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Inhibition of Photosynthesis by Azide and Cyanide and the Role of Oxygen in Photosynthesis[1]

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ABSTRACT

Cyanide and azide inhibit photosynthesis and catalase activity of isolated, intact spinach (*Spinacia oleracea*) chloroplasts. When chloroplasts are illuminated in the presence of CN^- or N_3^-, accumulation of H_2O_2 is observed, parallel to inhibition of photosynthesis. Photosynthetic O_2 evolution is inhibited to the same extent, under saturating light, whether CO_2 or phosphoglycerate is present as electron acceptor.

The illumination of chloroplasts with CN^- or N_3^- inactivates the NADPH- and ATP-dependent phosphoglycerate reduction. This enzyme system can be reactivated by dithiothreitol. In reconstituted, envelope-less chloroplasts, the phosphoglycerate-dependent and the ribose 5-phosphate-dependent O_2 evolution are inhibited to the same extent, while electron transport to NADP is unaffected.

It is concluded that the inhibition of photosynthesis by CN^- and N_3^- is due to H_2O_2 accumulation, which is a consequence of catalase inhibition.

The inhibition of phosphoglycerate reduction, but not of CO_2 reduction, is abolished under conditions where ATP is available in excess of NADPH (low light, supply of ATP). This is taken as an indication that electron flow from photosystem I is diverted to O_2 (Mehler reaction, which produces H_2O_2) when the unavailability of ATP is limiting the rate of reoxidation of NADPH. The Mehler reaction is considered a physiological process supplying ATP for photosynthesis.

Azide and cyanide inhibit both respiration and photosynthesis, *in vivo* as well as *in vitro*. While the inhibition of respiration is accounted for by the inhibition of metal-containing terminal oxidases, primarily cyt oxidase, the mechanism of inhibition of photosynthesis is controversial. It is well known that cyanide and azide do not inhibit photosynthetic electron transport from H_2O to NADP or to artificial electron acceptors, nor photosynthetic phosphorylation (1, 2). Therefore, the inhibition of photosynthesis by these poisons must be explained by an indirect mechanism of invoking the inhibition of one or more steps of the photosynthetic carbon cycle. Bamberger and Avron (3) have demonstrated that the steps between Rib5P[2] and 3PGA are not affected by azide, at concentrations that completely inhibit photosynthetic CO_2 fixation in isolated chloroplasts. These authors attribute the inhibition of photosynthesis by N_3^- to the inhibition of carbonic anhydrase, an enzyme abundant in chloroplast stroma (13), which has been proposed to have a function in the uptake of CO_2 into the chloroplast (5, 7). Although this function is under dispute (13), the importance of carbonic anhydrase in photosynthesis is beyond doubt.

The studies reported here on the mechanism of inhibition of photosynthesis by CN^- and N_3^- lead us to conclude that the inhibition of photosynthesis in isolated chloroplasts by these reagents can be accounted for by their inhibition of catalase and the consequent accumulation of H_2O_2, in addition to the inhibition of carbonic anhydrase. It is known that H_2O_2 inhibits enzymes dependent on —SH groups for activity.

The production of H_2O_2 by photosynthetic electron transport has been established by Mehler (11), and is the result of reoxidation by O_2 of the primary reductant of PSI. On the other hand, no H_2O_2 is formed when NADP is available as the electron acceptor. The Mehler reaction has been shown to be coupled to ATP formation (6) and it has been proposed that it has the function of supplying the ATP needed for the assimilation of CO_2 in excess of that provided by the electron transport to NADP under steady-state conditions (4) as well as during the onset of photosynthesis (8). Normally intact chloroplast preparations contain sufficient amounts of catalase to decompose any H_2O_2 formed (or most of it, see Fig. 3 and Table I). However, the inhibition of catalase leads to accumulation of H_2O_2 in broken (6) as well as in intact chloroplasts, as shown here. The production of H_2O_2 during photosynthesis in intact algae has also been demonstrated (12).

MATERIALS AND METHODS

Intact chloroplasts were prepared from spinach (*Spinacia oleracea*) leaves as previously described (9). Photosynthesis was measured as the evolution at O_2 at 20 C, by means of a Clark-type O_2 electrode. Illumination was provided by a 500 w tungsten lamp, filtered through 5 cm of water and a heath filter (Calflex C, Balzer). Unless otherwise indicated, light intensity was 1.4×10^6 ergs·sec^{-1}·cm^{-2}, which was saturating for photosynthesis. Catalase activity was measured as the initial rate of O_2 evolution, estimated at the O_2 electrode, upon addition of 1 mM H_2O_2 in the dark. The basic reaction mixture for all experiments with intact chloroplasts contained: 50 mM HEPES (pH 7.6), 0.33 M sorbitol, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 2 mM EDTA, 0.2 mM Pi. Other additions are indicated in the experimental section. H_2O_2 was determined enzymically, measuring O_2 evolution upon addition of catalase. The reaction mixture was acidified with H_2SO_4 to pH 1, stirred a few min to eliminate CN^- where present, then neutralized with NaOH. Crystalline catalase was then added. The rapid O_2 evolution, complete in few sec, was recorded. H_2O_2 concentration was calculated from the amount of O_2 evolved.

RESULTS

The inhibition of photosynthesis by increasing concentrations of cyanide is shown in Table I, as well as the inhibition of the

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[2] Abbreviations: PEP: phosphoenolpyruvate; 3PGA: 3-phosphoglycerate: Rib-5-P: ribose 5-phosphate; DTT; dithiothreitol.

H_2O_2-dependent O_2 evolution of the chloroplast preparation.

Figure 1 shows that inhibition by N_3^- of O_2 evolution by intact chloroplasts is not relieved by the addition of 3PGA. This rules out the possibility of an involvement of carbonic anhydrase, because 3PGA-dependent O_2 evolution does not require CO_2 or HCO_3^-. This point was investigated further using the reconstituted chloroplast system described by McC Lilley *et al.* (10). In this system, the chloroplast envelope is absent, and photosynthesis depends on the phosphorylating electron transport system of the *grana* and on the activity of the carbon cycle enzymes added as a concentrated extract obtained from intact chloroplasts disrupted by osmotic shock. So, transport of CO_2 or HCO_3^- through the envelope is not a factor in this system. The addition of a small amount of NADP produces a rapid ferredoxin-dependent O_2 evolution, which ceases when NADP is completely reduced (Fig. 2). The addition of ribose 5-phosphate restores O_2 evolution, which is stimulated by the further addition of ATP. NaN_3 addition completely inhibits O_2 evolution within about 90 sec, and the subsequent addition of 3PGA has no effect, indicating that 3PGA kinase and/or triosephosphate dehydrogenase are inactive. O_2 evolution can be restored to the initial rate by the addition of NADP, indicating that the electron transport system of thylakoids has not been damaged by N_3^- treatment. This experiment indicates that the inhibition by N_3^- and O_2 evolution is due primarily to the inactivation of the 3PGA-reducing system, rather than to inhibition of carbonic anhydrase, which would have no influence on 3PGA-dependent O_2 evolution.

The role of carbonic anhydrase inhibition in the block of Rib-5-P-dependent O_2 evolution is also unlikely, because the rate of spontaneous equilibration of HCO_3^- and CO_2 is largely sufficient to account for the rates of CO_2 fixation observed in our conditions (Fig. 2).

The inactivation of the 3PGA-reducing system in intact chloroplasts illuminated in the presence of NaN_3 was therefore investigated. Chloroplasts were illuminated in the presence or absence of 0.1 mM NaN_3 and samples were withdrawn at the times indicated in Table II and used for the estimation of 3PGA kinase and NADPH-dependent triophosphate dehydrogenase. Table II

Table I. Effect of KCN on Photosynthesis and Catalase Activity of Isolated, Intact Spinach Chloroplasts

$NaHCO_3$, 10 mM; chloroplasts containing 14 µg of Chl·ml^{-1}. KCN, at the concentration indicated, was added after 3.5 min of illumination. Light was turned off after additional 2.5 min. H_2O_2, (1mM) was then added and catalase activity measured as O_2 evolution.

KCN added	Photosynthetic activity	H_2O_2 dependent, O_2 evolution in the dark
µM	µmol O_2·mg^{-1} Chl·hr^{-1}	
None	77	175
10	71	98
20	69	74
50	55	51
250	29	27

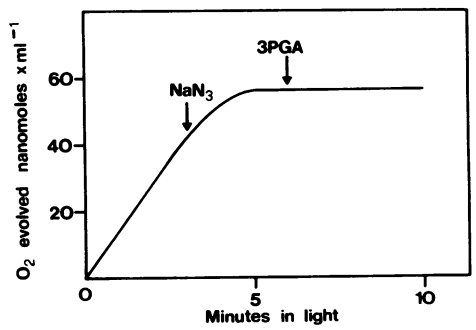

FIG. 1. Effect of NaN_3 on O_2 evolution in intact chloroplasts. Incubation: as under "Materials and Methods"; $NaHCO_3$, 6.8 mM; Chl, 61.4 µg·ml^{-1}. NaN_3 and 3PGA were added, where indicated, respectively, at the concentration of 0.1 mM and 1 mM.

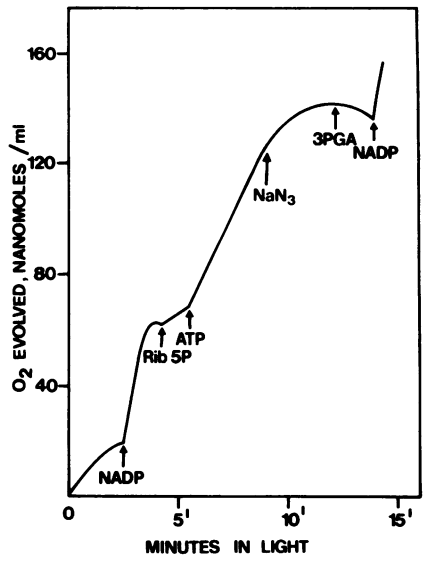

FIG. 2. Oxygen evolution in reconstituted chloroplasts. Incubation: as under "Materials and Methods"; $NaHCO_3$, 10 mM; $MgCl_2$, 5 mM; ferredoxin, 2.4 µM; chloroplast extract (see ref. 10) corresponding to 0.6 mg Chl·ml^{-1}; Chl, 146 µg·ml^{-1}. The following additions were made as indicated: NADP, 80 µM; ATP, 3PGA, and Rib5P, each 1 mM; NaN_3, 0.1 mM.

Table II. Effect of Illumination in the Presence of NaN_3 on Triosephosphate Dehydrogenase of Chloroplasts

Intact chloroplasts were illuminated in the standard reaction mixture with the additions of $NaHCO_3$, 10mM and NaN_3, 0.1 mM. Samples containing 6.6 µg Chl were pipetted into a spectrophotometer cuvette containing in 1 ml: tricine, 0.05M, pH 8; $MgCl_2$, 5mM; KCl, 5mM; 3PGA, 1 mM; ATP, 1 mM; PEP, 1 mM; pyruvate kinase in large excess and NADPH, 0.15 mM. The rate of oxidation of NADPH was recorded as the decrease of A at 340 nm. The rate of a control, illuminated or not for 12 min., without NaN_3, was 1.69 µmol·min^{-1} . mg^{-1} Chl.

Time of preillumination	NADPH oxidation Initial	After addition of 5mM DTT
min	µmol·min^{-1}·mg^{-1} Chl.	
3	1.32	1.77
6	1.06	1.77
12	0.42	1.34

shows that the ATP- and 3PGA-dependent oxidation of NADPH by the chloroplast extract was progressively inhibited by illumination of the intact chloroplasts in the presence of N_3^+, and that the activity was completely restored by addition of DTT. This indicates that N_3^- brings about the oxidation of —SH groups, leading to inactivation of the 3PGA-reducing system. This is consistent with the hypothesis that H_2O_2 accumulation is the cause of inhibition by N_3^-. On the other hand, it is well known that —SH is the catalytic group of triosephosphate dehydrogenase, and that this enzyme is very sensitive to —SH reagents.

The accumulation of H_2O_2 has been directly demonstrated during inhibition of photosynthesis by KCN in intact chloroplasts (Fig. 3). This figure shows that a small amount of H_2O_2 is produced in the chloroplasts, and its steady-state is about 14 µM. The addition of KCN, which inhibits catalase activity, causes a linear increase of H_2O_2 parallel to photosynthesis inhibition.

The experiments reported so far clearly show that production of H_2O_2 is a normal attribute of isolated chloroplasts in steady-state photosynthesis. If the Mehler reaction is the only mechanism leading to H_2O_2 production, one would expect that any condition which prevents the Mehler reaction would prevent H_2O_2 formation and therefore inhibition of the O_2 evolution. If

the Mehler reaction has the function of providing the ATP needed in excess of that produced during NADP reduction, one would expect that any condition in which ATP is in excess over the rate of NADPH generation would prevent the Mehler reaction. Such conditions were achieved in the experiments reported in Table III by essentially three methods: (*a*) providing 3PGA in saturating concentration: this reduces the ATP requirement for O_2 evolution to 2 ATP per O_2 evolved; (*b*) providing ATP in saturating concentration; (*c*) decreasing the light intensity in the presence of ATP, in order to make NADPH generation rate-limiting, rather than NADPH reoxidation. The results reported in Table III demonstrate that the inhibition by KCN disappears when ATP and 3PGA are supplied at low light intensity. At high light intensity, ATP does not overcome completely CN^- inhibition even in the presence of 3PGA (data not shown): however, under these conditions, O_2 evolution was also dependent on CO_2 fixation and not only on 3PGA reduction. Table III also shows that under no condition could the inhibition by CN^- (or N_3^-, not shown) of HCO_3^--dependent O_2 evolution be reversed by ATP. This might suggest that even in the absence of Mehler reaction (and H_2O_2 production), CN^- can still inhibit CO_2 fixation by a different mechanism, possibly inhibiting carbonic anhydrase, as proposed by Bamberger and Avron (3).

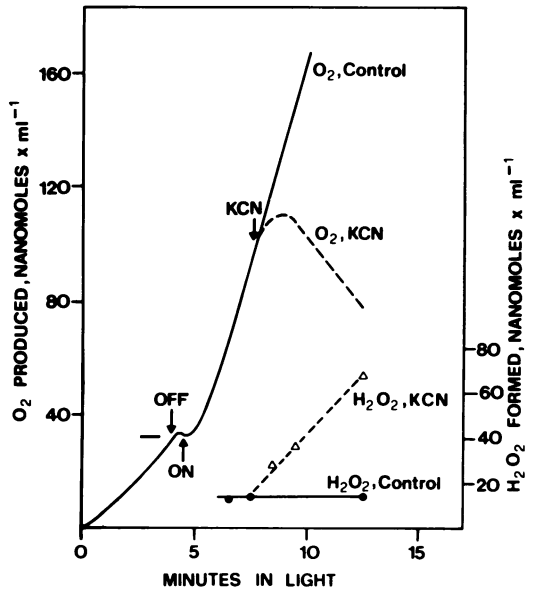

FIG. 3. Effect of KCN on O_2 evolution and H_2O_2 production. Incubation: as under "Materials and Methods"; $NaHCO_3$, 10 mM; Chl, 65 $\mu g \cdot ml^{-1}$. KCN was added, where indicated, at the concentration of 0.1 mM.

Table III. Effect of KCN on 3PGA Reduction and on Photosynthesis in Intact Chloroplasts as a Function of Light Intensity and ATP Concentration

Chl, 52 $\mu g \cdot ml^{-1}$. Illumination with red light: 610 nm $< \lambda <$ 720 nm KCN was added, where indicated, at the concentration of 0.2 mM; after 1 min of illumination. ATP and PGA, each 1 mM and $NaHCO_3$, 5mM were added before illumination.

Light intensity ergs·sec^{-1}·cm^{-2}	Additions	O_2 evolution after 3 min illumination $\mu mol \cdot mg^{-1}$ Chl·hr^{-1}
4×10^5	3PGA	30
4×10^5	3PGA + KCN	14.8
4.3×10^4	3PGA	10.1
4.3×10^4	3PGA + KCN	6.2
4.3×10^4	3PGA + ATP	12.3
4.3×10^4	3PGA + ATP + KCN	11.2
2.3×10^4	3PGA + ATP	4.1
2.3×10^4	3PGA + ATP + KCN	4.2
2.3×10^4	$NaHCO_3$	3.3
2.3×10^4	$NaHCO_3$ + KCN	0.0
2.3×10^4	$NaHCO_3$ + ATP	3.4
2.3×10^4	$NaHCO_3$ + ATP + KCN	0.0

DISCUSSION

The data presented here indicate that CN^- and N_3^- inhibit photosynthesis in isolated chloroplasts because they inhibit catalase, so causing the accumulation of H_2O_2 produced by the Mehler reaction. The evidence of this is based on the following observations: (*a*) catalase is inhibited and H_2O_2 is accumulated continuously upon addition of KCN, parallel to the inhibition of photosynthesis; (*b*) the reduction of 3PGA is inhibited as much as the reduction of CO_2, both in intact chloroplasts and in the envelope-less reconstituted chloroplasts, where any influence of transport of bicarbonate or CO_2 is not relevant to the activity measured. In this case, therefore, we can rule out the explanation suggested by Bamberger and Avron (3) *i.e.* that N_3^- inhibition could be the consequence of inhibition of carbonic anhydrase acting as a CO_2-HCO_3^- carrier across the envelope. Furthermore, this mechanism could not explain inhibition of 3PGA-dependent O_2 evolution, where the CO_2-HCO_3^- system is not required. Direct evidence (Table II) demonstrates that N_3^- treatment during illumination of chloroplasts inactivates the NADP-dependent triosephosphate dehydrogenase and PGA kinase system. This inhibition can be completely reversed by a sulfhydryl reagent such as DTT, indicating that inactivation was caused by —SH group(s) oxidation: a known effect of H_2O_2 on —SH groups.

Our results indicate that H_2O_2 is continuously generated in steady-state photosynthesis of isolated chloroplasts, in agreement with the observations of Patterson and Myers (12) in intact algae and of Egneus *et al.* (4) with isolated chloroplasts. In fact, we found that H_2O_2 is present at constant low concentration during steady-state photosynthesis and its concentration increases linearly upon addition of CN^-. This can explain the stimulation of photosynthesis by catalase observed by Egneus *et al.* (4) and by us (data not shown), as H_2O_2 is inhibitor of more than one enzyme of the carbon cycle. On the assumption that the only mechanism of generating H_2O_2 in photosynthetic electron transport is the reoxidation by O_2 of the primary reductant of PSI (Mehler reaction), one has to conclude that this process is an essential part of photosynthesis. This conclusion is made by Egneus *et al.* (4), on the basis of their measurements of $^{18}O_2$ exchange during photosynthesis. We can support this conclusion and their suggestion that the Mehler reaction has the role of providing the ATP needed for the operation of the carbon cycle in excess of that produced during NADP reduction. Indeed, evidence is presented here (Table III) that the Mehler reaction does not operate, H_2O_2 is not produced, and therefore CN^- does not inhibit PGA-dependent O_2 evolution whenever ATP availability does not limit the rate of NADPH reoxidation. This condition is achieved in isolated chloroplasts under low light intensity (so limiting the rate of NADP reduction) and with the addition of ATP and 3PGA in saturating concentrations. In our opinion, this result provides convincing evidence that the diversion of electron flow from the reducing end of PSI to either NADP or O_2 is regulated by the concentration of ATP in the chloroplasts, which determines the availability of diphosphoglycerate, and therefore the reoxidation of NADPH. Whenever NADP is available, the electron flow to O_2 is suppressed, and diverted to the faster reaction through ferredoxin and the flavoprotein toward NADP.

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