NITRATE AS A HILL REAGENT IN A RECONSTITUTED CHLOROPLAST SYSTEM

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1. Introduction

Nitrate is not only the principal source of nitrogen in higher plants but, as pointed out recently by Warburg [1,2], the first Hill reagent that was discovered when he and Negelein found in 1920 that living *Chlorella* cells suspended in solutions of nitrate to which no CO_2 was added, developed oxygen in the light with the concomitant reduction of nitrate to ammonia in accordance with the equation:

$$HNO_3 + H_2O = NH_3 + 2O_2 .$$

According to Warburg's original interpretation [1,2] the photochemical reduction of nitrate coupled to the evolution of oxygen is the result of two partial reactions: 1) a dark reaction in which the carbohydrate of the cells is oxidized to CO_2 by nitrate, and 2) a light reaction in which CO_2 is subsequently converted to carbohydrate and O_2. His conclusion is therefore that the balance reaction written above is deceiving in the sense that what appears to be a photolysis of water is in its mechanism a photolysis of CO_2.

The present report is concerned with the light reduction of nitrate to nitrite and ammonia coupled with the stoichiometric evolution of oxygen in a reconstituted chloroplast system under well characterized conditions which prevent the occurrence of CO_2 assimilation. The results corroborate previous work from our laboratory on this subject [3–8].

2. Materials and methods

The experiments were carried out under nitrogen at 18° in Warburg manometer flasks containing 0.1 ml of 20% KOH in the center well. Light was provided from below by a bank of 100 watt reflector flood lamps, illumination being of 10,000 luces at the level of the reaction vessels.

Once-washed broken chloroplast was prepared from spinach as described by Whatley and Arnon [9]. Spinach ferredoxin was obtained by the method of Tagawa and Arnon [10]. Ferredoxin-NADP reductase was purified from spinach according to Shin et al. [11] except for the omission of the last chromatographic step. Preparation of spinach NADH-nitrate reductase and ferredoxin-nitrite reductase as well as other technical details have been reported elsewhere [3–8].

3. Results

3.1. *Photoreduction of nitrate to nitrite*

Table 1 shows that, in the light and in the presence of the necessary electron carriers and enzymes, nitrate was reduced to nitrite by fresh spinach chloroplast fragments. Since water was the ultimate electron donor, the reduction of nitrate was not only light-dependent but coupled to the production of oxygen. The addition of low concentrations of DCMU to the system completely abolished both oxygen evolution and nitrate reduction. Ferredoxin could not by itself serve as electron carrier in mediating the transfer of electrons from illuminated grana to the nitrate-nitrate reductase system. It could only act by reducing first NAD with the aid of NADP reductase. No reduction of nitrate was observed when NADP substituted for NAD (cf. ref. [7]).

Table 1
Photoreduction of nitrate to nitrite coupled with oxygen evolution in a reconstituted chloroplast system.

System	NO_2^- formed (μmoles)	O_2 evolved (μatoms)
Complete	4.5	3.8
Minus nitrate reductase	0.0	0.0
Minus KNO_3	0.0	0.0
Minus NAD	0.0	0.0
Minus ferredoxin	1.9	1.3
Minus NADP reductase	0.6	0.5
Complete, dark	0.0	0.0
Complete, plus DCMU	0.0	0.0

The complete reaction mixture included, in a final volume of 3 ml, once-washed broken chloroplasts containing 0.4 mg chlorophyll; nitrate reductase, 0.6 mg; ferredoxin, 0.5 mg; NADP reductase, 3.9 mg; and the following in μmoles: Tris, pH 7.5, 200; NAD, 0.45; KNO_3, 10. Where indicated, 0.01 μmoles DCMU (dichlorophenyl dimethyl urea) were added. Reaction time was 21 min.

Table 2
Stoichiometry of nitrite formation and oxygen evolution in the photoreduction of nitrate to nitrite by a reconstituted chloroplast system.

NO_3^- added (μmoles)	NO_2^- produced (μmoles)	O_2 evolved μatoms)
0	0.0	0.2
1	0.9	1.1
2	1.8	1.8
3	2.8	2.8

The experimental conditions were the same as in the complete system of table 1, except that 0.2 mg nitrate reductase and 1.5 mg NADP reductase were used. KNO_3 was added as indicated. The reactions were run to completion.

As can be seen in table 2, substrate amounts of nitrate were reduced to nitrite and oxygen was evolved in accordance with the equation:

$$NO_3^- + H_2O \xrightarrow{\text{light}} NO_2^- + H_2O + \tfrac{1}{2}O_2 .$$

Under the appropriate experimental conditions and in the presence of orthophosphate and ADP, the photoreduction of nitrate and the coupled photooxidation of water were accompanied by the formation of ATP.

Table 3
Photoreduction of nitrate to ammonia coupled with oxygen evolution in a reconstituted chloroplast system.

System	O_2 evolved (μatoms)	NO_2^- formed (μmoles)	NH_3 formed μmoles)
Complete	7.0	0.5	1.8
Minus nitrate reductase	0.0	0.0	0.0
Minus NAD	0.0	0.0	0.0
Minus nitrite reductase	3.2	3.4	0.1

The experimental conditions were the same as in the complete system of table 1, except that 0.5 mg nitrite reductase was also added.

3.2. *Photoreduction of nitrate to ammonia*

Table 3 shows the gradual reduction of nitrate to nitrite and ammonia coupled to the evolution of oxygen by fresh iluminated grana. The total reaction proceeded in two steps: 1) the reduction of nitrate to nitrite, depending, as mentioned above, on ferredoxin, NADP reductase, NAD and nitrate reductase; and 2) the reduction of nitrite to ammonia, requiring, as described elsewhere [3,5,6], ferredoxin and nitrite reductase. If either the cofactors or the enzymes needed for the reduction of nitrate to nitrite were omitted from the complete reaction mixture, the first step was blocked and no reaction occurred. If, on the other hand, only nitrite reductase was absent, the reduction of nitrate went on up to nitrite but no ammonia was formed.

In conclusion, nitrate can act in the absence of CO_2 and outside of the living cell as a Hill reagent, according to the overall equation:

$$NO_3^- + 4H_2O + 2H^+ \xrightarrow{\text{light}} NH_4^+ + 3H_2O + 2O_2$$

4. Discussion

The experiments just described have demonstrated that, in the light and in the absence of added flavin nucleotides, spinach grana supplemented with ferredoxin, NAD and ferredoxin-NADP reductase can couple the oxidation of water with the reduction of nitrate to ammonia. This is catalysed in a stepwise

fashion by NADH-nitrate reductase and ferredoxin-nitrite reductase. Since the experiments have been carried out by using grana and under conditions that prevent CO_2 assimilation, the possibility of carbohydrates being first synthetized in light from CO_2 and then used as electron donors for the dark reduction of nitrate may be excluded. It might be thought, however, that even under these conditions, residual CO_2 has a catalytic function in the photochemical reaction itself, as pointed out by Hill [12]. The fact reported by Warburg et al. [2] that oxygen evolution by whole cells of *Chlorella* illuminated in the presence of nitrate completely ceases when CO_2 is taken away with KOH, remains very interesting and is probably related to an unknown metabolic control.

The question then arises whether the reactions shown to operate *in vitro* in a reconstituted chloroplast system are also active *in vivo* in the whole cell. Present evidence based on the intracellular localization and properties of the pertinent enzymes tends to support this view.

In previous work from our laboratory [5,6] it was shown that, in spinach, nitrate and nitrite reductases are located, at least partly, in the chloroplasts. It is, however, contradictory that whereas Ritenour et al. [13] have reported that nitrate reductase was not associated with chloroplasts isolated by aqueous or nonaqueous techniques from seedlings of corn and foxtail, Coupe et al. [14] estimated by using the second technique that nearly 60% of the total enzyme activity in barley leaves was present in the chloroplasts. In spinach, Heber and French [15] have communicated that when nitrate was added to intact chloroplasts, its reduction in the light was concomitant with the evolution of oxygen. In algae, Hattori and Myers [16] have obtained, by sonication of *Anabaena cylindrica* cells, a subcellular preparation which showed a high activity of photochemical system I associated with a high activity of nitrate reductase.

It is, finally, of interest to compare the role of ferredoxin as an electron mediator in the reduction of nitrate and nitrite by spinach chloroplast fragments and by *Anabaena* preparations. In spinach systems, photochemically reduced ferredoxin can donate electrons directly to nitrite reductase, but it can only react with nitrate reductase by first reducing NAD through the action of NADP reductase. In *Anabaena* preparations, however, both nitrate reductase and nitrite reductase can directly accept electrons from ferredoxin, although nitrate reductase prepared by acetone treatment can, in addition, be reduced by NADH [16].

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