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

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ABSTRACT

The catalase activity of unwashed preparations containing intact spinach (*Spinacia oleracea* L.) chloroplasts is inhibited both by cyanide and by azide at concentrations which also cause inhibition of photosynthetic CO_2-dependent O_2 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 CO_2 fixation. Aminotriazole may be used as a specific inhibitor of catalase in order to demonstrate inhibition of photosynthesis by added H_2O_2.

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 H_2O_2 under the best experimental conditions for CO_2 fixation.

Several investigators have recently described evidence that under certain circumstances O_2 may compete with NADP as an electron acceptor in intact chloroplasts (4, 10, 11, 15). Although three distinct mechanisms of photosynthetic O_2 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 O_2, and H_2O_2 has been known to be a product of this reaction (termed the "Mehler reaction") for some time (16, 17).

Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) causes dismutation of H_2O_2 to H_2O and O_2, so that on addition of catalase to illuminated, O_2-reducing chloroplasts, zero net O_2 exchange replaces the net O_2 uptake which occurs instead when H_2O_2 is allowed to accumulate as the product of the reaction. This effect of catalase on the stoichiometry of O_2 uptake in the Mehler reaction has been exploited to demonstrate that O_2 competes with NADP as the terminal electron acceptor in noncyclic electron transport in broken, washed chloroplasts, incapable of CO_2 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 CO_2 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 CO_2 fixation. It has been suggested, moreover, that H_2O_2 itself is inhibitory to CO_2 fixation (11, 15), so that if O_2 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 H_2O_2 on chloroplast CO_2 fixation it is nevertheless desirable to inhibit catalase in some way, in order that the H_2O_2 should not be quickly consumed in a purely enzymic reaction which itself would entail O_2 evolution at a rate many times greater than that associated with photosynthesis.

Two recent investigations of H_2O_2 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 CO_2 fixation. In their view, cyanide's inhibition of catalase causes a build-up of photosynthetically produced H_2O_2, which in turn inhibits CO_2 fixation by preventing reduction of P-glycerate to triose-P. Kaiser (15), however, has demonstrated an inhibition of CO_2 fixation by added H_2O_2, using cyanide (160 μM) in his reaction mixture to inhibit completely the breakdown of H_2O_2 by contaminant catalase. In Kaiser's experiment cyanide at the concentration used apparently had only a slight inhibitory effect on CO_2 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 H_2O_2 on photosynthesis, and would indicate that inhibitory concentrations of H_2O_2 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 $MgCl_2$, 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 5,000*g* 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}.

O_2 evolution was measured in twin O_2 electrodes (Hansatech, King's Lynn, Norfolk) of the type described by Delieu and Walker (9). A temperature of 20 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 μg ml^{-1}. Results were recorded on a Servoscribe dual channel chart recorder.

All reactions commenced at the "air-line" of the O_2 electrode, in a medium containing the common constituents 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 50 mM HEPES at pH 7.6, and with a final volume of 1 ml.

For measurements of catalase activity 4 mM H_2O_2 was also present, and a unit of catalase activity is here defined as the quantity of catalase required to liberate O_2 at a rate of 1 μmol min^{-1} under these conditions. Addition of chloroplast suspension (catalase) started the reaction.

For measurements of photosynthetic O_2 evolution the reaction medium contained, in addition to the common constituents (above): 2 mM ascorbate, 0.2 mM K_2HPO_4, 1 mM ribose-5-P, and 10 mM $NaHCO_3$. 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 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 $K_3Fe(CN)_6$, and 5 mM NH_4Cl. 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 (CO_2-dependent 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 μM, while 50% inhibition of photosynthesis occurs at 17 μM. For 90% inhibition, however, the situation is reversed: with catalase activity it occurs at 60 μM KCN; with photosynthesis at only 25 μM. 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 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 NaN_3 50% inhibition of catalase occurs at 3 μM, and of

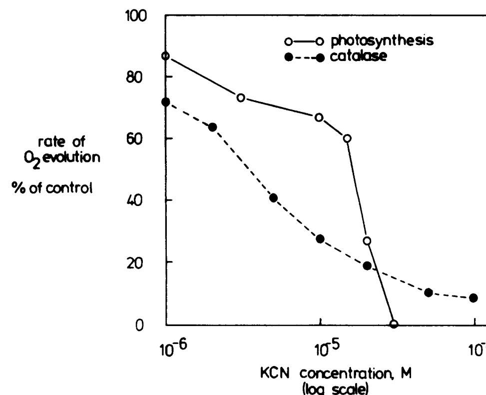

FIG. 1. Inhibition by KCN of photosynthetic O_2 evolution (CO_2-dependent) and of catalase in chloroplast preparations; Chl concentration was 20 μg ml^{-1}. Photosynthetic O_2 evolution: chloroplasts 58% intact; control rate was 25 μmol O_2 (mg Chl)$^{-1}$; unshocked chloroplast preparation had a catalase activity of 10 units (mg of Chl)$^{-1}$. Catalase activity: chloroplasts 40% intact; control activity: 13 units (mg of Chl)$^{-1}$.

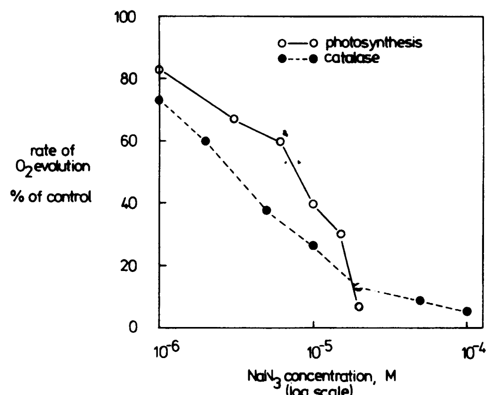

FIG. 2. Inhibition by NaN_3 of photosynthetic O_2 evolution (CO_2-dependent) and of catalase in the chloroplast preparation; Chl concentration was 20 μg ml^{-1}. Photosynthetic O_2 evolution: chloroplasts 58% intact; control rate: 25 μmol of O_2 (mg of Chl)$^{-1}$ hr^{-1}; catalase activity of unshocked chloroplast preparation: 10 units (mg Chl)$^{-1}$. Catalase activity: chloroplasts 40% intact; control activity: 12.4 units (mg of Chl)$^{-1}$.

photosynthesis at 8 μM, while 90% inhibition of catalase is at 40 μM NaN_3, and of photosynthesis at 20 μM.

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 mM, whereas 50% inhibition of photosynthesis does not occur until aminotriazole is present at 170 mM. At the aminotriazole concentration (68 mM) producing 90% inhibition of catalase, photosynthesis was inhibited by 32%.

Since the inhibitory effect of aminotriazole on catalase is likely to increase with decreasing H_2O_2 concentration, and since H_2O_2 has been reported (15) to be inhibitory to photosynthesis at concentrations much lower than the 4 mM used in the assay system, 50 mM 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 μg ml^{-1}.

In Figure 4, three O_2 electrode traces are reproduced. The two upper traces show the characteristic lag phase of CO_2-dependent 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 H_2O_2 (0.4 μmol) was added to one of the illuminated reaction mixtures (middle trace) and to the dark control (lower trace), and

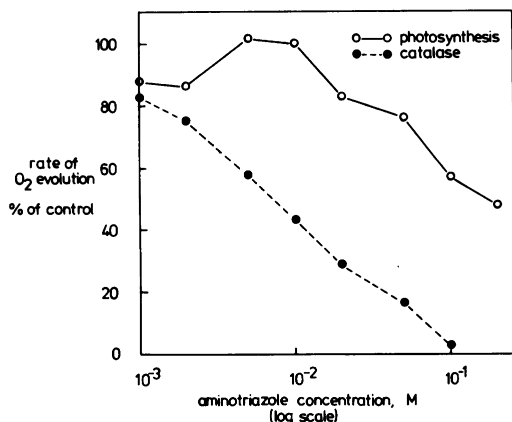

FIG. 3. Inhibition by aminotriazole of photosynthetic O_2 evolution (CO_2-dependent) and of catalase in the chloroplast preparation; Chl concentration was 20 μg ml^{-1}. Photosynthetic O_2 evolution: chloroplasts 59% intact; control rate: 35 μmol of O_2 (mg of Chl)$^{-1}$ hr^{-1}; catalase activity of chloroplast preparation: 10 units (mg of Chl)$^{-1}$. Catalase activity: chloroplasts 40% intact; control activity: 13 units (mg of Chl)$^{-1}$.

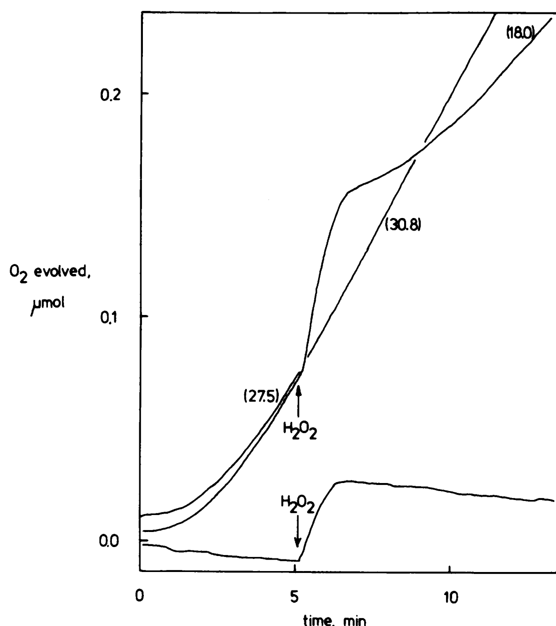

FIG. 4. O_2 electrode trace showing effect of H_2O_2 (0.4 μmol) on CO_2-dependent O_2 evolution by isolated chloroplasts. Middle trace shows this effect of H_2O_2. Lower trace is a dark control, and the upper trace is that of an illuminated reaction to which no addition of H_2O_2 was made. Bracketed figures are rates of O_2 evolution in μmol 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_2O_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_2O_2.

With 50 mM aminotriazole present in the reaction mixture, the corresponding results obtained are shown in Figure 5. Here addition of 0.4 μmol H_2O_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_2O_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_2O_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_2O_2.

In the traces reproduced in Figure 6 a larger quantity (0.8 μmol) of H_2O_2 was added after a 5-min illumination. In the absence of aminotriazole (upper trace) release of O_2 from H_2O_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_2O_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_2O_2 in the reaction mixture. Both in light and dark the O_2 uptake does not decay rapidly at higher H_2O_2 concentrations (results not shown). Addition of excess catalase eliminated this O_2 uptake as the H_2O_2 present was removed (Fig. 6). The low yield of O_2 on addition of catalase suggests that some H_2O_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_2O_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_2O_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 μM and 3.5 μM 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 μM cyanide) and inhibition of catalase (50% at 11 μM cyanide.). Kaiser (15) uses 160 μM 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

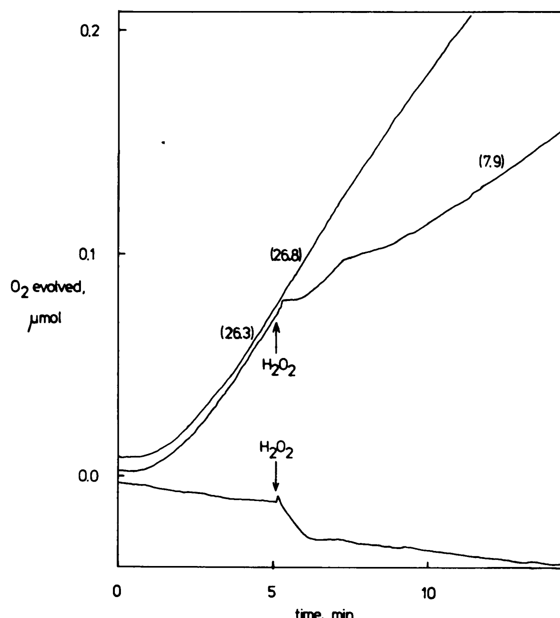

FIG. 5. O_2 electrode trace showing effect of H_2O_2 (0.4 μmol) on CO_2-dependent O_2 evolution by isolated chloroplasts in the presence of aminotriazole (50 mM). Middle trace shows this effect of H_2O_2. Lower trace is a dark control; upper trace is of an illuminated reaction to which no addition of H_2O_2 was made. Bracketed figures are rates of O_2 evolution in μmol of O_2 (mg of Chl)$^{-1}$ hr^{-1}. Chloroplasts 50% intact.

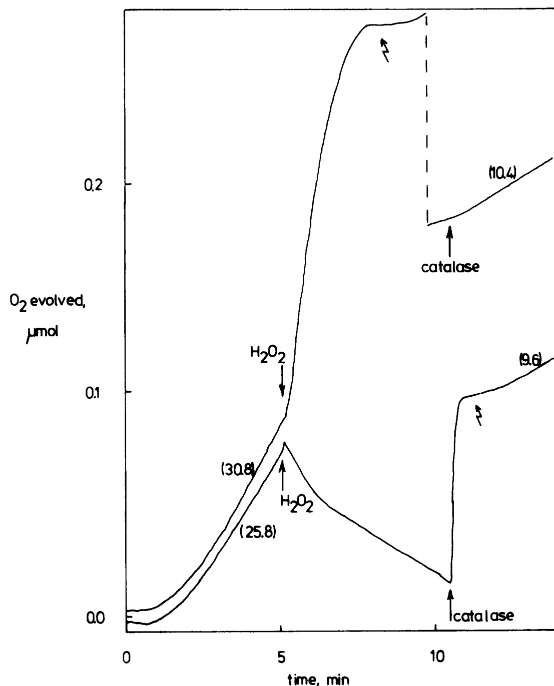

FIG. 6. Effect of H_2O_2 (0.8 μmol) on CO_2-dependent O_2 evolution by chloroplasts in the absence of a catalase inhibitor (upper trace) and in the presence of 50 mM aminotriazole (lower trace). Vertical broken line indicates adjustment of zero position on chart recorder. Bracketed figures are rates of O_2 evolution in μmol of O_2 (mg of Chl)$^{-1}$ hr^{-1}. Catalase added: 5×10^3 units. Chloroplasts 50% intact. Offset arrows ( ) indicate resumption of photosynthetic CO_2-dependent O_2 evolution after H_2O_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_2O_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_2O_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_2O_2 that has been independently produced. The additional factor of these inhibitors' effects on contaminant catalase ensures only that the end product of the Mehler reaction which they induce is H_2O_2 rather than H_2O.

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_2O_2 to chloroplasts in the absence of aminotriazole certainly leads to inhibition of photosynthesis (Fig. 4), although in the presence of aminotriazole the effect is enhanced (Fig. 5) because the added H_2O_2 is not then removed by the action of catalase. After addition of H_2O_2, the slow recovery of CO_2-dependent O_2 evolution is consistent with H_2O_2 having depleted the chloroplast of intermediates of the reductive pentose-P pathway. The second lag phase (which follows addition of H_2O_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_2O_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_2O_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_2O_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_2O_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_2O_2 production under conditions suboptimal for CO_2 fixation.

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