Effects of Inhibitors of Catalase on Photosynthesis and on Catalase Activity in Unwashed Preparations of Intact Chloroplasts

JOHN F. ALLEN AND F. R. WHATLEY
Botany School, South Parks Road, Oxford OX1 3RA, United Kingdom

ABSTRACT

The catalase activity of unwashed preparations containing intact spinach (Spinacia oleracea L.) chloroplasts is inhibited both by cyanide and azide at concentrations which also cause inhibition of photosynthetic CO₂-dependent O₂ evolution.

Aminotriazole can also be used to inhibit this contaminant catalase, and in this case inhibition of catalase can be achieved at aminotriazole concentrations which have little effect on the rate of photosynthetic CO₂ fixation. Aminotriazole may also be used as a specific inhibitor of catalase in order to demonstrate inhibition of photosynthesis by added H₂O₂.

It is therefore concluded that inhibition of photosynthesis by cyanide and azide does not necessarily result from inhibition of catalase in the chloroplast preparation, and that intact chloroplasts do not produce inhibitory concentrations of H₂O₂ under the best experimental conditions for CO₂ fixation.

Several investigators have recently described evidence that under certain circumstances O₂ may compete with NADP as an electron acceptor in intact chloroplasts (4, 10, 11, 15). Although three distinct mechanisms of photosynthetic O₂ reduction have been proposed (reviewed in ref. 5), the ferredoxin-mediated reaction (1) is the most important one in this context. In all cases, however, the net effect in the absence of catalase is divalent reduction of O₂, and H₂O₂ has been known to be a product of this reaction (termed the "Meehler reaction") for some time (16, 17).

Catalase (H₂O₂:H₂O oxidoreductase, EC 1.11.1.6) causes dismutation of H₂O₂ to H₂O and O₂, so that on addition of catalase to illuminated, O₂-reducing chloroplasts, zero net O₂ exchange replaces the net O₂ uptake which occurs instead when H₂O₂ is allowed to accumulate as the product of the reaction. This effect of catalase on the stoichiometry of O₂ uptake in the Mehler reaction has been exploited to demonstrate that O₂ competes with NADP as the terminal electron acceptor in noncyclic electron transport in broken, washed chloroplasts, incapable of CO₂ fixation (2). Manipulation of catalase activity is easily achieved in such a system, since catalase is absent from washed chloroplast lamellae (13), and since if catalase should happen to contaminate the system (because of incomplete washing, e.g.). it can be inhibited by mM concentrations of azide or cyanide, compounds which have no effect on electron transport itself (6).

With intact chloroplasts capable of CO₂ fixation the situation is more complex. The catalase activity of such chloroplast preparations is considerable (11, 15, 22). Since catalase is not a chloroplast enzyme but is localized in the peroxisome (12, 13, 18), its presence in intact chloroplast preparations must presumably result from contamination of the chloroplast suspension by peroxisomes. Removal of contaminant catalase by extensive washing of the chloroplasts, while easily carried out (3), is undesirable if high photosynthetic activity is to be maintained. Azide (8) and cyanide (23) are known to be inhibitory to photosynthetic CO₂ fixation. It has been suggested, moreover, that H₂O₂ itself is inhibitory to CO₂ fixation (11, 15), so that if O₂ reduction occurs in intact chloroplasts, inhibition of catalase in this system should also lead to inhibition of photosynthesis itself. To examine directly any effect of added H₂O₂ on chloroplast CO₂ fixation it is nevertheless desirable to inhibit catalase in some way, in order that the H₂O₂ should not be quickly consumed in a purely enzymic reaction which itself would entail O₂ evolution at a rate many times greater than that associated with photosynthesis.

Two recent investigations of H₂O₂ inhibition of chloroplast photosynthesis have been carried out. Forti and Gerola (11) have published data which suggest that inhibition of catalase in their preparations is inseparable from inhibition of CO₂ fixation. In their view, cyanide's inhibition of catalase causes a build-up of photosynthetically produced H₂O₂, which in turn inhibits CO₂ fixation by preventing reduction of P-glycerate to triose-P. Kaiser (15), however, has demonstrated an inhibition of CO₂ fixation by added H₂O₂, using cyanide (100 μM) in his reaction mixture to inhibit completely the breakdown of H₂O₂ by contaminant catalase. In Kaiser's experiment cyanide at the concentration used apparently had only a slight inhibitory effect on CO₂ fixation.

In view of the inconsistency of these authors' conclusions, it was decided to obtain and compare concentration curves for inhibition both of photosynthetic activity and of catalase by azide, cyanide, and by a third catalase inhibitor, aminotriazole. If all three catalase inhibitors were to inhibit photosynthesis over concentration ranges similar to those for inhibition of catalase, the conclusion of Forti and Gerola (11) would be corroborated. Alternatively, inhibition of catalase without simultaneous inhibition of photosynthesis, as reported by Kaiser (15), would provide a tool for the investigation of effects of H₂O₂ on photosynthesis, and would indicate that inhibitory concentrations of H₂O₂ do not accumulate quickly during photosynthesis by chloroplast preparations low in catalase activity.

MATERIALS AND METHODS

Chloroplasts were isolated from leaves of young, outdoor-grown spinach (Spinacia oleracea L.) by a method based on that of Walker (19). Spinach leaves (60 g) were washed and homogenized for 3 sec with 300 ml of slushy, partly thawed medium in a precooled domestic Braun liquidizer at the lowest speed setting. The homogenization medium contained 0.33 M sorbitol, 2 mM EDTA, 5 mM MgCl₂, 5 mM NaCl, 10 mM sodium ascorbate, and 50 mM MES, and had been adjusted to pH 6.5 with NaOH. The homogenate was squeezed through two layers of butter muslin into a beaker on ice, and then poured into a second beaker (also
on ice) through two layers of butter muslin, one on each side of a filtering pad of absorbent cotton wool about 10 mm thick. The cotton wool had previously absorbed 10 ml of the homogenization medium. The resulting chloroplast suspension was transferred to precooled glass centrifuge tubes and centrifuged at 5,000g for 30 sec (which includes an acceleration period of 20 sec; braking took a further 75 sec) in an MSE 6L centrifuge. After discarding the supernatant the chloroplast pellet was rinsed with resuspending medium to dislodge less firmly sedimented material, the rinsings were discarded, and the pellet resuspended in a few ml of a medium identical to that used for homogenization except for the presence of 50 mM HEPES instead of MES and for a pH of 7.6 instead of 6.5.

Chl estimation was performed by the method of Arnon (7). The Chl concentrations obtained were in the range 1 to 2 mg ml\(^{-1}\).

\( \text{O}_2 \) evolution was measured in twin \( \text{O}_2 \) electrodes (Hansatech, King's Lynn, Norfolk) of the type described by Delieu and Walker (9). A temperature of 20 \( ^\circ \text{C} \) was maintained with a Churchill thermocirculator with the electrode water jackets connected in series. Illumination for photosynthetic measurements was provided by Prinz 300-w (tungsten lamp) slide projectors (one/electrode) with built in heat filters and orange (Cinemoid 5A) filters in the slide compartments. Light intensity was saturating for noncyclic electron transport at Chl concentrations of up to 100 \( \mu \text{g ml}^{-1} \). Results were recorded on a Servoscribe dual channel chart recorder.

All reactions commenced at the “air-line” of the \( \text{O}_2 \) electrode, in a medium containing the common constituents 0.33 M sorbitol, 2 mM EDTA, 1 mM \( \text{MgCl}_2 \), 1 mM \( \text{MnCl}_2 \), and 50 mM HEPES at pH 7.6, and with a final volume of 1 ml.

For measurements of catalase activity 4 mM \( \text{H}_2\text{O}_2 \) was also present, and a unit of catalase activity is here defined as the quantity of catalase required to liberate \( \text{O}_2 \) at a rate of 1 \( \mu \text{mol min}^{-1} \) under these conditions. Addition of chloroplast suspension (catalase) started the reaction.

For measurements of photosynthetic \( \text{O}_2 \) evolution the reaction medium contained, in addition to the common constituents (above); 2 mM ascorbate, 0.2 mM \( \text{K}_2\text{HPO}_4 \), 1 mM ribose-5-P, and 10 mM \( \text{NaHCO}_3 \). For photosynthesis the reaction was started by switching on the light.

Estimates of intactness of chloroplast suspensions (14) are based on comparisons of rates of ferricyanide-dependent \( \text{O}_2 \) evolution in shocked and unshocked chloroplasts. In both cases the reaction medium eventually contained, besides the common constituents: 10 mM DL-glyceraldehyde, 5 mM \( \text{K}_3\text{Fe(CN)}_6 \), and 5 mM \( \text{NH}_4\text{Cl} \). Osmotic shock resulted from suspension of the chloroplasts in 10 mM DL-glyceraldehyde for 3 min. The addition of the other constituents then restored the shocked chloroplasts to an isotonic reaction medium identical to that in which the unshocked chloroplast had already been suspended.

Bovine catalase (crystalline suspension) was purchased from Boehringer Mannheim and 3-amino-1,2,4-triazole from Sigma.

**RESULTS**

Inhibition of catalase in the chloroplast preparation and of photosynthesis (\( \text{CO}_2 \)-dependent \( \text{O}_2 \) evolution) by KCN is shown in Figure 1. Although the two inhibition curves are accommodated on the same logarithmic scale of KCN concentration, the curves differ markedly in shape. Thus, 50% inhibition of catalase activity occurs at a KCN concentration of 3.5 \( \mu \text{M} \), while 50% inhibition of photosynthesis occurs at 17 \( \mu \text{M} \). For 90% inhibition, however, the situation is reversed: with catalase activity it occurs at 60 \( \mu \text{M} \) KCN; with photosynthesis at only 25 \( \mu \text{M} \). Under the experimental conditions of Figure 1 no single KCN concentration can be seen at which catalase is largely inhibited while photosynthesis is not.

The results obtained with \( \text{NaN}_3 \) as the inhibitor present a similar picture (Fig. 2) with perhaps an even smaller separation of catalase and photosynthetic activity than in the case of KCN. With \( \text{NaN}_3 \) 50% inhibition of catalase occurs at 3 \( \mu \text{M} \), and of photosynthesis at 8 \( \mu \text{M} \), while 90% inhibition of catalase is at 40 \( \mu \text{M} \) \( \text{NaN}_3 \), and of photosynthesis at 20 \( \mu \text{M} \).

Figure 3 shows that aminotriazole differs from azide and cyanide in that any given concentration of aminotriazole inhibits the contaminant catalase to a much greater extent than it inhibits photosynthesis. Thus, 50% inhibition of catalase occurs at an aminotriazole concentration of 7 \( \mu \text{M} \), whereas 50% inhibition of photosynthesis does not occur until aminotriazole is present at 170 \( \mu \text{M} \). At the aminotriazole concentration (68 \( \mu \text{M} \)) producing 90% inhibition of catalase, photosynthesis was inhibited by 32%.

Since the inhibitory effect of aminotriazole on catalase is likely to increase with decreasing \( \text{H}_2\text{O}_2 \) concentration, and since \( \text{H}_2\text{O}_2 \) has been reported (15) to be inhibitory to photosynthesis at concentrations much lower than the 4 \( \mu \text{M} \) used in the assay system, 50 \( \mu \text{M} \) aminotriazole (giving only 85% inhibition of catalase under the conditions of Fig. 3) was chosen as a suitable concentration at which catalase could be inhibited selectively to a greater extent than photosynthesis. For the experiments described in Figures 4 to 6 the Chl concentration was raised to 50 \( \mu \text{g ml}^{-1} \).

In Figure 4, three \( \text{O}_2 \) electrode traces are reproduced. The two upper traces show the characteristic lag phase of \( \text{CO}_2 \)-dependent \( \text{O}_2 \) evolution in illuminated intact chloroplasts (20, 21). The lower trace is a dark control, and for all three traces aminotriazole was absent from the reaction mixture. After 5 min of illumination \( \text{H}_2\text{O}_2 \) (0.4 \( \mu \text{M} \)) was added to one of the illuminated reaction mixtures (middle trace) and to the dark control (lower trace), and...
FIG. 3. Inhibition by aminotriazole of photosynthetic O$_2$ evolution (CO$_2$-dependent) and of catalase in the chloroplast preparation; Chl concentration was 20 $\mu$g ml$^{-1}$. Photosynthetic O$_2$ evolution: chloroplasts 59% intact; control rate: 35 nmol of O$_2$ (mg of Chl)$^{-1}$ hr$^{-1}$; catalase activity of chloroplast preparation: 10 units (mg of Chl)$^{-1}$. Catalase activity: chloroplasts 40% intact; control activity: 13 units (mg of Chl)$^{-1}$.

FIG. 4. O$_2$ electrode trace showing effect of H$_2$O$_2$ (0.4 $\mu$mol) on CO$_2$-dependent O$_2$ evolution by isolated chloroplasts. Middle trace shows this effect of H$_2$O$_2$. Lower trace is a dark control, and the upper trace is that of an illuminated reaction to which no addition of H$_2$O$_2$ was made. Bracketed figures are rates of O$_2$ evolution in nmol of O$_2$ (mg of Chl)$^{-1}$ hr$^{-1}$. Chloroplasts 50% intact.

in both cases O$_2$ was immediately evolved as a result of catalase being present. After release of O$_2$ from H$_2$O$_2$ had finished (1.5 min) the dark control's trace reverted to its original, slow O$_2$ uptake, while the illuminated reaction's trace showed a slow rate of O$_2$ evolution which gradually increased in a second lag phase to a second steady-state rate of O$_2$ evolution 34% lower than that which occurred before addition of H$_2$O$_2$.

With 50 mM aminotriazole present in the reaction mixture, the corresponding results obtained are shown in Figure 5. Here addition of 0.4 $\mu$mol H$_2$O$_2$ did not result in a sudden increase in the rate of O$_2$ evolution in the illuminated reaction (middle trace), and actually caused a short lived O$_2$ uptake in the dark control (lower trace). In fact, H$_2$O$_2$ immediately inhibited O$_2$ evolution in the illuminated reaction, and subsequent recovery of O$_2$ evolution took place in two phases. In the first phase (lasting for 2 min after addition of H$_2$O$_2$), O$_2$ evolution increased with the decay of the O$_2$-consuming reaction seen clearly in the dark control. The second recovery phase in the illuminated reaction was slower, and resembled the initial lag phase in onset of O$_2$ evolution. With aminotriazole present the final steady-state rate of O$_2$ evolution was 70% lower than that observed before addition of H$_2$O$_2$.

In the traces reproduced in Figure 6 a larger quantity (0.8 $\mu$mol) of H$_2$O$_2$ was added after a 5-min illumination. In the absence of aminotriazole (upper trace) release of O$_2$ from H$_2$O$_2$ was followed by a brief period (40 sec) of zero net O$_2$ exchange, and then by a slow increase to a rate of O$_2$ evolution 66% smaller than the first steady-state rate. In the presence of aminotriazole (lower trace) a rapid uptake of O$_2$ followed addition of H$_2$O$_2$. This O$_2$ consumption (seen also in the dark control in Fig. 5) is not a photosynthetic reaction, but requires the presence of aminotriazole, catalase, and H$_2$O$_2$ in the reaction mixture. Both in light and dark the O$_2$ uptake does not decay rapidly at higher H$_2$O$_2$ concentrations (results not shown). Addition of excess catalase eliminated this O$_2$ uptake as the H$_2$O$_2$ present was removed (Fig. 6). The low yield of O$_2$ on addition of catalase suggests that some H$_2$O$_2$ had been used up by reaction with aminotriazole, although the liberation of O$_2$ indicates that endogenous catalase had been largely inhibited. Resumption of photosynthetic activity (indicated by offset arrows in Fig. 6) occurred in the absence of aminotriazole after all of the H$_2$O$_2$ had been decomposed by the "endogenous" catalase. In the presence of aminotriazole photosynthetic CO$_2$-dependent O$_2$ evolution resumed only after the H$_2$O$_2$ had been decomposed by addition of excess catalase.

**DISCUSSION**

The similarity of the concentration ranges of inhibition by cyanide of photosynthesis and of catalase (Fig. 1) does not necessarily reflect the simple cause-and-effect relationship suggested by Forti and Gerola (11), since the shapes of the inhibition curves differ, as do the cyanide concentrations required for 50% inhibition (17 $\muM$ and 3.5 $\muM$ for photosynthesis and catalase, respectively). In fact Table I of Forti and Gerola (11) shows even less agreement between inhibition of photosynthesis (50% at 135 $\muM$ cyanide) and inhibition of catalase (50% at 11 $\muM$ cyanide.). Kaiser (15) uses 160 $\muM$ cyanide to inhibit catalase completely, in agreement with Figure 1 of the present paper, but reports that this cyanide concentration affects CO$_2$ fixation only slightly. Cyanide at 160...
leaves to inhibition of photosynthesis (Fig. 4), although in the presence of aminotriazole the effect is enhanced (Fig. 5) because the added H$_2$O$_2$ is not then removed by the action of catalase. After addition of H$_2$O$_2$, the slow recovery of CO$_2$-dependent O$_2$ evolution is consistent with H$_2$O$_2$ having depleted the chloroplast of intermediates of the reductive pentose-P pathway. The second lag phase (which follows addition of H$_2$O$_2$) presumably resembles the first lag phase in being a period in which autocatalysis of CO$_2$ fixation is taking place (20, 21). Depletion by H$_2$O$_2$ of Calvin cycle intermediates was proposed by Kaiser (15). An alternative explanation might be that the second lag phase is a period during which reduction and activation of regulatory steps of the cycle are occurring, inhibition by H$_2$O$_2$ having perhaps been caused by oxidation and consequent inhibition of regulatory enzymes.

The chief complication which results from the presence of aminotriazole in these experiments is the rapid nonphotosynthetic O$_2$ consumption which occurs at higher H$_2$O$_2$ concentrations (Fig. 6). The relative small effect of aminotriazole on photosynthesis per se makes it likely that any Mehler reaction in intact chloroplasts is not rapid enough to produce inhibitory concentrations of H$_2$O$_2$ under conditions optimal for CO$_2$ fixation. Endogenous peroxidase may nevertheless allow photosynthetic O$_2$ reduction to proceed under certain circumstances in vivo (4), and it may still be possible to demonstrate in vitro increased chloroplast H$_2$O$_2$ production under conditions suboptimal for CO$_2$ fixation.

Acknowledgment.—During the course of this work J. F. A. held a Postdoctoral Research Fellowship of the U.K. Science Research Council.

LITERATURE CITED

1. ALLEN JF 1975 A two-step mechanism for the photosynthetic reduction of oxygen by ferredoxin. Biochem Biophys Res Commun 66: 36–43

![Fig. 6. Effect of H$_2$O$_2$ (0.8 µmol) on CO$_2$-dependent O$_2$ evolution by chloroplasts in the absence of a catalase inhibitor (upper trace) and in the presence of 50 mM aminotriazole (lower trace). Vertical broken line indicates adjustment of zero position on chart recorder. Bracketed figures are rates of O$_2$ evolution in µmol of O$_2$ (mg of Chl) $^{-2}$ hr $^{-1}$. Catalase added: 5 × 10$^{-5}$ units. Chloroplasts 50% intact. Offset arrows (↓) indicate resumption of photosynthetic CO$_2$-dependent O$_2$ evolution after H$_2$O$_2$ treatment. µM would inhibit photosynthesis by slightly more than 50% according to the results of Forti and Gerola (11), while in the experiment presented here in Figure 1 no CO$_2$-dependent O$_2$ evolution could be detected at cyanide concentrations greater than 30 µM. It is difficult to reconcile this disparity. Since cyanide would perhaps be expected to have a smaller inhibitory effect on more active chloroplasts the explanation may in part be associated with differences in the photosynthetic activities of the chloroplast preparations being studied.

Azide also produces simultaneous inhibition of catalase and photosynthesis (Fig. 2), although the similarity of the concentration curves could well be fortuitous. Azide has been reported to inhibit photosynthetic CO$_2$ fixation by virtue of inhibiting carbonic anhydrase, with less than 10 µM azide causing 50% inhibition of carbonic anhydrase in lettuce chloroplasts (8). Cyanide is likely to have an even more direct effect on CO$_2$ fixation, since it is known to be an inhibitor of ribulose bisP carboxylase (23), and with a $K_i$ of 16 µM this property of cyanide may be a sufficient explanation of the inhibition of photosynthesis seen in Figure 1.

It is possible to reinterpret the finding that cyanide and azide not only inhibit CO$_2$ fixation by intact chloroplasts, but also lead to photosynthetic production of H$_2$O$_2$ (11). If it is assumed that on addition of cyanide or azide CO$_2$ fixation is inhibited directly, then NADPH oxidation by triose-P dehydrogenase will also be inhibited, and as NADP ceases to be available as the electron acceptor for PSI O$_2$ will replace it in this role. The production of H$_2$O$_2$ would then be a direct consequence of addition of azide or cyanide and not merely a result of inhibition of breakdown by catalase of H$_2$O$_2$ that has been independently produced. The additional factor of these inhibitors' effects on contaminants catalase ensures only that the end product of the Mehler reaction which they induce is H$_2$O$_2$ rather than H$_2$O.

This conclusion is strengthened by the ability of aminotriazole to inhibit catalase in unwashed chloroplast preparations without having an equal effect on photosynthesis (Fig. 3). Addition of H$_2$O$_2$ to chloroplasts in the absence of aminotriazole certainly