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Light-dependent Assimilation of Nitrite by Isolated Pea Chloroplasts[1]

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ABSTRACT

Chloroplasts were prepared from peas (*Pisum sativum*) in glucose-phosphate medium. In the presence of DL-glyceraldehyde, they catalyzed nitrite-dependent O_2 evolution (mean of 13 preparations, 17.5 μmole per mg chlorophyll per hour, SD 3.64). The optimum concentration of nitrite was 0.5 mM; 0.12 mM nitrite supported $V_{max}/2$. The reaction was accompanied by the consumption of nitrite; 55 to 80% of the nitrite-N consumed was recovered as ammonia. In short experiments (less than 10 minutes) the O_2 to nitrite ratio approached 1.5, but thereafter decreased. There was no nitrite-dependent O_2 evolution with chloroplasts from plants grown without added nitrate but such chloroplasts could assimilate ammonia at about the usual rate. The results are consistent with the reduction of nitrite to ammonia involving nitrate-induced nitrite reductase and a reductant generated by the chloroplast electron transport chain.

In the presence of ADP, pyrophosphate, and $MgCl_2$ the O_2 to nitrite ratio was typically 0.5 to 0.6 and the recovery of nitrite-N as ammonia about 60%. Under these conditions, α-ketoglutarate increased the O_2 to nitrite ratio (0.9–1.35) and the recovery of nitrite-N as ammonia decreased to 27%. These data and the results of nitrite plus ammonia addition experiments (with and without α-ketoglutarate) are attributed to incorporation of nitrite-N into glutamate via the chloroplast enzymes nitrite reductase, glutamine synthetase, and glutamate synthetase.

The enzymes glutamine synthetase and glutamate synthetase, which catalyze reactions now known to be basic for N assimilation, have been located in chloroplasts (7, 11, 13) and shown to be coupled to light-dependent electron transport. Thus, glutamate synthetase activity can be monitored by (glutamine plus α-ketoglutarate)-dependent O_2 evolution (2). In the presence of the cofactors ADP, PPi, and Mg^{2+}, pea chloroplasts also catalyze (ammonia plus α-ketoglutarate)-dependent O_2 evolution which we attributed to assimilation of ammonia into glutamate via photosynthetically coupled[4] glutamine synthetase and glutamate synthetase (1).

Spinach chloroplasts in the light actively reduce nitrite (9, 10, 14); approximately 60 to 90% of the nitrite-N consumed is incorporated into amino-N (9, 10). Spinach chloroplasts also catalyze nitrite-depentent O_2 evolution (10). According to current theory, nitrate-N is reduced to nitrite in the cytoplasm and further reduced to ammonia by nitrite reductase within the chloroplast. The ammonia so formed is incorporated via glutamine into glutamate in the chloroplast in reactions catalyzed by glutamine synthetase and glutamate synthase. In this event, nitrite-N should compete with glutamine-N and ammonia-N in the latter stages of the N assimilation pathway. We previously reported, however, that nitrite-dependent and (glutamine plus α-ketoglutarate)-dependent O_2 evolution activities catalyzed by pea chloroplasts did not compete (2) implying that ammonia produced from nitrite was not assimilated by pea chloroplasts under the prevailing experimental conditions. We give a report in this paper of a study of light-dependent reduction and assimilation of nitrite by pea chloroplasts under conditions which (*a*) support and (*b*) do not support (ammonia plus α-ketoglutarate)-dependent O_2 evolution.

MATERIALS AND METHODS

Seedlings of pea (*Pisum sativum* cv. Feltham First) were used. The methods for growing peas, measurement of Chl and chloroplast preparation (referred to in this paper as method 1) were as described previously (2). O_2 evolution was measured polarographically and calibrated against air-saturated water at 25 C as before (2). Chloroplasts prepared by method 1 were incubated in sorbitol-HEPES medium (2) and were known to catalyze (glutamine plus α-ketoglutarate)-dependent O_2 evolution and, in the presence of ADP, $MgCl_2$ and PPi (ammonia plus α-ketoglutarate)-dependent O_2 evolution (1, 2). Some aspects of nitrite reduction by method 1 chloroplasts were compared with chloroplasts used in a previous study of nitrite reduction (10). Method 2 chloroplasts were prepared essentially as described by Miflin (10) except that sorbitol was replaced by sucrose and with L-cysteine at 2 mM. Chloroplasts prepared by method 2 were incubated in sucrose-PPi medium (10). The composition of the extracting media were as follows: method 1: 0.33 M glucose, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, 5 mM $MgCl_2$, 0.1% (w/v) NaCl, 0.2% (w/v) sodium isoascorbate, 0.1% (w/v) BSA (type V) adjusted to pH 6.5 with KOH; method 2: 0.33 M sucrose, 1 mM $MgCl_2$, 2 mM EDTA, 2 mM L-cysteine, 10 mM $Na_4P_2O_7$ adjusted to pH 7 with HCl. As summarized above, the incubating media were also different for the two methods (2, 10). Chloroplast intactness was determined by the ratio of the O_2 evolution rates of shocked and unshocked chloroplasts using ferricyanide as electron acceptor (8).

Nitrite-dependent O_2 evolution was measured at 25 C under two conditions. Condition 1 did not support (ammonia plus α-ketoglutarate)-dependent O_2 evolution. Reaction mixtures contained 0.5 mM sodium nitrite, 10 mM DL-glyceraldehyde and chloroplasts (50–150 μg of Chl) in a volume of 2 ml. Condition 2 supported (ammonia plus α-ketoglutarate)-dependent O_2 evolution (1). Incubation mixtures (2 ml) containing 4.5 mM ADP, 5 mM PPi, 10 mM $MgCl_2$, and 10 mM DL-glyceraldehyde and chloroplasts (100–150 μg of Chl) were preincubated for 10 min with or without 2.5 mM α-ketoglutarate and the reaction initiated

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[4] Definition: the term "photosynthetically coupled" is used throughout this paper to refer to the generation of a reductant by photosystems I and II (*e.g.* reduced ferredoxin or NADPH) which is used as a substrate in the associated reaction.

with 0.5 mM sodium nitrite. (Ammonia plus α-ketoglutarate)-dependent O_2 evolution and all other substrate-dependent O_2 evolution activities were measured at 25 C as before (1, 2).

The procedure for estimation of ammonia was based on a method adopted for the determination of relatively small amounts of free ammonia in samples containing amides (3). A very slow stream of acid-washed N_2 gas was used to transfer 3 ml of saturated sodium tetraborate (adjusted to pH 10 with NaOH) from a tall tube (approximately 25 × 2.5 cm) into a similar one containing 2 to 4 ml of a mixture of sample with four times its volume of ethanol. The gas was next led into 5 ml of 0.02 M H_2SO_4 in another tube. Froth formation was controlled by addition of approximately 50 μl of octanol. Standard amounts of ammonia, estimated by the method of Kaplan (6), were recovered quantitatively after 30-min rapid gas flow.

Nitrite was measured by the method of Miflin (10). The decrease in nitrite in reaction mixtures containing this substrate was taken as a measure of nitrite reduction. Nitrate reductase was measured in crude extracts (without an osmoticum) as described by Scholl *et al.* (16).

RESULTS

O_2 Evolution and Nitrite Reduction for Condition 1. DL-Glyceraldehyde (10 mM) was usually added to reaction mixtures. It inhibited endogenous O_2 evolution and enhanced the rate of nitrite-dependent O_2 evolution (Fig. 1). In the absence of glyceraldehyde, the rate of nitrite-dependent O_2 evolution (which was always measured after endogenous O_2 evolution ceased, Fig. 1) gradually decreased. In the presence of glyceraldehyde, however, the rate of nitrite-dependent O_2 evolution was constant for at least 5 min (Figs. 1 and 2). Provided measurements were taken during this period the molar ratio of O_2 evolved to nitrite reduced approached the theoretical value of 1.5 (Fig. 2). Molar ratios substantially less than this value were found (in the presence of glyceraldehyde) for some experiments (Table I). In general, low ratios were associated with those experiments which either exhibited a large decrease in the rate of O_2 evolution during the incubation or were measured over a long time period. This is shown by the data in Figure 3; the molar ratio decreased from approximately 1.2 at 10 min to about 0.7 after 50 min. Certain of the results in Table I show that glyceraldehyde also led to higher rates of nitrite reduction and higher molar ratios. Nitrite-dependent O_2 evolution ceased abruptly in the dark and was completely inhibited by 1.7 μM DCMU (Fig. 2). Chloroplasts also failed to catalyze nitrite reduction under these conditions.

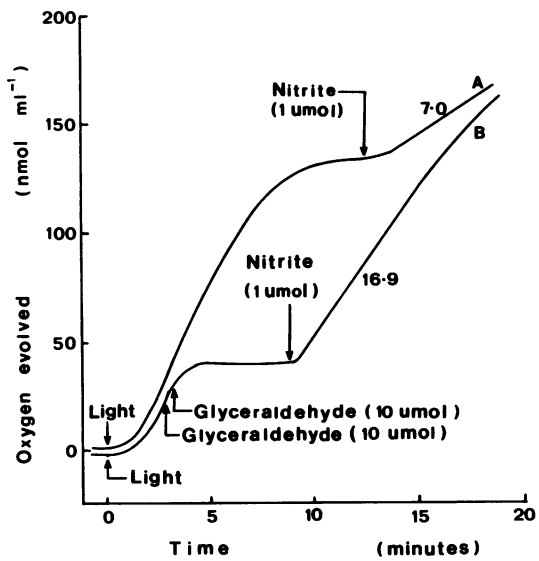


FIG. 1. Effect of DL-glyceraldehyde and nitrite on O_2 evolution by method 1 chloroplasts for condition 1. The reaction mixtures (A and B) initially contained chloroplasts only; additions were made as shown. Reactions were timed from the moment of illumination. Values beside the curves represent rates of O_2 evolution in μmol mg of Chl^{-1} hr^{-1}. Chl concentration, 50 μg ml^{-1}; chloroplast intactness, 74%; volume, 2 ml.

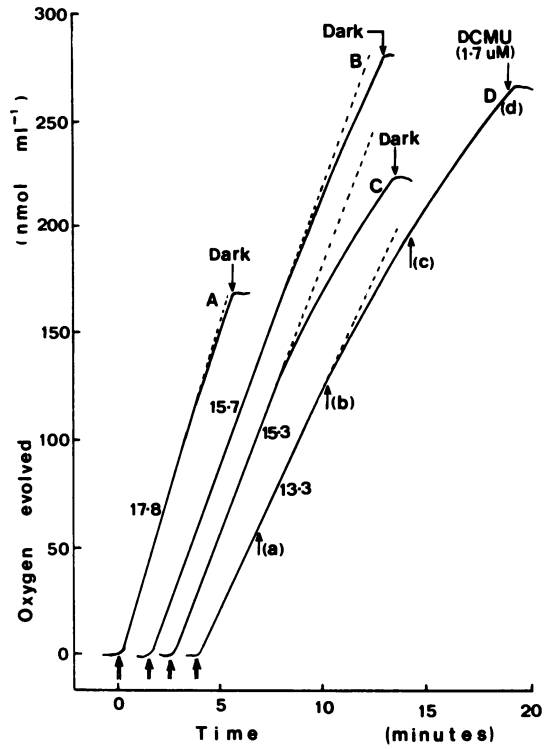


FIG. 2. Typical nitrite-dependent O_2 evolution curves for short term experiments and the associated nitrite reduction catalyzed by method 1 chloroplasts under condition 1. Double-tailed arrows show the time of illumination. Samples were removed for nitrite estimations immediately prior to illumination and again at the times indicated by single-tailed arrows. Other treatments were as shown. Appropriate details for each experiment (A to D) were as follows:

Experiment	Chl concentration	Intactness	Time of sampling for nitrite	Nitrite consumed	O_2:nitrite ratio
	(μg ml^{-1})	(%)	(min)	(nmol ml^{-1})	
A	100	69	5.7	103	1.62
B	100	78	11.5	232	1.19
C	100	75	10.8	223	1.18
D	95.2	62	3.0(a)	47	1.25
			6.5(b)	82	1.52
			10.5(c)	127	1.52
			15.2(d)	183	1.43

All reactions mixtures contained 0.5 mM nitrite and 10 mM DL-glyceraldehyde. Values beside the curves represent rate of O_2 evolution in μmol mg of Chl^{-1} hr^{-1}.

When the nitrite concentration was progressively increased, the rate of O_2 evolution increased sharply with nitrite concentration up to 0.5 mM ($V_{max}/2$ = 0.12 mM) followed by a more gradual decrease (Fig. 4). Osmotically shocked chloroplasts did not catalyze nitrite-dependent O_2 evolution either in the light or in the dark.

We found no correlation of nitrite-dependent O_2 evolution rates with the observed percentage intactness of the unshocked preparations (62–85% intact). However, the initial nitrite-dependent O_2 evolution rates for method 1 chloroplasts (mean of 13 determinations, 17.5 μmol mg of Chl^{-1} hr^{-1}, SD 3.64) were correlated with rates of ferricyanide-dependent O_2 evolution catalyzed by shocked chloroplasts uncoupled with 5 mM ammonia (557.3 μmol mg of

Table I. Estimates of the O_2 nitrite ratio by method 1 and method 2 chloroplasts incubated under condition 1

Glyceraldehyde (10 mM), a standard component for condition 1, was omitted in some experiments. Abbreviations: n.d., not determined; Gald, glyceraldehyde.

Experiment	Chloroplast intactness	Chloroplast preparation method	Additions	Pre-illumination time	Illumination time	Rate of nitrite consumption	O_2:nitrite ratio
	(%)			(min)	(min)	(μmol mg Chl^{-1} hr^{-1})	
1	n.d.	1	nil	11.2	22.0	5.7	0.79
2	n.d.	1	nil	11.6	13.3	10.9	0.79
3A	73	1	nil	8.8	7.5	4.4	1.16
B		1	Gald	nil	3.6	8.8	1.09
4A	74.5	1	nil	20.9	13.1	6.0	1.16
B		1	Gald	nil	11.1	11.4	1.07
5A	75.5	2	nil	22.8	11.2	2.8	0.59
B		2	Gald	nil	11.1	5.2	0.75

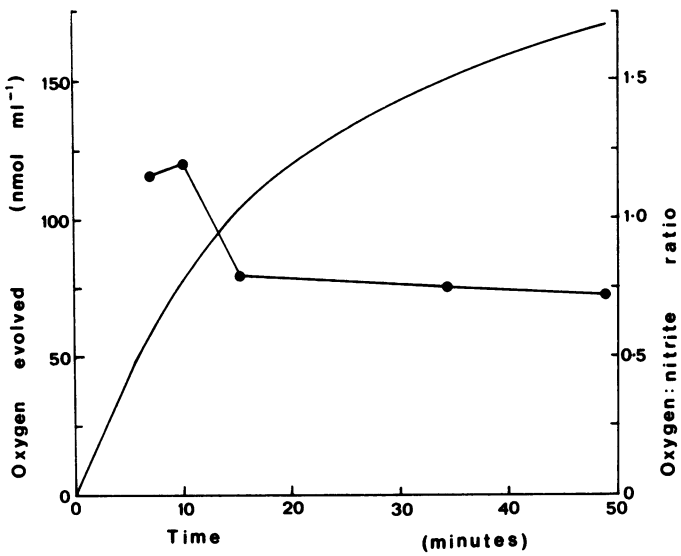


FIG. 3. Nitrite-dependent O_2 evolution (continuous curve) and the O_2 to nitrite ratio (●) in a long term experiment catalyzed by method 1 chloroplasts under condition 1. Chl concentration, 50 μg ml^{-1}; chloroplast intactness, 49%.

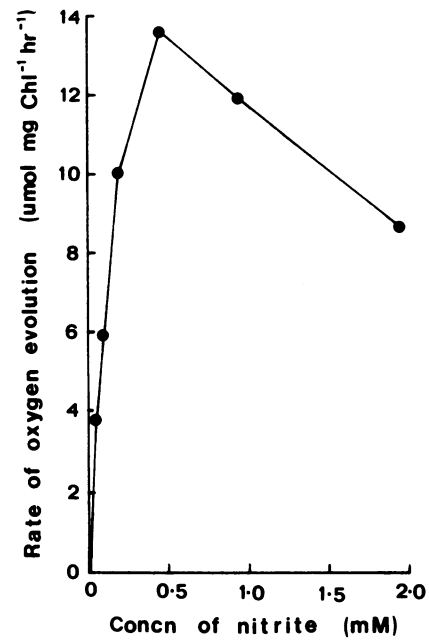


FIG. 4. Effect of nitrite concentration on the rate of O_2 evolution catalyzed by method 1 chloroplasts under condition 1.

Chl^{-1} hr^{-1}, SD 76.2). Both the product-moment correlation coefficient and the Spearman rank correlation coefficient were significant at $P = 0.05$.

In some experiments, chloroplasts were supplied with limiting amounts of nitrite (typically 100 nmol ml^{-1}). Under these conditions O_2 evolution commenced in the usual way, but the rate gradually declined and finally ceased. When a further addition of nitrite was made, O_2 evolution recommenced. This sequence, like that for (α-ketoglutarate plus glutamine)-dependent O_2 evolution (2) could be repeated several times. The molar ratio of O_2 evolved to nitrite supplied was calculated for each successive addition of nitrite for several experiments (Table II). Addition of α-ketoglutarate (2.5 mM) did not affect the molar ratio for chloroplasts incubated under condition-1.

Chloroplasts prepared by method 1 or 2 did not catalyze O_2 evolution under condition 1 when nitrate was replaced by nitrate (0.05–1.6 mM) either in the absence or presence of 10 mM DL-glyceraldehyde. Addition of α-ketoglutarate (2.5 mM) and/or azaserine (0.5 mM) did not affect the rates of nitrite-dependent O_2 evolution or nitrite reduction catalyzed by method 1 chloroplasts under condition 1.

Method 2 chloroplasts, incubated in sucrose-PPi medium in the absence of glyceraldehyde, consistently yielded higher rates of nitrite reduction in the presence of 10 mM α-ketoglutarate and 10 mM pyruvate (Table III). However, neither α-ketoglutarate nor pyruvate enhanced the rate of nitrite-dependent O_2 evolution (results not shown). Glyceraldehyde enhanced the rate of nitrite-dependent O_2 evolution by method 2 chloroplasts (Table I).

Oxygen Evolution and Nitrite Reduction for Condition 2. Method 1 chloroplasts catalyzed nitrite-dependent O_2 evolution under condition 2 at about 80% of the rate for condition 1. We employed the technique of measuring O_2 evolution for each addition of a limiting amount of nitrite to determine the molar ratio of O_2 evolved to nitrite supplied. In the absence of α-ketoglutarate the ratio was typically 0.5 to 0.6 (Fig. 5B). In the presence of 2.5 mM α-ketoglutarate, however, the ratio for the first addition of nitrite was 0.90 and thereafter increased with each successive addition (Fig. 5A). Furthermore, whereas the rate in the control decreased with each successive addition of nitrite, the rate of the reaction mixture containing α-ketoglutarate (initially about 10% greater than the control) did not.

The effect of nitrite on the rate of O_2 evolution in the presence of 1 mM ammonia and 2.5 mM α-ketoglutarate was also studied. Chloroplasts were preincubated in the presence of α-ketoglutarate and O_2 evolution initiated with ammonia. Subsequent addition of nitrite enhanced the rate by 3.6 and 3.9 times in separate experi-

Table II. Ratio of O_2 evolved:nitrite supplied as determined by supplying method 1 chloroplasts (under condition 1) with limiting amounts of nitrite

O_2 evolution was initiated by the addition of nitrite (100 nmol ml^{-1} for experiments 1-3A; 50 nmol ml^{-1} for experiment 3B). In some cases, further additions of nitrite were made after O_2 evolution ceased in a manner analogous to that shown in Fig. 5. Abbreviation: α-KG, 2.5 mM α-ketoglutarate.

Experiment	Chloroplast intactness	Chl concentration	Additions	Molar ratio (Addition) 1	2	3
	(%)	(μg ml^{-1})				
1	62	50	nil	0.81	1.12	
2	85	150	nil	0.78		
3A	76	150	nil	1.02		
B	76	150	α-KG	0.93	0.97	0.94

Table III. Effect of 10 mM α-ketoglutarate and 10 mM pyruvate on the rate of reduction of nitrite by method 2 chloroplasts in the absence of glyceraldehyde under condition 1

Values in parentheses denote the rate relative to the control without any additions. Abbreviation: n.d., not determined.

Experiment	Rate of nitrite reduction: No additions	With α-ketoglutarate	With pyruvate
	(μmol mg Chl^{-1} hr^{-1})		
1	1.23	2.19 (1.78)	n.d.
2	2.95	6.28 (2.13)	3.87 (1.31)
3	1.64	2.80 (1.71)	2.17 (1.33)
4	1.34	2.33 (1.62)	1.98 (1.43)

ments (Table IV). However, when the order of addition of ammonia and nitrite was reversed, the enhancement in the rate was much less than that predicted from the sum of the two independent activities when measured alone.

Ammonia Formation from Nitrite for Conditions 1 and 2. For condition 1, illuminated method 1 chloroplasts catalyzed the reduction of nitrite with the concomitant production of ammonia. The proportion of nitrite-N recovered as ammonia was typically 55 to 80% (Table V). For condition 2 in the absence of α-ketoglutarate, similar recoveries were obtained. In the presence of α-ketoglutarate, however, the proportion of nitrite-N recovered as ammonia was only 27% (Table V).

Nitrogen Assimilation by Method 1 Chloroplasts from Seedlings Grown With and Without Added Nitrate. An adequate supply of nitrate (10 mM) was routinely supplied to the seedlings to ensure establishment of the nitrite-reducing system. We investigated some reactions of N metabolism previously shown to be coupled to light-dependent electron transport in chloroplasts prepared from seedlings raised in Vermiculite with and without nitrate. The results (Table VI) show that the virtual absence of nitrite-dependent O_2 evolution in the chloroplasts of peas grown without nitrate was not accompanied by absence of activity with α-ketoglutarate plus ammonia, glutamine or aspartate, respectively. Crude extracts of peas grown without nitrate contained negligible nitrate reductase activity.

DISCUSSION

The results of the study of nitrite consumption, ammonia production, and O_2 evolution by method 1 chloroplasts for condition 1 in the presence of glyceraldehyde are in broad agreement with the operation of photosynthetically coupled nitrite reductase:

$$H_2O + HNO_2 \xrightarrow{h\nu} NH_3 + 1\tfrac{1}{2}O_2 \quad (1)$$

The requirement for light and the sensitivity of the reaction to DCMU (Fig. 1) demonstrate that light-dependent electron transport was involved. We presume that reduced ferredoxin, generated by the light reactions, serves as the reductant (4). For short term experiments the O_2 to nitrite ratio approached the theoretical value of 1.5 (Fig. 1) and approximately 55 to 80% of the nitrite-N

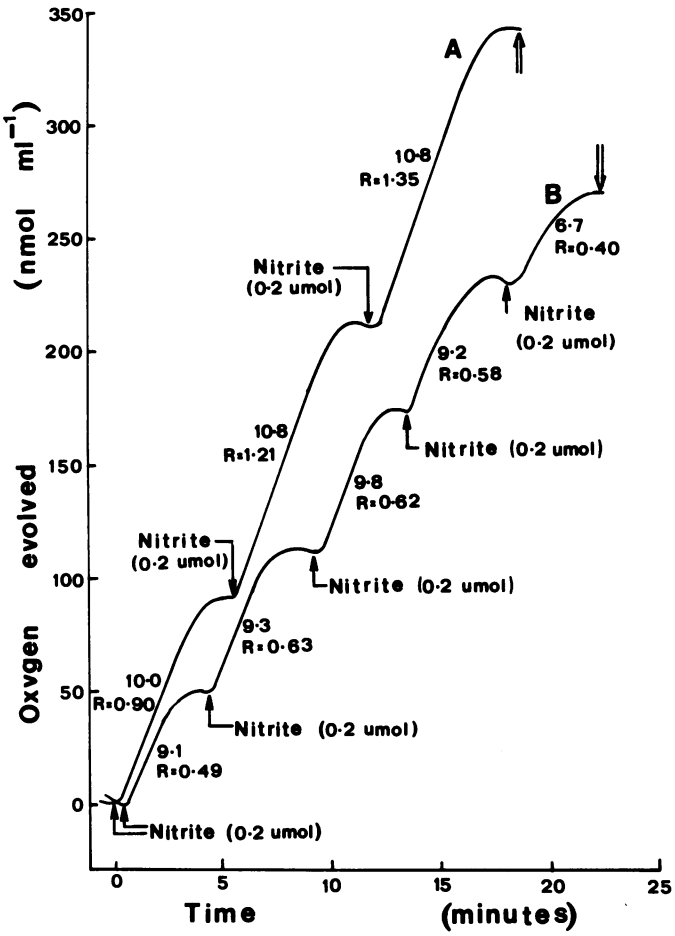


FIG. 5. Effect of α-ketoglutarate on nitrite-dependent O_2 evolution catalyzed by method 1 chloroplasts under condition 2. Chloroplasts (300 μg of Chl, 76% intact) were preincubated as described under "Materials and Methods" with (A) and without (B) 2.5 mM α-ketoglutarate in a volume of 2 ml and nitrite added as shown. Values beside the curves represent rates of O_2 evolution in μmol mg of Chl^{-1} hr^{-1}. The molar ratios of O_2 evolved per nitrite supplied for each successive addition of nitrite are also shown (R). Double-tailed arrows indicate the time at which the reaction mixtures were analyzed for ammonia (see Table V).

Table IV. Effect of 0.5 mM nitrite and 1 mM ammonia on the rate of O_2 evolution by method 1 chloroplasts under condition 2 in the presence of 2.5 mM α-ketoglutarate

Values in parentheses represent the rate of O_2 evolution calculated by summing the rates for each substrate when measured in the absence of the other substrate.

Experiment	Chloroplast intactness	Additions	Rate of O_2 evolution	Rate with both substrates / Rate with first substrate
	(%)		(μmol mg Chl^{-1} hr^{-1})	
1A	70	NH_3	4.9	
		NH_3+Nitrite	19.1 (20.5)	3.9
B		Nitrite	15.6	
		Nitrite+NH_3	16.8 (20.5)	1.1
2A	78	NH_3	5.2	
		NH_3+Nitrite	18.8 (14.9)	3.6
B		Nitrite	9.7	
		Nitrite+NH_3	10.9 (14.9)	1.1

Table V. Production of ammonia and O_2 in relation to nitrite consumed by method 1 chloroplasts under conditions 1 and 2

For condition 1, chloroplasts were incubated in the presence of 10 mM DL-glyceraldehyde. The initial concentration of nitrite for all experiments was 0.5 mM. Experiments 1-3 were initiated by illumination. Experiments 4A and 4B were as shown in Fig. 5B and Fig. 5A respectively and were initiated with nitrite. The reaction mixtures shown in Fig. 5 were analyzed for ammonia at the times indicated by the double tailed arrows.

Experiment	Incubation condition	Chl concn.	Chloroplast intactness	Additions	Period of illumination	Rate of nitrite consumption	NH_3 produced / Nitrite consumed	O_2 evolved / Nitrite consumed
		($\mu g\ ml^{-1}$)	(%)		(min)	($\mu mol\ mg\ Chl^{-1}\ hr^{-1}$)		
1	1	100	68.4	nil	0 - 3.5	21.0	0.66	0.82
					3.5 - 7.43	13.8		1.05
2	1	100	78.4	nil	0 - 11.4	11.7	0.81	1.22
					11.4 - 32.5	6.47	0.74	0.68
3	1	83	n.d.	nil	0 - 3.5	20.1	0.56	1.13
					3.5 - 11.7	18.2		0.72
4A	2	150	76.3	nil	0 - 21.3	(See Fig. 5B)	0.58	(See Fig. 5B)
B	2	150	76.3	α-KG	0 - 18.9	(See Fig. 5A)	0.27	(See Fig. 5A)

Table VI. Activity of some photosynthetically coupled reactions of nitrogen metabolism in chloroplasts from pea seedlings grown with and without nitrate

Pea seedlings were grown with and without 10 mM KNO_3 in vermiculite for 11 days in a growth cabinet at 20 C and 12 hr day length. Chloroplasts were prepared by method 1. The nitrate reductase activity associated with the soluble fraction was also measured. Abbreviation: α-KG, α-ketoglutarate.

Experiment	Activity	With Nitrate	Without Nitrate
1	Chloroplasts		
	Intactness	68%	71%
	Nitrite-dep. O_2 evolution	18.4 $\mu mol\ mg\ Chl^{-1}\ hr^{-1}$	0.3 $\mu mol\ mg\ Chl^{-1}\ hr^{-1}$
	(Glutamine + α-KG)-dep. O_2 evolution	10.6 " "	9.5 " "
	Soluble fraction		
	Nitrate reductase	3.6 $\mu mol\ g\ FW^{-1}\ hr^{-1}$	0 $\mu mol\ g\ FW^{-1}\ hr^{-1}$
2	Chloroplasts		
	Intactness	85%	71%
	Nitrite-dep. O_2 evolution	8.0 $\mu mol\ mg\ Chl^{-1}\ hr^{-1}$	0 $\mu mol\ mg\ Chl^{-1}\ hr^{-1}$
	(Glutamine + α-KG)-dep. O_2 evolution	10.1 " "	5.7 " "
	(Ammonia + α-KG)-dep. O_2 evolution	4.0 " "	3.7 " "
	(Aspartate + α-KG)-dep. O_2 evolution	9.8 " "	5.2 " "
	Soluble fraction		
	Nitrate reductase	4.3 $\mu mol\ g\ FW^{-1}\ hr^{-1}$	0 $\mu mol\ g\ FW^{-1}\ hr^{-1}$

Table VII. Summary of the N-flux capacity of the component reactions of N-assimilation of isolated pea chloroplasts as determined from O_2 evolution

The rates of O_2 evolution are abstracted from this and previous reports (1,2). Abbreviations: α-KG, α-ketoglutarate; Gln, glutamine.

Substrate(s)	Enzyme activities	Rate of O_2 evolution	Theoretical N-flux per mol O_2	N-flux capacity
		($\mu mol\ mg\ Chl^{-1}\ hr^{-1}$)		($\mu g\ atoms\ mg\ Chl^{-1}\ hr^{-1}$)
Nitrite	Nitrite reductase	17.5	0.67	11.7
Gln + α-KG	Glutamate synthase	10.6	2.0	21.2
NH_3 + α-KG	Glutamine synthetase + glutamate synthase	8.3	2.0	16.6

consumed was recovered as ammonia (Table V). The kinetics of nitrite-dependent O_2 evolution (Fig. 4) is in approximate agreement with that for purified nitrite reductases (5); the concentration of nitrite for $V_{max}/2$ (value for purified enzymes in parentheses) was 120 μM (3–200 μM). We did not find the marked substrate inhibition of nitrite reduction or nitrite-dependent O_2 evolution observed for spinach (10) or the disproportionate O_2 to nitrite ratio found with spinach (10), *Wolfia* (18), and *Ankistrodesmus* (12), but not for *Chlorella* (12).

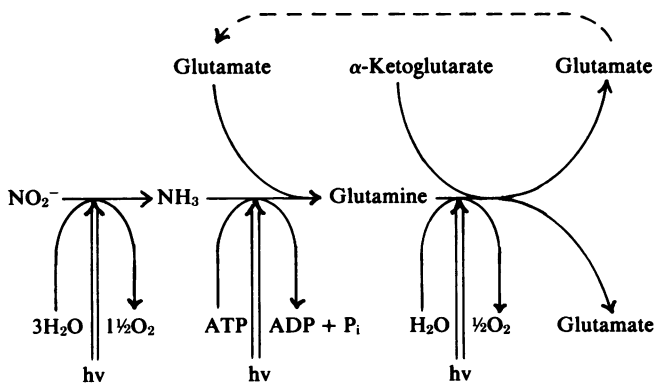
Certain results for condition 1 are not in complete agreement with the theoretical equation. The decrease in the O_2 to nitrite ratio as the incubation proceeds (Fig. 3) might suggest that some process other than the direct formation of reduced ferredoxin by the light reactions (also) operates for the synthesis of this reductant in the later stages of prolonged experiments. We presume that some nitrite-N is incorporated into organic-N since 20 to 45% of nitrite-N consumed was not recovered as ammonia. Although ammonia accumulated in chloroplasts under condition 1 this is unlikely to explain the decreased rates and lower O_2 to nitrite ratios in the latter stages of an incubation. The decreased rate of nitrite-dependent O_2 evolution as ammonia accumulated (Table V) suggests that under the prevailing experimental conditions the small amounts of ammonia produced did not cause uncoupling of the chloroplast electron transport chain.

The rates of light-dependent nitrite-reduction catalyzed by intact pea chloroplasts for condition 1 approximate those found for spinach (9, 10, 14) and maize (15), but the rate of nitrite-dependent O_2 evolution (17.5 μmol mg of $Chl^{-1}\ hr^{-1}$) was somewhat greater (10) presumably due to differences in species, chloroplast quality, and the use of glyceraldehyde. Method 1 pea chloroplasts, incubated under condition 1, appear to differ from spinach in that most of the nitrite-N consumed is recovered in ammonia. This implies that under condition 1 ammonia is not incorporated into amino-N to any great extent and would explain why α-ketoglutarate did not enhance the rate of nitrite reduction as reported for spinach (10) or nitrite-dependent O_2 evolution, or enhance the O_2 to nitrite ratio (Table II). However, the rate of nitrite reduction catalyzed by method 2 chloroplasts prepared and incubated in medium containing PPi was enhanced by α-ketoglutarate (Table III). Although the rates relative to those for method 1 chloroplasts are low, the results are consistent with the requirement for PPi for

the incorporation of ammonia into glutamine catalyzed by method 1 chloroplasts (1) as used in condition 2. Perhaps it is significant that experiments in which α-ketoglutarate has been shown to affect nitrite reduction (10) and incorporation of nitrite-N into amino-N (9) have been conducted in the presence of PPi buffer (10) or 0.5 mM Pi (9).

Plaut *et al.* (14) recently reported that pretreatment of intact and reconstituted chloroplasts systems with CO_2 and various intermediates of the CO_2 reduction pathway enhanced the rate of nitrite assimilation. They concluded that carbon cycle intermediates regulate, to some extent, nitrite reduction *in vivo*. Although we found that glyceraldehyde, an inhibitor of CO_2 assimilation (17) enhanced the rate of nitrite-dependent O_2 evolution and nitrite reduction (Table I), this apparent anomaly could be due to the enhanced availability of reductant (generated by the chloroplast electron transport chain) for nitrite reduction in the absence of any competing reactions of carbon metabolism. Alternatively, glyceraldehyde might interfere with the regulatory mechanisms for nitrite reduction.

Several features of the reactions catalyzed by chloroplasts incubated under conditions favorable for the operation of photosynthetically coupled glutamine synthetase and glutamate synthase (condition 2) differ from those for condition 1. Under condition 1 α-ketoglutarate did not affect the O_2 to nitrite ratio or the rates of nitrite-dependent O_2 evolution and nitrite reduction. For condition 2, however, α-ketoglutarate enhanced the O_2 to nitrite ratio, slightly enhanced the rate of O_2 evolution, and caused a decrease in the recovery of nitrite-N as ammonia. Taken collectively these results suggest that for condition 2 in the presence of α-ketoglutarate, nitrite-N is assimilated into glutamate via ammonia and glutamine, and involves the enzyme sequence nitrite reductase, glutamine synthetase, and glutamate synthase as follows:



The involvement of the latter enzyme would enhance the theoretical O_2 to nitrite ratio to 2. The enhancement in the rates of O_2 evolution following the addition of nitrite to chloroplasts previously supplied with ammonia (Table IV) is in approximate agreement with the value of 4 predicted from theory on the assumption that no single reaction is rate-limiting (theory predicts for ammonia to glutamate, O_2:ammonia = 0.5; for nitrite to glutamate, O_2:nitrite = 2). Conversely, the small enhancement in the rate of O_2 evolution caused by ammonia when nitrite was supplied first (Table IV) suggests that the ammonia-dependent reactions were virtually saturated by ammonia produced from nitrite. The data summarized in Table VII indicate that the N-flux capacities of the component reactions of N-assimilation in chloroplasts as judged by O_2 evolution are of a similar order of magnitude, but that nitrite reductase is marginally rate-limiting. The data in Table IV are consistent with this possibility.

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