.

Total chlorophyll was determined as described [27]. Cyclic photophosphorylation was determined as previously described [26] except that chloroplasts (50 μg of chlorophyll) were preincubated at 25 °C in a final volume of 0.5 ml of 250 mM sucrose, 20 mM TES/NaOH buffer (pH 7.8), 5 mM $MgCl_2$ and 50 μM pyocyanine in dark or light for 1 min, and with the additions stated. After preincubation, 0.5 mM dithioerythritol was added in the dark and aliquots of 0.1 ml (10 μg of chlorophyll) were transferred to 0.9 ml of the reaction medium for photophosphorylation. Reaction was started by turning on the light and was stopped after 2 min with 0.1 ml of 50 % (w/v) trichloroacetic acid.

The trypsin-activated Ca^{2+}-ATPase of chloroplasts was determined as described by Lien and Racker [28]. The reaction medium (1 ml) was 50 mM Tris · HCl buffer (pH 7.5), 5 mM $CaCl_2$, 5 mM ATP and pretreated chloroplasts (20 μg of chlorophyll). Chloroplasts (200 μg of chlorophyll) were pretreated with DTNP in dark or light for 2 min as indicated above; then they were centrifuged at 12 000 $\times$ *g* in an Eppendorf Microcentrifuge at room temperature in the dark for 2 min, and the chloroplasts were resuspended in 0.1 ml of 50 mM Tris · HCl buffer (pH 7.5). The activation of the latent Ca^{2+}-ATPase with trypsin was carried out in a reaction medium (1 ml) containing 50 mM Tris · HCl buffer (pH 7.5), 2 mM EDTA, 2 mM ATP and 800 μg of trypsin freshly dissolved; after 10 min at 25 °C, 1.6 mg of trypsin inhibitor was added. Then, aliquots of 0.2 ml (20 μg of chlorophyll) were transferred to 0.8 ml of reaction medium (50 mM Tris · HCl (pH 7.5), 5 mM $CaCl_2$, 5 mM ATP). After 10 min at 37 °C the reaction was stopped with 0.1 ml of 50 % (w/v) trichloroacetic acid and the P_i liberated was determined as described [29].

DTNP, trypsin, trypsin inhibitor, TES, dithioerythritol and nucleotides were obtained from Sigma Chemical Co (USA). FCCP, Dio-9 and discarine B were generous gifts from Dr. P. G. Heytler, E. I. Du Pont de Nemours & Co (Wilmington, USA), Dr. P. L. Hoogland, Gist-Brocades N.V. (Delft, The Netherlands) and Dr.

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All others chemicals were of analytical grade.

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