MECHANISM OF NITRITE REDUCTION IN CHLOROPLASTS

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In a previous paper, Paneque, del Campo, and Losada (1963) have shown that chloroplasts isolated from spinach leaves can use nitrite as terminal electron acceptor in the electron transport chain characteristic of the non-cyclic photophosphorylation reaction (Arnon et al., 1961). The photoreduction of nitrite required in addition to grana the presence of chloroplast extract and did not proceed when the latter was heated. No external substrates (except water which acted as electron donor) or cofactors were needed for the light reaction to take place. After blocking the system involved in the photooxidation of water (Losada, Whatley and Arnon, 1961) the couple ascorbate-dichlorophenol indophenol could be used as electron donor instead of water.

It has been shown that ferredoxins can be reduced, under suitable conditions, either in the dark or in the light (Mortenson et al., 1962; Tagawa and Arnon, 1962), and that in green plants the reduction of nitrite itself is a dark process (Roussos and Nason, 1960; Hageman et al., 1962; Paneque et al., 1963).

The present report is concerned with the mechanism involved in the photosynthetic and dark reduction of nitrite, and confirms the suggestion that ferredoxin is the natural electron carrier of the reaction.

METHODS

The experiments were carried out under argon at 20°C in Warburg vessels kept in the dark or illuminated from below by a 100 watt fluorescent lamp providing approximately 20,000 luces.

Once washed chloroplast fragments and chloroplast extract were prepared from spinach according to Whatley et al. (1959). Spinach ferredoxin was obtained in a highly purified form as described by Tagawa and Arnon (1962). Ferredoxin-free chloroplast extract was prepared by passing 5 ml. of fresh extract in 0.07 M Tris buffer, pH 7.3, (equivalent to about 8 mg. chlorophyll) through a 3 x 1 cm. column of diethylaminoethyl-cellulose (DEAE) equilibrated with the same buffer. Inactivation of DEAE-treated chloroplast extract was brought about by heating 5 minutes in a boiling water bath.

Nitrite and hydroxylamine were estimated in the supernatant, after spinning down the green particles, by the method of Novak and Wilson (1948). The oxidation of reduced triphosphopyridine nucleotide was measured by the change in optical density at 340 millimicrons, in cuvettes with a light path of 1 cm.

RESULTS

As shown in Table I, the photoreduction of nitrite requires in addition to washed chloroplasts fragments, ferredoxin and a thermolabile substance (presumably nitrite reductase) contained in the chloroplast extract. This latter factor remains in the chloroplast extract even when it has been passed through the DEAE column which adsorbs the ferredoxin.

TABLE I

EFFECT OF FERREDOXIN AND CHLOROPLAST EXTRACT IN THE PHOTOREDUCTION OF NITRITE

	Treatment	NO_2^- reduced (micromoles)	O_2 evolved (micromoles)
1.	Complete	1.2	1.7
2.	Fd omitted	0.3	0.2
3.	Fd-free CE omitted	0.7	0.7
4.	Fd-free CE heated	0.3	0.4

The reaction mixture includes in a final volume of 3 ml.: once washed chloroplast fragments containig 1 mg. chlorophyll, 150 micromoles Tris buffer, pH 8.0, 6 micromoles Na NO_2, and when indicated, 0.05 ml. of a ferredoxin (Fd) purified solution, and normal or heated ferredoxin-free chloroplast extract (Fd-free CE) equivalent to 1.3 mg chlorophyll. Incubation time, 25 minutes. Other conditions as described under METHODS.

The reduction of nitrite in the light is accompanied by the evolution of oxygen in a ratio of about 1 micromole of nitrite reduced per 1.5 micromoles of oxygen evolved. This seems to indicate that nitrite is being reduced up to the level of ammonia, a conclusion supported by the fact that hydroxylamine is also reduced by the chloroplast fragments in the presence of

ferredoxin and a factor (probably hydroxylamine reductase) present in the chloroplast extract which passes through the DEAE column (Table II).

TABLE II

EFFECT OF FERREDOXIN AND CHLOROPLAST EXTRACT IN THE PHOTOREDUCTION OF HYDROXYLAMINE

	Treatment	NH_2OH reduced (micromoles)	O_2 evolved (micromoles)
1.	Complete	6.1	2.2
2.	Fd omitted	1.7	1.2
3.	Fd-free CE omitted	2.4	1.3

Experimental conditions as in Table I except that 18 micromoles of hydroxylamine were added instead of nitrite.

The fact that chloroplasts can reduce nitrite up to ammonia completes the findings of Losada, Trebst and Arnon (1959) that chloroplast extract can incorporate ammonia into glutamate, glutamine, aspartate and alanine as it contains the enzymes glutamate dehydrogenase, glutamine synthetase, aspartate glutamate transaminase and alanine-glutamate transaminase.

In some experiments we have observed that nitrite and hydroxylamine are appreciably reduced by a system containing chloroplast fragments and ferredoxin even in the absence of the DEAE-treated chloroplast extract (treatment 3 in Tables I and II). This suggests that the factors required for NO_2^- or NH_2OH reduction are partially bound to the chloroplast fragments, as happens in the similar case found by Tagawa and Arnon (1962) of

triphosphopyridine nucleotide reduction by a flavoprotein present in the broken, washed chloroplasts.

TABLE III

EFFECT OF FERREDOXIN AND CHLOROPLAST EXTRACT IN THE DARK OXIDATION WITH NITRITE OF REDUCED TRIPHOSPHOPYRIDINE NUCLEOTIDE

	Treatment	$TPNH_2$ oxidized (micromoles)
1.	Complete	2.6
2.	NO_2^- omitted	0.8
3.	Fd omitted	0.5
4.	Fd-free CE omitted	1.2
5.	Fd-free CE heated	0.8

Experimental conditions as in Table I except that the reactions were carried out in the absence of chloroplast fragments, 5 micromoles of reduced triphosphopyridine nucleotide ($TPNH_2$) were added as the electron donor, and chloroplast extract equivalent to 1.9 mg. chlorophyll was used.

The dark reduction of nitrite with reduced triphosphopyridine nucleotide in the presence of chloroplast extract reported by Paneque *et al.* (1963) is also ferredoxin dependent, as shown in Table III. It may be suggested that in these experiments, reduced triphosphopyridine nucleotide transfers its electrons to ferredoxin with the aid of the flavoprotein present in the chloroplasts through a reaction which would be the reverse of that studied by Tagawa and Arnon (1962). Once ferredoxin has been reduced it can donate its electrons to nitrite by the mechanism discussed above.

REFERENCES

Arnon, D. I., M. Losada, F. R. Whatley, H. Y. Tsujimoto, D. O. Hall, and A. A. Horton, Proc. Nat. Acad. Sci. 47, 1314 (1961).
Hageman, R. H., C. F. Cresswell, and W. J. Hewitt, Nature 193, 247 (1962).
Losada, M., A. V. Trebst, and D. I. Arnon, Presented by D. I. Arnon at the 9th Inter. Bot. Congr., Montreal, 1959.
Losada, M., F. R. Whatley, and D. I. Arnon, Nature 190, 606 (1961).
Mortenson, L. E., R. C. Valentine, and J. E. Carnahan, Biochem. Biophys. Res. Comm. 7, 448 (1962).
Novak, R., and P. W. Wilson, J. Bact. 55, 517 (1948).
Paneque, A., F. F. del Campo, and M. Losada, submitted to Nature.
Roussos, G. G., and A. Nason, J. Biol Chem. 235, 2997 (1960).
Tagawa, K., and D. I. Arnon, Nature 195, 537 (1962).
Whatley, F. R., M. B. Allen, and D. I. Arnon, Biochim. et Biophys. Acta 32, 32 (1959).