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THE EFFECT OF HYDROGEN PEROXIDE ON CO_2 FIXATION OF ISOLATED INTACT CHLOROPLASTS

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SUMMARY

Low concentrations of hydrogen peroxide strongly inhibit CO_2 fixation of isolated intact chloroplasts (50 % inhibition at 10^{-5} M hydrogen peroxide). Addition of catalase to a suspension of intact chloroplasts stimulates CO_2 fixation 2–6 fold, indicating that this process is partially inhibited by endogenous hydrogen peroxide formed in a Mehler reaction.

The rate of CO_2 fixation is strongly increased by addition of Calvin cycle intermediates if the catalase activity of the preparation is low. However, at high catalase activity addition of Calvin cycle intermediates remains without effect. Obviously the hydrogen peroxide formed at low catalase activity leads to a loss of Calvin cycle substrates which reduces the rate of CO_2 fixation.

3-Phosphoglycerate-dependent O_2-evolution is not influenced by hydrogen peroxide at a concentration ($5 \cdot 10^{-4}$ M) which inhibits CO_2 fixation almost completely. Therefore the inhibition site of hydrogen peroxide cannot be at the step of 3-phosphoglycerate reduction. Dark CO_2 fixation of lysed chloroplasts in a hypotonic medium is not or only slightly inhibited by hydrogen peroxide ($2.5 \cdot 10^{-4}$ M), if ribulose-1,5-diphosphate, ribose 5-phosphate or xylulose 5-phosphate were added as substrates. However, there is a strong inhibition of CO_2 fixation by hydrogen peroxide, if fructose 6-phosphate together with triose phosphate are used as substrates. This indicates that hydrogen peroxide interrupts the Calvin cycle at the transketolase step, leading to a reduced supply of the CO_2-acceptor ribulose 1,5-diphosphate.

INTRODUCTION

Broken chloroplasts are able to reduce oxygen in the light via superoxide to hydrogen peroxide in a so-called "pseudo-cyclic" electron transport [1–7]. Such a light-dependent oxygen uptake was shown to occur also in isolated intact chloroplasts [8, 9] or in intact cells [10, 11]. The observed rates of oxygen uptake are considerably lower than those of CO_2 assimilation [8–11] or of endogenous cyclic photophosphorylation [12]. Therefore one would expect that due to the low rates of oxygen uptake the hydrogen peroxide formed in the light might be quickly destroyed by endogenous catalase. However, there is strong evidence that catalase is localized

mainly in the extrachloroplastic part of the cell and not inside the chloroplast [13, 14]. Therefore, even if the rates of oxygen reduction are low, enough hydrogen peroxide might accumulate in the chloroplast to cause metabolic changes of the Calvin cycle such as the proposed formation of glycolate from sugar phosphates [15–18]. In this paper we will investigate whether and at which site hydrogen peroxide inhibits CO_2 fixation of isolated intact spinach chloroplasts.

MATERIALS AND METHODS

1. Chloroplasts were isolated from freshly harvested spinach leaves (*Spinacea oleracea* var. "Atlanta") according to Cockburn et al. [19], with the following modifications: the grinding medium contained 0.33 M sorbitol, 0.05 M MES/NaOH pH 6.5, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA. Instead of muslin, a pad of cotton wool sandwiched between two layers of nylon cloth was used for the filtration of the spinach homogenate and the chloroplasts were washed once in the suspending medium.

2. The percentage of intact chloroplasts in the suspension was routinely controlled by comparing the rates of ferricyanide reduction of normal and osmotically shocked chloroplast preparations [20] and is given for each experiment in the legends of the figures and tables.

3. Incorporation and measurement of $^{14}CO_2$ was carried out as described elsewhere [12].

4. Chlorophyll was determined according to Arnon [21].

5. The Calvin cycle intermediates were obtained from Sigma GmbH, München. Catalase from beef liver (50 000 units/mg protein) was a product of Boehringer, Mannheim.

Further experimental details are given in the legends to the figures and tables.

RESULTS AND DISCUSSION

Fig. 1 shows the effect of hydrogen peroxide on CO_2 fixation of isolated intact chloroplasts. To avoid decomposition of the added hydrogen peroxide, KCN was present in the reaction medium at a concentration which was sufficient for a complete inhibition of endogenous catalase (not shown), but which had only a slight inhibitory effect on CO_2 fixation. Under these conditions, even low concentrations of hydrogen peroxide ($5 \cdot 10^{-6}$ M) cause a significant inhibition of CO_2 fixation. However, the concentration of hydrogen peroxide necessary for a total inhibition of CO_2 fixation (10^{-3} M) was considerably higher than reported by other authors (compare ref. 9).

The following experiments should show (i) whether CO_2 fixation is influenced by endogenous hydrogen peroxide formed inside the chloroplast and (ii) how and at which site hydrogen peroxide inhibits CO_2 fixation.

If CO_2 fixation is partially inhibited by endogenous hydrogen peroxide accumulated during illumination, this inhibition should be reversed by addition of an excess of catalase to the reaction medium. In agreement with Egneus et al. [9] we found that catalase indeed stimulates CO_2 fixation (Table I). The extent of stimulation varies with the physiological activity of the chloroplast preparation. It is high, if the control rate of CO_2 fixation is low and vice versa. The rate of CO_2 fixation as well

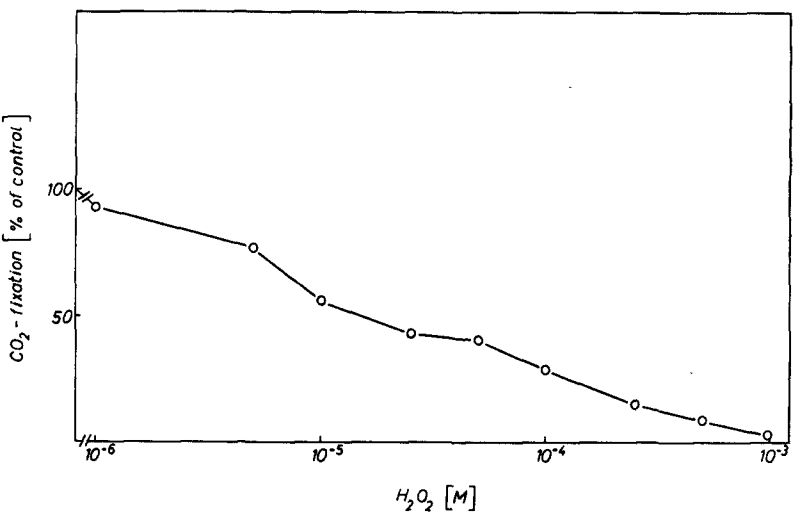

Fig. 1. Inhibition of CO_2 fixation of isolated intact chloroplasts by hydrogen peroxide. The reaction medium contained in a final volume of 1 ml: 0.33 M sorbitol, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 2 mM EDTA, 0.05 M HEPES/NaOH, pH 7.6, 0.1 mM KH_2PO_4, 10 mM $KHCO_3$, 10 μCi $NaH^{14}CO_3$ and chloroplasts (50 μg chlorophyll). In this experiment (and in the experiment shown in Fig. 3) the reaction medium contained additionally $1.6 \cdot 10^{-4}$ M KCN. Under these conditions catalase activity was completely abolished (not shown), while the rate of CO_2 fixation was only slightly reduced. The reaction was started by addition of chloroplasts (0.2 ml) in the light and stopped after 6 min by addition of 0.3 ml trichloroacetic acid (20 %). CO_2 fixation of the control, corrected for 28 % of broken chloroplasts in the preparation: 47 μmol $CO_2 \cdot mg^{-1}$ chlorophyll · h. Light intensity 40 klux, temperature 18 °C.

as the degree of stimulation by catalase seems to be independent from the percentage of intact chloroplasts in the preparation, which was relatively similar in the experiments shown in Table I. Obviously the hydrogen peroxide formed by the portion of broken chloroplasts in the preparation does not contribute significantly to the inhibition of CO_2 fixation of the intact chloroplasts. However, it should be kept in mind that the measurement of intactness by the ferricyanide method (see Materials and Methods) does not distinguish between intact (Class A according to Hall [22]) and resealed chloroplasts [23]. The latter one might have a much lower content of intermediates

TABLE I

STIMULATION OF CO_2 FIXATION BY CATALASE

10 μl of catalase (5000 units) were added to 1 ml of the reaction medium. Percentage of intact chloroplasts in the preparations: Expt. 1 = 70 %, 2 = 72 %, 3 = 76 %. For further details see Fig. 1.

Expt. No.	CO_2 fixation (μmol · mg^{-1} chlorophyll · h^{-1})		B/A
	Control (A)	+Catalase (B)	
1	10.4	63.0	6.1
2	31.1	128.5	4.1
3	77.4	175.2	2.3

and enzymes and therefore a strongly diminished capacity for CO_2 fixation. Consequently the rate of NADPH reoxidation would be low in these chloroplasts. Therefore the flow of electrons should be directed to oxygen, leading to an increased rate of the formation of hydrogen peroxide. A preparation of intact chloroplasts with a high portion of resealed chloroplasts might therefore show a low control rate of CO_2 fixation and a high degree of stimulation by catalase. Furthermore, the catalase activity of different chloroplast preparations might vary considerably, as also proposed by other authors [9], and only those preparations with sufficient catalase activity might be able to fix CO_2 with reasonable rates.

It has been discussed by several authors that hydrogen peroxide might oxidize a transketolase-glycolaldehyde complex derived from sugar phosphates yielding glycolate [15–18]. This hydrogen peroxide-dependent glycolate formation would use Calvin cycle intermediates and thus might lead to a reduced supply of the CO_2 acceptor ribulose 1,5-diphosphate and consequently to a lower rate of CO_2 fixation. If this assumption is true, the loss of substrates for CO_2 fixation should occur only at low catalase activity and it should be abolished by addition of Calvin cycle intermediates as well as by an excess of catalase. Fig. 2 shows the effect of 3 different Calvin cycle intermediates on CO_2 fixation in presence and absence of exogenous catalase. In the absence of exogenous catalase CO_2 fixation is indeed strongly stimulated by ribose 5-phosphate, fructose 6-phosphate and dihydroxyacetone phosphate, while in presence of catalase addition of Calvin cycle intermediates is without effect. The relative stimulation of CO_2 fixation by the different intermediates is strongest in presence of KCN (10^{-4} M), i.e. under conditions where the catalase activity is

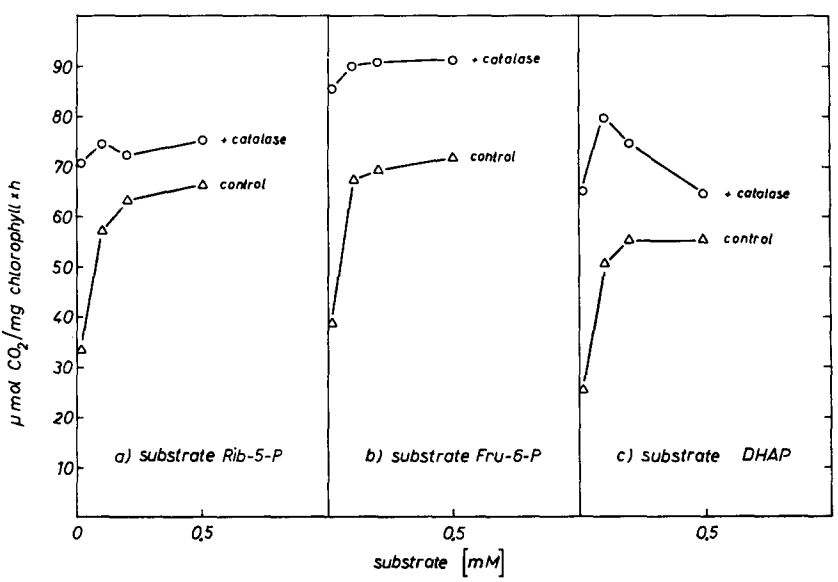

Fig. 2. Effect of Rib-5-*P* (ribose 5-phosphate), Fru-6-*P* (fructose 6-phosphate) and DHAP (dihydroxyacetone phosphate) on CO_2 fixation of isolated intact chloroplasts with and without exogenous catalase. The rates are corrected for the percentage of broken chloroplasts in the preparation, which was in (a) 8 %, in (b) 38 % and in (c) 40 %.

TABLE II

STIMULATION OF CO_2 FIXATION (RELATIVE VALUES) BY DIFFERENT CALVIN CYCLE SUBSTRATES UNDER NORMAL CONDITIONS (= CONTROL), IN THE PRESENCE OF KCN AND IN THE PRESENCE OF EXCESS CATALASE

For further details see Fig. 1. Abbreviations: Rib-5-*P*, ribose 5-phosphate; Fru-6-*P*, fructose-6-phosphate; DHAP, dihydroxyacetone phosphate.

Substrate (1 mM)	Relative rates of CO_2 fixation (%)		
	Control	+KCN (10^{-4} M)	+Catalase (5000 units)
–	100	100	100
+Rib-5-*P*	200	312	105
+Fru-6-*P*	179	250	105
+DHAP	195	382	84

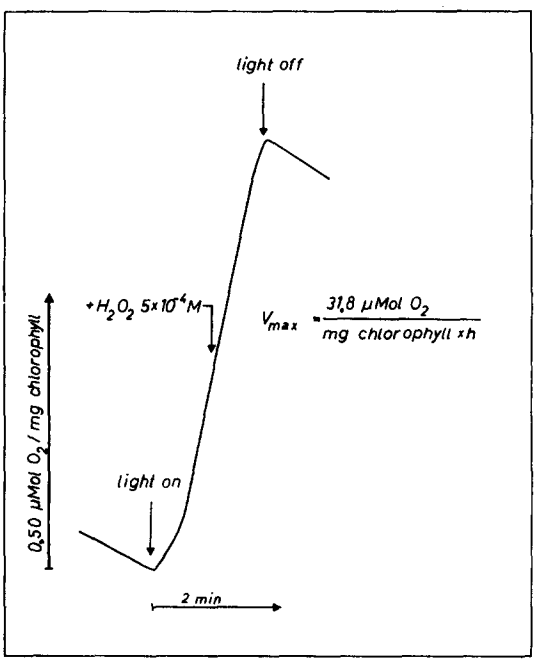

Fig. 3. Effect of hydrogen peroxide ($5 \cdot 10^{-4}$ M) on 3-phosphoglycerate dependent O_2-evolution of isolated intact chloroplasts. Polarographic measurement of O_2-evolution was performed with a Beckmann electrode in a temperature-controlled cuvette (volume 2.5 ml), which was illuminated from the side with a slide projector. The reaction mixture was the same as described in Fig. 1 (KCN included), but contained 2.5 mM PGA (3-phosphoglycerate) instead of $KHCO_3$. Hydrogen peroxide (20 µl) was added with a microliter syringe to give a final concentration of $5 \cdot 10^{-4}$ M, where indicated in the figure. The percentage of intact chloroplasts was 90 %. For further details see Fig. 1.

extremely low (Table II). These results suggest that the reduced rates of CO_2 fixation under conditions of low catalase activity are indeed due to a diminished supply of Calvin cycle intermediates. Furthermore we assume that the well known stimulation of chloroplast photosynthesis by addition of Calvin cycle intermediates [24, 25] might be caused under certain conditions by a reversal of the hydrogen peroxide inhibition of CO_2 fixation.

While CO_2 fixation is almost completely inhibited by $5 \cdot 10^{-4}$ M hydrogen peroxide (compare Fig. 1), this concentration of hydrogen peroxide has no influence on 3-phosphoglycerate-dependent oxygen evolution (Fig. 3). This confirms similar results obtained by Egneus et al. [9]. In agreement with these authors we conclude that the site of inhibition of photosynthesis by hydrogen peroxide cannot be at the step of 3-phosphoglycerate reduction.

In order to localize the inhibition site more exactly, we investigated the effect of hydrogen peroxide on dark CO_2 fixation of lysed chloroplasts [26]. In these chloroplasts CO_2 fixation depends strictly on both the addition of suitable substrates and of ATP. This type of CO_2 fixation should be inhibited by hydrogen peroxide only, if the reaction sequence leading from an added substrate to the formation of the CO_2 acceptor ribulose 1,5-diphosphate would include a hydrogen peroxide sensitive step. As shown in Table IIIa, an inhibition of CO_2 fixation by hydrogen peroxide is observed only with fructose 6-phosphate+dihydroxyacetone phosphate as substrates. If the substrate concentrations are rate limiting (Table IIIb), a smaller but significant inhibition is also observed with xylulose 5-phosphate or with sedoheptulose 7-phos-

TABLE III

EFFECT OF HYDROGEN PEROXIDE ON DARK CO_2 FIXATION OF CHLOROPLASTS ISOLATED INTACTLY AND SUSPENDED IN A HYPOTONIC MEDIUM

The hypotonic medium contained in a final volume of 1.0 ml: 50 mM HEPES/NaOH pH 8.0, 10 mM $MgCl_2$, 5 mM ATP, 0.1 mM KCN and substrates as indicated in the table. Percentage of intact chloroplasts in (a) 72 %, in (b) 88 %. The reaction was started by addition of 0.1 ml of intact chloroplasts containing 100 μg chlorophyll and stopped after 1.5 min in the dark by addition of 0.3 ml trichloroacetic acid (20 %). Abbreviations: Rbu-1,5-P_2, ribulose 1,5-diphosphate; Rib-5-*P*, ribose 5-phosphate; Xyl-5-*P*, xylulose 5-phosphate; Sed-7-*P*, sedoheptulose 7-phosphate; Fru-6-*P*, fructose 6-phosphate; DHAP, dihydroxyacetone phosphate.

Substrate	CO_2 fixed in the dark (μmol · mg^{-1} chlorophyll · h^{-1})		Inhibition (%)
	Control	+H_2O_2 ($2.5 \cdot 10^{-4}$ M)	
(a) 2 mM			
—	—	—	0
Rbu- ,5-P_2	156	148	5
Rib-5-*P*	123	133	0
Xyl-5-*P*	134	118	12
Sed-7-*P*+DHAP	55	40	27
Fru-6-*P*+DHAP	19	4	79
(b) 0.05 mM			
Rib-5-*P*	9	10	0
Xyl-5-*P*	9	4	55
Sed-7-*P*+DHAP (1.5 mM)	10	8	20

phate+dihydroxyacetone phosphate as substrates. Therefore the site at which hydrogen peroxide inhibits CO_2 fixation seems to be mainly at the transketolase step of the Calvin cycle, but there might be some further steps (epimerase?) with a lower sensitivity to hydrogen peroxide. At present we cannot decide from our results whether the inhibition is due to hydrogen peroxide-dependent glycolate formation as proposed by other authors [15–18], or only to the inhibition of an enzyme. Furthermore it remains unsolved whether the inhibition of photosynthesis by hydrogen peroxide takes place also in vivo, where the catalase activity in the peroxisomes [13, 14] might be high enough for a quick and complete decomposition of hydrogen peroxide.

REFERENCES

1 Mehler, A. H. (1951) Arch. Biochem. Biophys. 33, 65–77
2 Mehler, A. H. (1951) Arch. Biochem. Biophys. 34, 339–351
3 Mehler, A. H. and Brown, A. H. (1952) Arch. Biochem. Biophys. 38, 365–370
4 Allen, J. F. and Hall, D. O. (1973) Biochem. Biophys. Res. Commun. 52, 856–862
5 Epel, B. L. and Neumann, J. (1973) Biochim. Biophys. Acta 325, 520–529
6 Elstner, E. F. and Kramer, R. (1973) Biochim. Biophys. Acta 314, 340–353
7 Allen, J. F. (1975) Biochem. Biophys. Res. Commun. 66, 36–43
8 Whitehouse, D. G., Ludwig, J. L. and Walker, D. A. (1971) J. Exp. Bot. 22, 772–791
9 Egneus, H., Heber, U., Matthiesen, U. and Kirk, M. (1975) Biochim. Biophys. Acta 408, 252–268
10 Patterson, C. O. P. and Myers, J. (1973) Plant Physiol. 51, 104–109
11 Glidewell, S. M. and Raven, J. A. (1975) J. Exp. Bot. 26, 479–488
12 Kaiser, W. and Urbach, W. (1976) Biochim. Biophys. Acta 423, 91–102
13 Tolbert, N. E., Oeser, A., Kisaki, T., Hagemann, R. H., Yamazaki, R. H. (1968) J. Biol. Chem. 243, 5179–5184
14 Frederick, S. E. and Newcomb, E. H. (1969) J. Cell. Biol. 43, 343–353
15 Coombs, J. and Wittingham, C. P. (1966) Proc. R. Soc. B. 164, 511–520
16 Plaut, Z. and Gibbs, M. (1970) Plant Physiol. 45, 470–474
17 Shain, Y. and Gibbs, M. (1971) Plant Physiol. 48, 325–330
18 Robinson, J. M. and Gibbs, M. (1974) Plant Physiol. 53, 790–797
19 Cockburn, W., Walker, D. A. and Baldry, C. W. (1968) Biochem. J. 107, 89–95
20 Heber, U. and Santarius, K. A. (1970) Z. Naturforsch. 25b, 718–728
21 Arnon, D. I. (1949) Plant Physiol. 24, 1–5
22 Hall, D. A. (1972) Nat. New Biol. 235, 125–126
23 Lilley, R. McC., Fitzgerald, M. P., Rienitz, K. G. and Walker, D. A. (1975) New Phytol. 1–10
24 Bamberger, E. S. and Gibbs, M. (1969) Plant Physiol. 40, 919–926
25 Bucke, C., Walker, D. A. and Baldry, C. W. (1966) Biochem. J. 101, 636–641
26 Jensen, R. G. (1971) Biochim. Biophys. Acta 234, 360–370