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Reversible Inhibition of the Calvin Cycle and Activation of Oxidative Pentose Phosphate Cycle in Isolated Intact Chloroplasts by Hydrogen Peroxide*

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Abstract. Hydrogen peroxide (6×10^{-4} M) causes a 90% inhibition of CO_2-fixation in isolated intact chloroplasts. The inhibition is reversed by adding catalase (2500 U/ml) or DTT (10 mM). If hydrogen peroxide is added to a suspension of intact chloroplasts in the light, the incorporation of carbon into hexose- and heptulose bisphosphates and into pentose monophosphates is significantly increased, whereas; carbon incorporation into hexose monophosphates and ribulose 1,5-bisphosphate is decreased. At the same time formation of 6-phosphogluconate is dramatically stimulated, and the level of ATP is increased. All these changes induced by hydrogen peroxide are reversed by addition of catalase or DTT. Additionally, the conversion of [^{14}C]glucose-6-phosphate into different metabolites by lysed chloroplasts in the dark has been studied. In presence of hydrogen peroxide, formation of ribulose-1,5-bisphosphate is inhibited, whereas formation of other bisphosphates, of triose phosphates, and pentose monophosphates is stimulated. Again, DTT has the opposite effect. The release of $^{14}CO_2$ from added [^{14}C]glucose-6-phosphate by the soluble fraction of lysed chloroplasts via the reactions of oxidative pentose phosphate cycle is completely inhibited by DTT (0.5 mM) and re-activated by comparable concentrations of hydrogen peroxide. These results indicate that hydrogen peroxide interacts with reduced sulfhydryl groups which are involved in the light activation of enzymes of the Calvin cycle at the site of fructose- and sedoheptulose bisphosphatase, of phosphoribulokinase, as well as in light-inactivation of oxidative pentose phosphate cycle at the site of glucose-6-phosphate dehydrogenase.

Key words: Chloroplast metabolism – Hydrogen peroxide – Regulation.

Introduction

It was recently shown that CO_2-fixation of isolated intact chloraplasts is strongly inhibited by hydrogen peroxide which was either formed endogenously by pseudocyclic electron transport or added to the chloroplast suspension (Egneus et al., 1975; Kaiser, 1976; Forti and Gerola, 1977). This inhibition was shown to be due to an interaction of hydrogen peroxide with some intermediates or enzymes of the Calvin cycle (Kaiser, 1976). The site of inhibition was suggested to be located somewhere in the reaction sequence leading from triose phosphate to the formation of pentose monophosphates (Kaiser, 1976). A possible mechanism for the inhibitory action of hydrogen peroxide was thought to be an oxidation of a glycolaldehyde-transketolase complex leading to glycolate formation, as proposed by Gibbs and coworkers (Plaut and Gibbs, 1970; Shain and Gibbs, 1971). However, more recent studies indicated that there is no relationship between the action of hydrogen peroxide on CO_2-fixation and the formation of glycolate (Kirk and Heber, 1976). Therefore it seemed necessary to study and characterize the interaction of hydrogen peroxide with CO_2-fixation more extensively.

* Dedicated to Prof. Dr. W. Simonis on the occasion of his 70th birthday

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Abbreviations: ADPG = ADP-glucose; DHAP = dihydroxyacetone phosphate; DTT = dithiothreitol; FBP = fructose-1,6-bisphosphate; HEPES = N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; HMP = hexose monophosphates (fructose-6-phosphate, glucose-6-phosphate, glucose-1-phosphate); 6-PGl = 6-phosphogluconate; PMP = pentose monophosphates (xylulose-5-phosphate, ribose-5-phosphate, ribulose-5-phosphate); RuBP = ribulose-1,5-bisphosphate; S7P = sedoheptulose-7-phosphate; SBP = sedoheptulose-1,7-bisphosphate

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Materials and Methods

Plant Material

Spinach was grown in vermiculite fertilized with Hoaglands solution under artificial light (33,000 lx) with an 8 h light period and a 16 h dark period at a temperature of 13° C. Chloroplasts were isolated from young leaves according to Jensen and Bassham, (1966), and chlorophyll was determined following the procedure of Arnon (1949).

Incorporation of $^{14}CO_2$ or ^{32}Pi

50 µl of a suspension of intact chloroplasts (100 µg chlorophyll) were added to solution C (Jensen and Bassham, 1966), containing 7 mM $NaHCO_3$ and either 100 µC $NaH^{14}CO_3$ or 50 µCi $KH_2{}^{32}PO_4$ (0.5 mM) in a total volume of 1 ml. The suspension was assayed in serum-stoppered flasks in a water bath illuminated from below with fluorescent tubes, providing a light intensity of 450 µmol photons $m^{-2} s^{-1}$. At given intervalls 50 µl were removed with microsyringes and injected into 250 µl methanol. For measuring total CO_2-fixation, aliquots were removed from the chloroplast-methanol mixture, acidified with 200 µl glacial acetic acid, dried by flushing with nitrogen, and counted by liquid scintillation.

Separation of Chloroplasts from the Medium

In some experiments the chloroplast pellet was separated from the medium by the method of Bassham et al. (1968), but with minor modifications. Intact chloroplasts (150 µg chlorophyll) were incubated in a total volume of 3 ml solution C, and labelled with 150 µCi ^{32}Pi (0.5 mM) under the conditions mentioned above. At given times in the light, 200 µl of the suspension was quickly transferred into ice-cold microfuge tubes and centrifuged for about 12 s in a microfuge (Beckmann Instruments). Aliquot samples (150 µl) of the supernatant were removed and injected into 300 µl methanol. The rest of the supernatant was discarded. The pellet was inactivated by injecting 200 µl of methanol into the microfuge tubes. The average time from sampling to deactivation was about 40 s.

Experiments with Lysed Chloroplasts or Stroma Enzymes

For preparing lysed chloroplasts or membrane-free stroma enzymes, a suspension of freshly isolated intact chloroplasts containing about 2 mg chlorophyll was centrifuged for about 1 min at 1,000 *g* (4° C). The pellet was resuspended in 1.4 ml of a lysing solution containing 0.025 M HEPES-NaOH pH 8.0 and 5 mM $MgCl_2$. The resulting suspension of lysed chloroplasts was either used directly, or centrifuged for 15 min at 27,000 *g* (4° C) in order to obtain membrane-free stroma enzymes. The resulting clear supernatant had an average protein content of 5–10 mg protein in 1.2 ml. 300–600 µl of the lysed chloroplast suspension or of stroma enzymes were added to a reaction medium (0.5–1.0 ml) which was identical with the lysing solution, but contained in addition 2 mM U-labelled [^{14}C]glucose-6-phosphate (specific activity 50 µCi/µmol), 1 mM $NADP^+$ or 1 nM ATP, and other components, as indicated in the legends of the figures and tables.

For studying the conversion of [^{14}C]glucose-6-phosphate into other products of the Calvin cycle, aliquots (50 µl) of the reaction mixture were sampled with micro-syringes and injected into 250 µl of methanol. Separation of products was carried out as described below.

For measuring the release of $^{14}CO_2$ from the added [^{14}C]glucose-6-phosphate via the reactions of oxidative pentose phosphate cycle, the flasks were continuously flushed with nitrogen. The gas outlet was connected to 25-ml scintillation vials, containing 20 ml of a quaternary ammonium hydroxid solution (Protosol, New England Nuclear). The reaction was usually started by adding the stroma enzymes, and stopped after 10 min by adding 100 µl of 2 M HCl in order to get a quantitative release of CO_2. Gasing was usually continued for 5 min. An aliquot of the trapping solution was counted by liquid scintillation.

Analysis of Products

Chloroplast metabolites were separated by descending paper chromatography and radioautography (Jensen and Bassham, 1966). Aliquots of the chloroplast-methanol mixture were spotted on Whatman Nr. 1 paper and developed in two dimensions; first for 60 h with a phenol-water-acetic acid solvent, and then for 48 h in the second direction with butanol-water-propionic acid. For a further separation of the bisphosphates, the corresponding spot was eluted with water, phosphatased in acetate buffer (pH 5), and rechromatographed for 24 h in each direction in the solvent system mentioned above. The resulting spots of the free sugars were cut into small pieces and eluted with 5 ml of water in 25 ml-scintillation vials for about 4 h. After that, 15 ml of scintillation fluid were added and the sample was counted by liquid scintillation.

Results

Effect of Hydrogen Peroxide on Carbon Metabolism of Intact Chloroplasts

When hydrogen peroxide is added to a suspension of illuminated intact chloroplasts in concentrations of between $3 \cdot 10^{-4}$ M and $7 \cdot 10^{-4}$ M, CO_2-fixation is strongly inhibited, despite the presence of some catalase activity which contaminates most chloroplast preparations (Fig. 1). The inhibition is almost fully reversed in the light by addition of either catalase (2,500 U) or DTT (10 mM).

Subsequent addition of hydrogen peroxide and catalase to intact chloroplasts in the light causes characteristic changes in the labelling pattern of different metabolites (Fig. 2A–D). After adding hydrogen peroxide, formation of DHAP stops and the level of DHAP decreases slightly. PGA is still formed, but with a much lower rate. The level of FBP+SBP increases further, whereas the level of HMP is slightly decreased. Formation of PMP is also stimulated. The ^{14}C-labelling of 6-PGl is usually very low in the light. However, after addition of hydrogen peroxide, formation of 6-PGl is dramatically stimulated. At the same time, the level of RuBP drops down. All these changes in the flow of carbon into different metabolites are usually reversed after addition of catalase or DTT (Fig. 2A–D). However, some individual effects have to be considered more extensively. After addition of catalase, the level of DHAP is transiently decreased

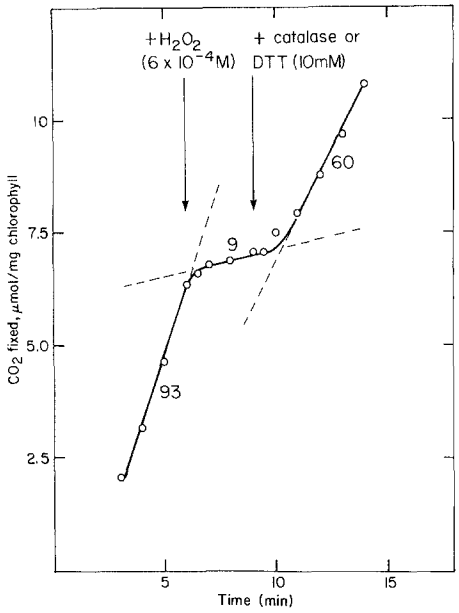

Fig. 1. Inhibition of CO_2-fixation of isolated intact chloroplasts by hydrogen peroxide, and its reversal by catalase (2,500 U/ml) or DTT (10 mM). 20 µl of hydrogen peroxide or 10 µl of catalase were added as indicated to give the final concentrations shown in the Figure. Numbers next to the curves give the rates of CO_2-fixation in µmol/mg chlorophyll · h. For further details see Materials and Methods

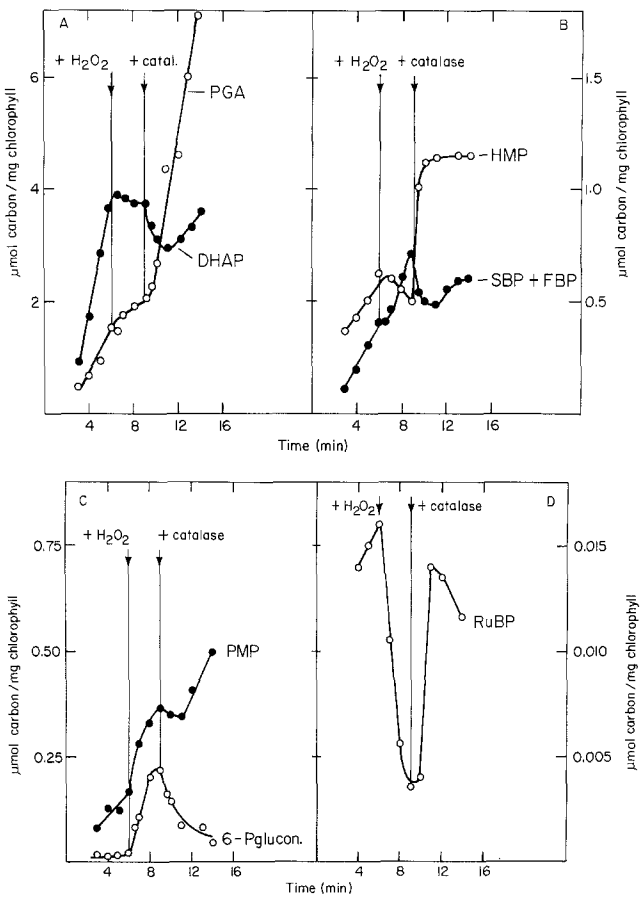

Fig. 2A–D. Effect of subsequent addition of hydrogen peroxide and catalase on incorporation of carbon into different metabolites by intact chloroplasts. Same experiment as in Fig. 1. For details see Materials and Methods

during the first two minutes, parallelled by a corresponding change in the level of FBP+SBP. After 1–3 min, carbon incorporation into all three compounds increases again but with a much slower rate than in the first phase proceeding the addition of hydrogen peroxide. At the same time, much more carbon is fixed in the form of PGA, resulting in a lower ratio of [DHAP]/[PGA]. Similar effects of catalase on the [DHAP]/[PGA]-ratio have been reported by Egneus et al. (1975).

It has been shown that changes in the levels of DHAP and PGA (e.g. induced by light-dark transition) inside the chloroplasts are very different from those in the surrounding medium, and also differ from changes in total [PGA] and [DHAP] (Kaiser and Bassham, 1979a). Therefore, it seemed necessary to investigate the effects of hydrogen peroxide and catalase on the concentrations of ^{32}P-labelled DHAP and PGA in the chloroplast pellet only. As expected we found the level of PGA inside the chloroplasts to be considerably decreased after addition of hydrogen peroxide, and increased after addition of catalase (Table 1). The level of DHAP inside the chloroplasts was found to be much lower than the PGA-level, and it was only slightly influenced by hydrogen peroxide or catalase. Consequently, the [DHAP]/[PGA]-ratio inside the chloroplasts was increased by hydrogen peroxide and decreased by catalase. These changes were accompanied by corresponding changes of the total ATP-level in chloroplasts and supernatant (Table 1). The data fully supported similar findings reported recently by Heldt et al. (1978).

Table 1. Levels of PGA and DHAP in the chloroplast pellet in the light, as compared to the level of ATP. A suspension of intact chloroplasts was illuminated in the presence of 0.5 mM ^{32}Pi. After 6 min, sample a (control) was taken and subjected to centrifugation. At the same time, hydrogen peroxide (7×10^{-4} M) was added to the reaction flask. After 4 more min, sample c (+hydrogen peroxide) was taken, and catalase was added (2500 U). 2 min later, sample c (+catalase) was taken. The level of ATP was measured under identical conditions without separating pellet and supernatant. Metabolite levels were calculated by accepting one ^{32}Pi/PGA or DHAP, and 2 ^{32}Pi/ATP. For further details see Materials and Methods

Conditions	nmol/mg chlorophyll			[DHAP]/[PGA]
	DHAP	PGA	ATP	
a) control	10.0	57.0	6.6	0.175
b) $+H_2O_2$	7.5	29.0	11.5	0.259
c) +catalase	7.0	85.0	3.5	0.082

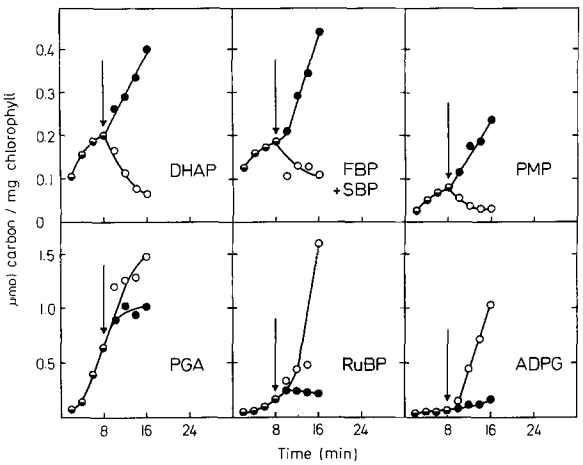

Fig. 3. Effect of hydrogen peroxide (1 mM) or DTT (10 mM) on the conversion of [^{14}C]glucose-6-phosphate into Calvin cycle intermediates by lysed chloroplasts. 2 parallel samples (●—●) were incubated in the dark under nitrogen as described in Materials and Methods. At the time indicated by an arrow, hydrogen peroxide was added to one flask (●—●), and DTT to the other (○—○)

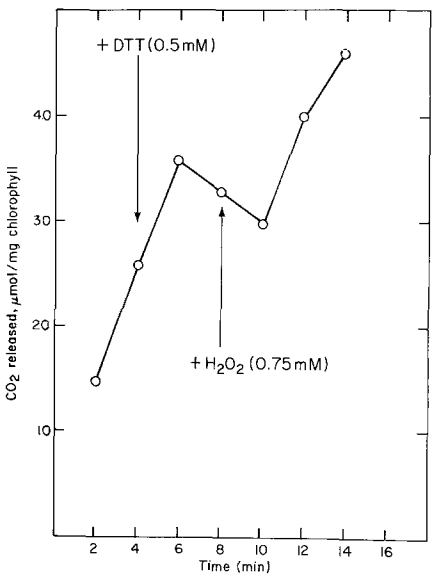

Fig. 4. Effect of DTT (0.5 mM) and hydrogen peroxide (0.75 mM) on the release of $^{14}CO_2$ from added [^{14}C]glucose-6-phosphate by the soluble fraction of lysed chloroplasts. The reaction medium was identical with the lysing solution, but contained in addition in a total volume of 500 µl: 2 mM $NADP^+$, 2 mM glucose, 5 U hexokinase and 2 mM [^{14}C]glucose-6-phosphate with a specific activity of 50 µCi/µmol. Glucose and hexokinase were added as an ATP-trapping system in order to avoid consumption of glucose-6-phosphate by phosphofructokinase

Effects of Hydrogen Peroxide and DTT on Dark Metabolism of Lysed Chloroplasts

The occurrence and interpretation of changes of carbon incorporation into different metabolites by intact chloroplasts is complicated by factors such as a multiple interconnections of different metabolite pools via freely reversible reactions, or by export of only some metabolites into the medium. Furthermore, if a reaction at an earlier step of the Calvin cycle (e.g. at the level of fructose bisphosphatase) is inhibited, subsequent reactions will also be indirectly influenced due to a lack of substrate. In order to avoid some of these disadvantages, additional experiments have been carried out with an open system by feeding [^{14}C]G6P and ATP to lysed chloroplasts in the dark and following its conversion into other metabolites. It has been shown recently that lysed chloroplasts are able to convert [^{14}C]G6P into different compounds of the Calvin cycle, depending on conditions (Kaiser and Bassham, 1979c).

The effects of hydrogen peroxide or DTT on the conversion of [^{14}C]G6P into other products by lysed chloroplasts in the dark (under nitrogen) are shown in Fig. 3. Hydrogen peroxide (1 mM) inhibits the formation of RuBP, and to a smaller extent also of PGA. The formation of all other products (DHAP, SBP+FBP, PMP) is stimulated. In contrast to this, formation of RuBP is dramatically stimulated by DTT, together with a less significant increase in the rate of PGA-formation (which, in the absence of CO_2, is formed via triose phosphate oxidation). The levels of DHAP, FBP+SBP and PMP are considerably decreased after addition of DTT. Another striking effect of DTT is the strong stimulation of ADP-glucose formation, which was recently described in more detail (Kaiser and Bassham, 1979b).

Reversible Activation of Oxidative Pentose Phosphate Cycle by Hydrogen Peroxide

The level of 6-PGl, an intermediate of oxidative pentose phosphate cycle, is usually extremely low in the light (Fig. 2C). However, after addition of hydrogen peroxide to a suspension of intact chloroplasts the level of 6-PGl increases dramatically until it reaches a steady state. After addition of catalase it decreases slowly to the original level (Fig. 2C). It is assumed that an increase or decrease in the level of 6-PGl reflects an activation or inactivation of G6P-dehydrogenase, resp.

The occurrence of oxidative pentose phosphate cycle can also be followed quantitatively by trapping the $^{14}CO_2$ released from [^{14}C]G6P by the soluble fraction of lysed chloroplasts in the presence of substrate amounts of $NADP^+$ (Kaiser and Bassham, 1979c). It was found that the release of CO_2 was completely inhibited by DTT (0.5 mM) and fully reactivated by hydrogen peroxide (0.75 mM, Fig. 4). For

unknown reasons both the inhibition and the reactivation occurred with a lag-phase of about 2 min.

Discussion

It is now generally accepted that reduced sulfhydryl groups are involved in light-activation of certain Calvin cycle enzymes, such as the fructose- and sedoheptulose bisphosphatases, phosphoribulokinase, and perhaps also of GAP-dehydrogenase (for review see Kelly et al., 1976). Furthermore, it has been shown that dithiols at low concentrations inactivate the key enzyme of oxidative pentose phosphate cycle, G6P-dehydrogenase (Anderson et al., 1974). Recently it was proposed by Wolosiuk and Buchanan (1977) that some enzymes of the Calvin cycle are regulated by the ratio of oxidized/reduced glutathione, involving the ferredoxin-thioredoxin system. In this system, glutathione might be reduced via NADPH-dependent glutathione reductase and oxidized in the presence of hydrogen peroxide via glutathione peroxidase. Thereby the level of hydrogen peroxide inside the chloroplasts might play an important role for regulating the activity of light-modulated enzymes.

Our results obtained with intact chloroplasts and, more significantly, with a lysed chloroplast system, show that hydrogen peroxide stimulates the formation of FBP, SBP and PMP, and inhibits the formation of HMP (also containing S7P) and RuBP. These data support other findings on an increased level of bisphosphates in intact chloroplasts after addition of hydrogen peroxide (Heldt et al., 1978). Similar changes in metabolite levels have been observed in *Chlorella* cells and isolated intact spinach chloroplasts after addition of Vitamin K_5 (Krause and Bassham, 1969). They were attributed to an inhibition of noncyclic electron transport by Vitamin K_5 with a subsequent inhibition of light-activation of Calvin cycle enzymes (Krause and Bassham, 1969). Considering the above-mentioned models on the regulation of the bisphosphatases and phosphoribulokinase, we are in agreement with Heldt et al. (1978) that hydrogen peroxide inhibits these enzymes by oxidizing light-generated SH-groups. This would also explain the the complete reversal of the inhibition of CO_2-fixation by reduced DTT. However, it cannot be decided whether DTT acts simply by removing hydrogen peroxide, as does catalase, or by reducing endogenous SH-groups which have been oxidized by hydrogen peroxide.

The reversal of the inhibitory effect of hydrogen peroxide by catalase cannot be caused by a direct interaction of the enzyme with SH-groups. But it is reasonable to assume that after removal of hydrogen peroxide, oxidized SH-groups are quickly re-reduced in the light by photosynthetic electron transport.

Recent experiments with lysed chloroplasts showed that CO_2-fixation in the dark was inhibited by hydrogen peroxide only with F6P and DHAP, but not with PMP as substrate (Kaiser, 1976). At that time it was assumed that hydrogen peroxide might oxidize a glycolaldehyde-transketolase complex, as proposed by Gibbs and coworkers (Plaut and Gibbs, 1970; Shain and Gibbs, 1971), thereby depleting the chloroplasts of photosynthetic intermediates. Our present data offer another explanation: formation of RuBP from added F6P and DHAP includes two SH-regulated steps, the sedoheptulose bisphosphatase and phosphoribulokinase. Inactivation of these enzymes by addition of hydrogen peroxide should prevent formation of RuBP and CO_2-fixation, as previously observed (Kaiser, 1976).

According to the results described above one would also expect an inhibition of CO_2-fixation of lysed chloroplasts with PMP as substrate, since this would still include one SH-regulated step. However, preliminary experiments (not shown here) indicate that even in the complete absence of SH-groups there is still considerable phosphoribulokinase activity left (10–30% of maximal activity) which is not influenced by additional hydrogen peroxide. Since our earlier experiments were done in the absence of dithiols, addition of hydrogen peroxide could hardly decrease the phosphoribulokinase activity further, as previously observed (Kaiser, 1976).

It cannot be decided from our present data whether hydrogen peroxide inhibits GAP-dehydrogenase. The changes of the [DHAP]/[PGA] ratio inside the chloroplasts after addition of hydrogen peroxide might merely reflect the changes of the ATP-level, which in turn might be due to changes in the activity of phosphoribulokinase. Also, recent experiments showed that PGA-reduction by intact chloroplasts is less sensitive to hydrogen peroxide than the other reactions (Kaiser, 1976).

Our experiments also gave clear evidence for an activation of oxidative pentose phosphate cycle by hydrogen peroxide, which can be reversed in the light by adding catalase or in the dark by adding DTT. Since the key enzyme of oxidative pentose phosphate cycle, G6P-dehydrogenase, has been shown to be inactivated by reduced dithiols (Anderson et al., 1974), this fits again very well into the concept of an interaction of hydrogen peroxide with light-generated SH-groups which are involved in light-dark regulation of chloroplast metabolism.

Since hydrogen peroxide is a natural product of photosynthetic electron transport in vivo as well as in vitro (Patterson and Myers, 1973; Egneus et al.,

1975; Radmer and Kok, 1976), one might speculate about a possible physiological role of hydrogen peroxide in the regulation of photosynthesis. It can be assumed that hydrogen peroxide is formed in the light, primarily if the re-oxidation of NADPH is somehow limited, e.g. under conditions of low CO_2-partial pressures (Radmer and Kok, 1976). In this case an increased formation of hydrogen peroxide would tend to decrease the activities of the bisphosphatases and of phosphoribulokinase, thus adapting these enzyme activities to the availability of CO_2.

It is also only a matter of speculation whether hydrogen peroxide might be involved in light-dark regulation of photosynthesis, as proposed by Wolosiuk and Buchanan (1977). If a certain steady-state level of hydrogen peroxide would be maintained in the light, it could serve to oxidize light-generated SH-groups following a light-dark transition. At the same time, oxidative pentose phosphate cycle would be activated. So far, the data presented above support the attractive model of a counter-regulation of Calvin cycle versus oxidative pentose phosphate cycle via hydrogen peroxide and light-generated SH-groups. However, further investigations will be necessary until a final decision can be made about the physiological significance of this system in vivo.

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