MECHANISM OF NITRATE REDUCTION IN CHLOROPLASTS[x]

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The effect of light in the reduction of nitrate by green plants is well known, but the explanation of the phenomenon is an unresolved question. Evans and Nason (1953) and Jagendorf (1956) concluded that photochemically reduced triphosphopyridine nucleotide provided the reducing power, but Stoy (1956) proposed riboflavin as the light-absorbing catalyst, and Kessler (1959) considered the action of light to be a complicated phenomenon contributing to the supply of hydrogen donors, energy-rich phosphate bonds, and carbon compounds.

In recent work from this laboratory (Losada et al. 1963; Paneque et al. 1964), it was shown that nitrite could act in the light and in the presence of grana, ferredoxin, and nitrite reductase from spinach chloroplasts, as terminal electron acceptor in a new type of non-cyclic photophosphorylation, similar but not identical to that studied by Arnon

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and his associates with TPN[x] (Arnon 1963; Tagawa and Arnon 1962). The light reduction of nitrite to ammonia did not require pyridine nucleotide and was accompanied by the formation of stoichiometric amounts of ATP. It was also shown (Del Campo et al., 1963) that, under similar conditions, ferredoxin, either alone or plus TPN, was uneffective in mediating the reduction of nitrate, and that benzyl viologen could act as electron carrier in the process.

The present communication reports the finding of a new type of non-cyclic photosynthetic electron flow, in which flavin-nucleotides, the wellknown natural cofactors of cyclic photophosphorylation (Arnon, 1963), mediate the direct transfer of electrons from illuminated grana to nitrate with the aid of nitrate reductase, and shows that neither ferredoxin nor pyridine nucleotide nor ATP are required for the photoreduction of nitrate to take place. A comparative study of the light and dark reduction of nitrate by spinach enzymes will be given elsewhere.

METHODS.- The experiments were carried out under argon in Warburg manometer flasks illuminated from below by a 100 watt fluorescent lamp and from above by a set of reflector flood lamps at a distance of 3 and 14 cm, respectively, from the reaction vessels. Reaction time, 30 minutes.

Nitrate reductase was purified from spinach leaves by a procedure which included the following steps: a) preparation of a crude homogenate by grinding the leaves in 25 mM

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x Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; ATP, adenosine triphosphate; TPN, oxidized triphosphopyridine nucleotide; Fd, spinach ferredoxin; BV, benzyl viologen; DPIP, 2, 6-dichlorophenolindophenol.

Tris buffer, pH 8.0, containing 0.66 mM cysteine; b) centrifugation at 27,000 g for 20 min to get rid of the particles; c) removal of ferredoxin from the supernatant by passing through a DEAE-cellulose bed (Losada et al., 1963); d) adsorption of the nitrate reductase which came through the column on calcium phosphate gel (1 mg per mg of protein); e) washing of the gel with 0.1 M phosphate buffer, pH 7.0; f) elution of nitrate reductase from the gel with 0.1 M sodium pyrophosphate, pH 7.0; g) concentration of the enzyme by precipitation with ammonium sulfate at 50 per cent saturation.

Washed broken chloroplasts were prepared from spinach according to Whatley et al. (1959). When the system involved in the photooxidation of water was blocked by heating the grana at 55° for 5 min (Losada et al., 1961; Paneque and Arnon, 1962), the system cystein- or ascorbate-DPIP was used as electron donor instead of water. Spinach ferredoxin was obtained by the method of Tagawa and Arnon (1962). Nitrite was estimated according to Novak and Wilson (1948) with alkaline iodine to eliminate interference by ascorbate (Hewitt and Betts, 1963). Ammonia was determined by nesslerization after distillation and absorption of the gas in 0.01 N sulfuric acid in Conway units (Conway, 1957). Protein was assayed by the method of Warburg and Christian (1941).

RESULTS.- Table I shows the light-dependent reduction of nitrate by a chloroplast system in which the photoevolution of oxygen had been suppressed and the electrons were supplied by the couple ascorbate-DPIP. The reaction did not proceed in the dark and required FAD in addition to nitrate reductase. The product of nitrate reduction was found to be nitrite.

TABLE I

CHARACTERIZATION OF NITRATE PHOTOREDUCTION IN A RECONSTITUTED CHLOROPLAST SYSTEM

System	Nitrite formed (micromoles)
Complete	5.02
Nitrate omitted	0
Ascorbate-DPIP omitted	0.06
Grana omitted	0.06
FAD omitted	0
Nitrate reductase heated	0
Complete, dark	0

The reaction mixture included in a final volume of 3 ml: once washed broken chloroplasts, heated at 55° for 5 min, containing 0.2 mg chlorophyll; nitrate reductase, 4 mg, and the following in micromoles: sodium phosphate buffer, pH 7.0, 200; FAD, 0.5; ascorbate, 20; DPIP, 0.2; potassium nitrate, 10. Temperature, 27°. Other conditions as described under METHODS.

Table II shows the effect of different catalysts of photosynthetic phosphorylation on the photoreduction of nitrate by a chloroplast system in which the electrons were supplied by water. FMN or FAD were found to be the physiological cofactors which mediated the transfer of electrons from illuminated grana to the nitrate-nitrate reductase system. Menadione and ferredoxin were not capable of replacing the flavin nucleotides, but BV (or methyl viologen) was an effective electron carrier in the system. Similar results were obtained when the couple ascorbate-DPIP or cysteine-DPIP were substituted for water as electron donor after blocking by heating the first light reaction.

TABLE II

EFFECT OF DIFFERENT COFACTORS OF PHOTOSYNTHETIC PHOSPHORYLATION ON THE REDUCTION OF NITRATE BY ILLUMINATED CHLOROPLASTS

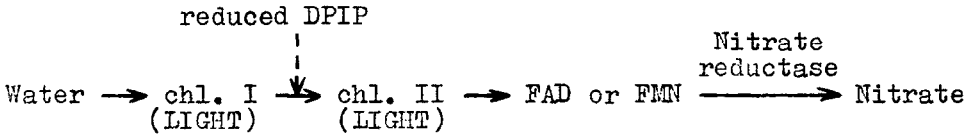
Addenda	Nitrite formed (millimicromoles)
BV	615
FMN	660
FAD	585
Menadione	60
Fd	38
None	0

The reaction mixture included in a final volume of 3 ml: once washed broken chloroplasts containing 0.2 mg chlorophyll; nitrate reductase, 4 mg; sodium phosphate buffer, pH 7.0, 200 micromoles; potassium nitrate, 10 micromoles, and where indicated, BV, 1 micromole; FMN, 0.2 micromoles; FAD, 0.5 micromoles; menadione, 0.5 micromoles; Fd, 0.9 mg. Temperature, 20° C. Other conditions as described under METHODS.

Shin et al. (1963) have shown that spinach TPN reductase can catalyze the oxidation of reduced triphosphopyridine nucleotide by FMN or FAD. The experiments just described have revealed that these flavin nucleotides can mediate the transfer of electrons from illuminated grana to the nitrate-nitrate reductase system in the absence of pyridine nucleotides. It seems, therefore, likely that the flavin enzyme from green leaves known as pyridine nucleotide-nitrate reductase (Nason, 1963) may be in fact a mixture of TPN reductase and nitrate reductase itself, as was found to be the case for the similar enzyme, pyridine nucleotide-nitrite reductase, now known to be

ferredoxin dependent (Losada et al., 1963; Paneque et al., 1964).

According to present knowledge, the results discussed above on the photosynthetic reduction of nitrate can be schematically represented by the following sequence of reactions:



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