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Light and Dark Reduction of Nitrite in a Reconstituted Enzymic System*

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Kessler (1) has studied the reduction of nitrite to ammonia by the green alga *Ankistrodesmus* both in the light in an atmosphere of nitrogen and in the dark under hydrogen. No specific conclusion could be drawn as to the mechanism of the process, although it was assumed that reduced pyridine nucleotides might serve as the immediate hydrogen donors for the reduction of nitrite and that energy-rich phosphate bonds were specifically required for the occurrence of the reaction. The production of "assimilatory power" by noncyclic photophosphorylation (2) was, therefore, considered a prerequisite for nitrite reduction. Nicholas (3), working with cell-free enzyme preparations from Neurospora, found also that phosphorylations were involved in the reduction of nitrite.

Roussos and Nason (4) purified from extracts of soybean leaves a soluble "pyridine nucleotide-nitrite enzyme" which, in the presence of an unidentified, heat-stable, organic factor obtained from the same extracts, required nitrite in order to catalyze the oxidation of reduced di- or triphosphopyridine nucleotide. However, they could not demonstrate any disappearance of nitrite concomitant with the enzymic oxidation of pyridine nucleotide, and the fate of nitrite remained unknown. More recently, Hageman, Creswell, and Hewitt (5) have shown that the reduction of nitrite to ammonia by marrow leaf enzymes occurs with reduced benzyl viologen alone or in catalytic amounts with DPNH, but not with TPNH or DPNH in the absence of the dye.

Mortenson, Valentine, and Carnahan (6) obtained from *Clostridium pasteurianum* a water-soluble, nonheme, nonflavin, iron-containing protein, named ferredoxin, which linked hydrogenase with a variety of electron acceptors, among them nitrite. According to Valentine *et al.* (7), spinach ferredoxin substituted for *Clostridium* ferredoxin in the reduction of nitrite by extracts of this bacterium with hydrogen gas as the electron donor.

Huzisige and Satoh (8) isolated from spinach leaves a soluble enzyme preparation, "photosynthetic nitrite reductase," which was required in addition to grana for the photochemical reduction of nitrite. In previous work from our laboratory (9, 10), it has been shown, however, in spinach chloroplasts, that the reduction of nitrite itself is a dark reaction. The mechanism involved in the process was found to be similar to the one implicated in the photosynthetic reduction of TPN^+ (11). To take place, the reduction of nitrite required, in addition to light- or dark-reduced spinach ferredoxin, a thermolabile factor (nitrite reductase) also present in the chloroplast extract.

The present report is concerned with the purification of the enzymes involved in the dark and light reduction of nitrite, and with the characterization of the process in a reconstituted enzymic system. Of particular interest in the present investigation is that energy-rich phosphate is not required for the reduction of nitrite; but when nitrite is reduced in the light, adenosine triphosphate is produced simultaneously in stoichiometric amounts.

EXPERIMENTAL PROCEDURE

Preparation of Chloroplast Fragments and Ferredoxins—Broken chloroplasts or once-washed broken chloroplasts were prepared from spinach according to Whatley, Allen, and Arnon (12). Spinach and *C. pasteurianum* ferredoxins were obtained as described by Tagawa and Arnon (11).

Purification of Spinach Nitrite Reductase—A crude homogenate of spinach leaves was prepared as described by San Pietro and Lang (13) except that, as indicated by Tagawa and Arnon (11), the pH was adjusted with Tris buffer to pH 7.3. Precipitation with acetone, extraction of the precipitate, and dialysis were also carried out according to San Pietro and Lang (13), but at pH 7.3.

The dialyzed extract was supplemented with sufficient NaCl to give a final concentration of 0.2 M, and passed through a DEAE-cellulose bed, 3 × 6 cm, equilibrated with 0.15 M Tris (pH 7.3)-0.2 M $[Cl^-]$ in order to adsord the ferredoxin. The nitrite reductase fraction which went through the column was precipitated with ammonium sulfate between 0 and 70% saturation. After standing for 20 minutes, the suspension was centrifuged for 10 minutes at 27,000 × *g*, and the supernatant solution was discarded. The sediment was suspended in Tris 0.05 M, pH 7.3, and dialyzed overnight against the same buffer in the cold. This purified spinach nitrite reductase preparation evidenced ferredoxin-TPN reductase activity (14) as assayed by the method of Avron and Jagendorf (15), but was free of ferredoxin.

Purification of C. pasteurianum Hydrogenase—*C. pasteurianum* strain W-5 was obtained from the American Type Culture Collection, Washington, D. C. The lyophilized culture was transferred to the potato medium of Jensen and Spencer (16) and then to the medium of Carnahan and Castle (17), with ammonia as the nitrogen source. A crude extract of *C. pasteurianum* was prepared by autolysis of the dried cells according to the technique of Carnahan *et al.* (18), except that 0.05 M Tris buffer, pH 7.3, was used. In order to adsorb the ferredoxin, the crude

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extract was passed through a DEAE-cellulose bed equilibrated with the same buffer. The hydrogenase-containing fraction which came through the column was treated with calcium phosphate gel (2 mg per mg of protein), allowed to stand for 10 minutes, and centrifuged for 5 minutes at 2000 × *g*. The supernatant liquid was discarded, and the sediment washed by suspending it in Tris, 0.7 M, pH 7.3. The suspension was allowed to stand for 10 minutes with occasional mixing and centrifuged as before, and the supernatant solution was discarded. The pellet was suspended in 0.1 M sodium pyrophosphate buffer, pH 7.0, in order to elute the hydrogenase, and the suspension was allowed to stand for 10 minutes with occasional stirring. After centrifugation at 2000 × *g* for 5 minutes, the sediment was discarded; the resulting supernatant solution contained the hydrogenase. All steps were performed at 0–4° in the presence of 3 mM cysteine. In the presence of ferredoxin the purified *Clostridium* hydrogenase did not catalyze either TPN^+ or nitrite reduction with molecular hydrogen (6, 7, 19). The enzyme was also unable to reduce methylene blue; however, it liberated H_2 in the presence of reduced methyl viologen.

Chemicals—ADP, TPN^+, TPNH, DEAE-cellulose, and L-cysteine were purchased from Sigma Chemical Company. Methyl viologen was purchased from Mann Research Laboratories. $Na_2H^{32}PO_4$ was obtained from the J. E. N., Madrid, Spain. The calcium phosphate gel was prepared according to Keilin and Hartree (20), but Na_2HPO_4 was used instead of Na_3PO_4, and NaOH was added to adjust the pH to 7.4.

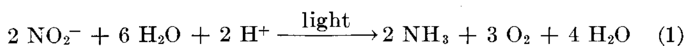
Analytical Methods—Oxygen evolution or hydrogen uptake was estimated in Warburg manometer flasks. When the reactions were carried out in the light, illumination was provided from below by a 100-watt fluorescent lamp and from above by a bank of 100-watt reflector flood lamps at distances of 3 and 14 cm, respectively, from the reaction vessels.

Nitrite was estimated by the method of Novak and Wilson (21). Ammonia was determined by nesslerization after diffusion and absorption of the gas in 0.01 N H_2SO_4 in Conway units (22). Radioactive adenosine triphosphate was measured by the technique of Arnon, Whatley, and Allen (23). Protein concentration was assayed by the method of Lowry *et al.* (24).

RESULTS AND DISCUSSION

The experiments to be described on the mechanism of nitrite reduction in a reconstituted enzymic system confirm previous investigations from this laboratory (9, 10) and reveal that ferredoxin is always involved in the process. Ferredoxin itself is not the enzyme which catalyzes the reduction of nitrite, but the electron carrier which functions in collaboration with the spinach nitrite reductase in the transfer of electrons to nitrite.

Photochemical Reduction of Nitrite—The reduction of nitrite in the light required spinach ferredoxin and spinach nitrite reductase in addition to illuminated grana (Table I). No stimulation of nitrite reduction by Mn^{++} ions was observed (4, 5). Substrate amounts of nitrite were reduced to ammonia, and oxygen was evolved, in accordance with Equation 1 (Table II).



$$2\ NO_2^- + 6\ H_2O + 2\ H^+ \xrightarrow{\text{light}} 2\ NH_3 + 3\ O_2 + 4\ H_2O \quad (1)$$

Under the appropriate experimental conditions and in the presence of orthophosphate and ADP, the reduction of 2 moles of nitrite to ammonia was accompanied by the production of 6 moles of ATP and the evolution of 3 moles of oxygen, in accordance with Equation 2 (Fig. 1).

TABLE I

Characterization of nitrite photoreduction in reconstituted chloroplast system

The complete reaction mixture included, in a final volume of 3 ml, once washed broken chloroplasts containing 0.2 mg of chlorophyll; spinach ferredoxin, 1 mg; spinach nitrite reductase, 2 mg; Tris buffer, pH 8.0, 150 μmoles; and sodium nitrite, 6 μmoles. The gas phase was argon. The mixture was incubated at 20° for 15 minutes. Other experimental conditions are indicated under "Experimental Procedure."

Reaction system	NO_2^- reduced	Oxygen evolved
	μmoles	*μatoms*
Complete	1.4	4.8
Spinach ferredoxin omitted	0.2	0.7
Nitrite reductase omitted	0.1	0.9
Nitrite omitted	0	0.7
Complete, dark	0.1	0

TABLE II

Equivalence of oxygen evolution and ammonia formation in photoreduction of nitrite

The experimental conditions were the same as in Table I, except that 0.5 mg of spinach ferredoxin was used, and nitrite was added as indicated. The reaction was run to completion.

NO_2^- added	Oxygen evolved	Ammonia produced
μmoles	*μatoms*	*μmoles*
0	0	0
0.5	1.3	0.4
1.5	4.2	1.3

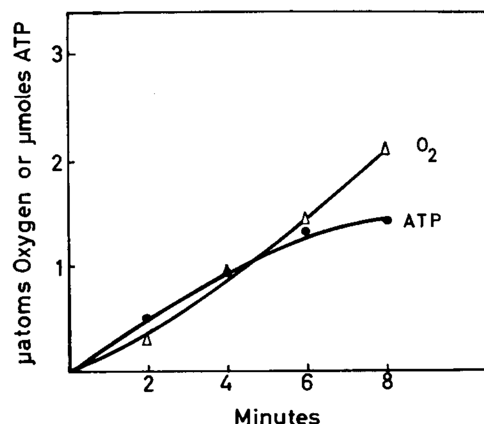
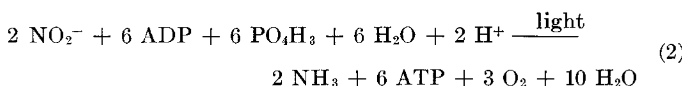


FIG. 1. Stoichiometry of oxygen evolution and ATP formation in noncyclic photophosphorylation with nitrite as the terminal electron acceptor. The experimental conditions were the same as in Table I, except that broken chloroplasts containing 0.2 mg of chlorophyll were used, and the following were added: $MgCl_2$, 10 μmoles; $Na_2H^{32}PO_4$, 10 μmoles; and ADP, 10 μmoles.



$$2\ NO_2^- + 6\ ADP + 6\ PO_4H_3 + 6\ H_2O + 2\ H^+ \xrightarrow{\text{light}} 2\ NH_3 + 6\ ATP + 3\ O_2 + 10\ H_2O \quad (2)$$

The decrease of the rate of ATP formation as compared with O_2

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evolution is explained by the uncoupling effect of ammonia on ATP formation during the photochemical reduction of nitrite. Nitrite itself does not affect the photophosphorylation coupled to ferricyanide reduction, but its reduction product, ammonia, effectively uncouples photosynthetic phosphorylation from ferricyanide (25) and TPN^+ reduction (26).

The reduction of nitrite in the light is thus a new type of noncyclic, ferredoxin-dependent photophosphorylation reaction in chloroplasts, similar to but not identical with the one studied by Arnon *et al.* (2, 27) with TPN^+ as the terminal oxidant. At the electron donor end, the similarities are total, since both TPN^+ (27, 28) and NO_2^- (9, 10) can use either water or ascorbate-dichlorophenol indophenol as the reductant. At the electron acceptor end the difference is most pronounced since, as shown in Table III, the noncyclic electron flow was not affected by 1 mM KCN in the TPN^+ system (*cf.* Avron and Jagendorf (15)), whereas it was completely inhibited in the NO_2^- system (*cf.* Roussos and Nason (4) and Hageman, Creswell, and Hewitt (5)). Both systems are, therefore, identical up to the ferredoxin level. Reduced ferredoxin has been recently identified by Whatley, Tagawa, and Arnon (29) as the earliest chemically isolated reductant formed at the expense of radiant energy trapped during photosynthesis. Once ferredoxin has been reduced, the electrons can be transferred either to TPN^+ by TPN reductase (14) or to nitrite by nitrite reductase.

Tagawa and Arnon (11) found that the proteins from green plants (13) and photosynthetic bacteria (27), previously known as photosynthetic pyridine nucleotide reductases, are ferredoxins which share chemical and functional similarities with the ferredoxins from nonphotosynthetic microorganisms (19), and are capable of transferring the electrons coming from hydrogen gas or from light-activated chlorophyll to the chloroplast TPN^+-TPN reductase system. It was interesting, therefore, to determine whether *Clostridium* ferredoxin is capable of replacing spinach ferredoxin in the photochemical reduction of nitrite. As shown in Fig. 2, *Clostridium* ferredoxin can substitute for spinach ferredoxin but is a far less effective electron carrier.

Dark Reduction of Nitrite—The investigation of the dark reduction of nitrite to ammonia with the hydrogen gas-*Clostridium* hydrogenase system as the source of electrons also showed that the process was ferredoxin-dependent and required the presence of spinach nitrite reductase (Fig. 3). In this dark system, by contrast with the photochemical one, *Clostridium* ferredoxin was more active than spinach ferredoxin in mediating the transfer of electrons (*cf.* Valentine *et al.* (7)). The specificity of *Clostridium* and spinach ferredoxin is, therefore, more rigid for the electron donor system than for the nitrite-spinach nitrite reductase system.

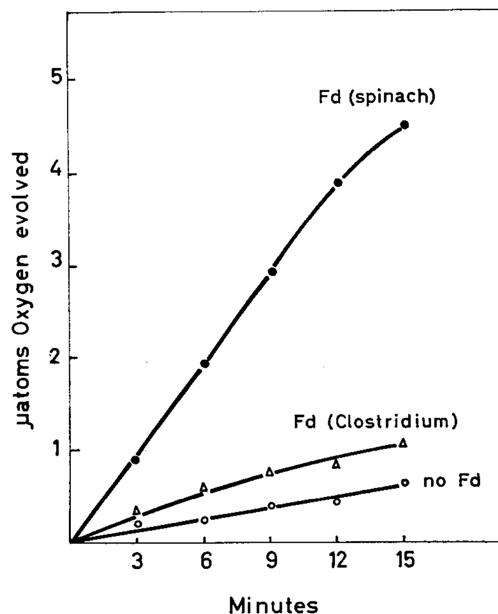

FIG. 2. Ferredoxins (*Fd*) as electron carriers in the light reduction of nitrite by chloroplasts. Experimental conditions were as in Table I, except that 1.5 mg of *Clostridium* ferredoxin were used where indicated.

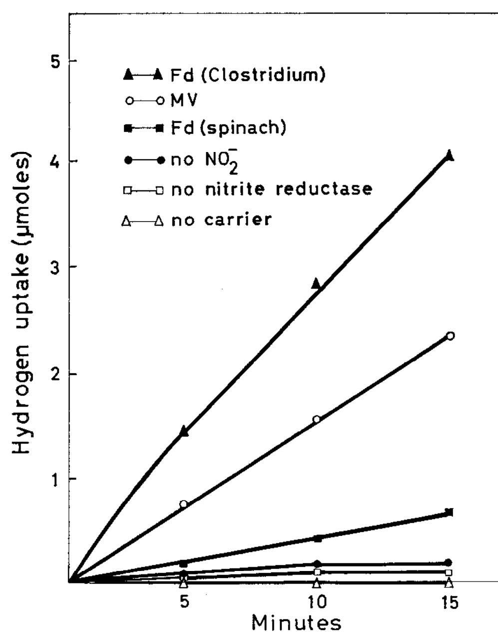

FIG. 3. Ferredoxins (*Fd*) and methyl viologen (*MV*) as electron carriers in the dark reduction of nitrite with hydrogen gas as the electron donor. The reaction mixture contained, in a final volume of 3 ml, *Clostridium* hydrogenase, 1 mg; spinach nitrite reductase, 10 mg; Tris buffer, pH 8.0, 200 µmoles; cysteine, 10 µmoles; and sodium nitrite, 1.5 µmoles. Where indicated, 1 mg of spinach ferredoxin, 1.5 mg of *Clostridium* ferredoxin, or 1 µmole of methyl viologen was added. Gas phase, hydrogen. Temperature, 26°.

TABLE III

Effect of cyanide on noncyclic electron flow with TPN^+ and NO_2^- as terminal electron acceptors

The reaction mixture included, in a final volume of 3 ml, once washed broken chloroplasts containing 0.3 mg of chlorophyll, 0.5 mg of spinach ferredoxin, 4 mg of spinach nitrite reductase, and 200 µmoles of Tris buffer, pH 8.0. Where indicated, 4 µmoles of $NaNO_2$, 4 µmoles of TPN^+, or 3 µmoles of KCN were added. Other experimental conditions were the same as in Table I.

Acceptor	Addition	Oxygen evolved
		µatoms
NO_2^-		3.1
NO_2^-	Cyanide	0
TPN^+		3.2
TPN^+	Cyanide	3.1

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With the hydrogen-*Clostridium* hydrogenase system, it was possible to demonstrate that spinach nitrite and TPN^+ reductases differed not only in their sensitivities to KCN (Table III), but also in their inactivation by heating. As shown in Table IV, nitrite reductase activity disappeared after the enzyme was heated for 10 minutes at 60°, whereas TPN reductase was not affected by the same treatment.

Losada *et al.* (10) found that the dark reduction of nitrite with TPNH, catalyzed by the spinach chloroplast extract, was ferredoxin-dependent. They suggested that TPNH transferred its electrons to ferredoxin with the TPN reductase also present in the chloroplast extract in a reaction which would be the reverse of that studied by Tagawa and Arnon (11); *i.e.* spinach ferredoxin could mediate the transfer of electrons from the TPNH-TPN reductase system to the nitrite-nitrite reductase system. Recent reports from Arnon's laboratory (14) have shown that spinach TPN-reductase in fact catalyzes the reversible reduction of TPN^+ by ferredoxin. In *C. pasteurianum*, however, the reaction seems not to be reversible. According to Valentine ferredoxin does not mediate the evolution of H_2 (19) or the reduction of nitrite (30) from TPNH. Since our spinach nitrite reductase preparation contained also TPN reductase, we have tested its ability to reduce nitrite from TPNH. As Table V shows, the enzymic oxidation of TPNH by nitrite required the presence of spinach ferredoxin.

TABLE IV

Effect of heating on spinach nitrite reductase and TPN reductase

The experimental conditions were the same as in Fig. 3, except that all the vessels included 1 mg of *Clostridium* ferredoxin, and 4 μmoles of $NaNO_2$ or TPN^+ were added as indicated. The heat treatment of the spinach nitrite reductase preparation containing TPN reductase was carried out at 60° for 10 minutes.

Acceptor	Treatment	H_2 uptake
		μmoles
NO_2^-	None	2.6
NO_2^-	Heat	0.3
TPN^+	None	3.0
TPN^+	Heat	3.0
TPN^+		0.0

TABLE V

Ferredoxin as electron carrier in dark oxidation of TPNH with nitrite as electron acceptor

The reaction mixture included, in a final volume of 3 ml, spinach ferredoxin, 0.5 mg; spinach nitrite reductase (containing TPN reductase) 2 mg; Tris buffer, pH 8.0, 150 μmoles; sodium nitrite, 6 μmoles; and TPNH, 4 μmoles. The reaction was run at 20°, under argon, for 20 minutes. The oxidation of TPNH was measured by the change in optical density at 340 mμ. The endogenous oxidation in the absence of nitrite was subtraced from the observed rates.

Reaction system	TPNH oxidized
	μmoles
Complete	1.0
Spinach ferredoxin omitted	0
Nitrite reductase omitted	0.2

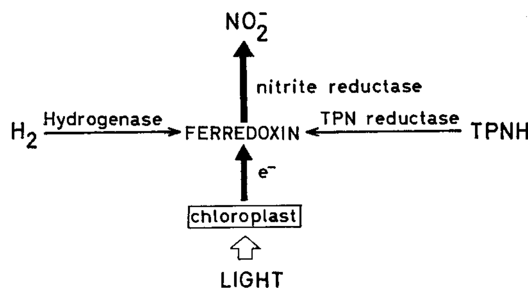

FIG. 4. Diagrammatic representation of the role of ferredoxin in the reduction of nitrite. The transfer of electrons from ferredoxin to nitrite is a dark reaction catalyzed by the chloroplast nitrite reductase. Ferredoxin itself can be reduced either in the light (by grana) or in the dark (by hydrogen gas or reduced triphosphopyridine nucleotide).

The role of ferredoxin in the photochemical and dark reduction of nitrite catalyzed by spinach nitrite reductase is diagrammatically represented in Fig. 4.

SUMMARY

Reduction of nitrite by a reconstituted enzymic system both in the dark and in the light has been investigated.

In the light, and in the presence of spinach grana, ferredoxin, and spinach nitrite reductase, nitrite can act as the terminal electron acceptor in a new type of noncyclic photophosphorylation. Substrate amounts of nitrite are reduced to ammonia, and this reaction is accompanied by the evolution of oxygen and the formation of adenosine triphosphate. The relation between moles of nitrite reduced, moles of ammonia produced, moles of orthophosphate esterified, and atoms of oxygen evolved is 1:1:3:3.

In the dark, and in the presence of ferredoxin and spinach nitrite reductase, nitrite can be reduced either by the hydrogen-*Clostridium* hydrogenase system or by the reduced triphosphopyridine nucleotide-spinach triphosphopyridine nucleotide reductase system.

Clostridium ferredoxin substitutes for spinach ferredoxin in the reduction of nitrite by spinach nitrite reductase, the first being a more effective electron carrier in the dark with the hydrogen-hydrogenase system, and the second in the light, when the electrons are supplied by the illuminated grana.

Noncyclic photoreduction of TPN^+ is not affected by cyanide under conditions which completely inhibit the electron flow to nitrite.

Spinach nitrite reductase is destroyed by heating at 60° for 10 minutes, whereas spinach TPN reductase is not affected by the same treatment.

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