OXYGEN EVOLUTION IN THE DARK FOLLOWING ILLUMINATION OF CHLOROPLASTS IN THE PRESENCE OF ADDED MANGANESE

D.A.WALKER and L.J.LUDWIG
Botany Department, Imperial College, London, S.W.7

D.G.WHITEHOUSE
Chemistry and Biology Department, Barking Regional College of Technology, Essex

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1. Introduction

In the Hill reaction [1] illuminated chloroplast catalyse the transfer of electrons from water to an oxidant (A) and there is an evolution of oxygen (reaction 1)

$$H_2O + A \rightarrow \tfrac{1}{2} O_2 + AH_2 \quad (1)$$

Mehler [2, 3] showed that molecular oxygen can also act as the oxidant (reaction 2) so that there is a net uptake of oxygen.

$$H_2O + O_2 \rightarrow \tfrac{1}{2} O_2 + H_2O_2 \quad (2)$$

Following this reaction, the hydrogen peroxide formed may give rise to an oxygen evolution in an ensuing dark period if there is sufficient endogenous, or added, catalase to promote an appreciable rate of dismutation (reaction 3). Each molecule of hydrogen peroxide then gives rise to half a molecule of oxygen so that the net oxygen uptake in reaction 2 is exactly equivalent to that released in reaction 3.

$$H_2O_2 \rightarrow \tfrac{1}{2} O_2 + H_2O \quad (3)$$

In carefully washed, envelope-free, chloroplasts the endogenous catalase is so low that reaction 3 is completely inhibited by the addition of 10^{-4} M cyanide or azide. We wish to report that despite this lack of measurable catalase activity, preillumination with $MnCl_2$ can still lead to an O_2 evolution in the dark.

2. Materials and methods

Spinach chloroplasts were isolated in sorbitol-pyrophosphate medium without ascorbate [4]. They were stripped of their envelopes by osmotic shock [5] in grinding medium diluted ten fold and then washed twice in full strength medium to resuspension and assay in a solution containing sorbitol, 0.33 M; $MgCl_2$, 1.0 mM; and HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid), 50 mM; adjusted to pH 7.6 with NaOH. Reaction mixtures contained 100 μg chlorophyll in 2 ml resuspending medium and (in μmoles) FMN, 0.04; NH_4Cl, 40; KCN, 0.4; $MnCl_2$, 2.0, as indicated. In fig. 2, the manganese was chelated with EDTA (4 μmoles) and in 2A, FMN and KCN were omitted and potassium ferricyanide (2 μmoles) used as the oxidant. Commercial catalase (Sigma) was added in excess (approx. 240 units) where indicated.

O_2 was measured simultaneously in twin cells fitted with Clark-type electrodes and illuminated, at 20°C, by quartz-iodine slide-projectors (150 W, 24 V) fitted with heat filters and a broad red filter transmitting light above 600 nm [5].

3. Results and discussion

Stimulation, by manganese, of O_2 uptake by chloroplasts was discovered by Gerretsen [6, 7] and confirmed by Mehler [3] and by Good and Hill [8]. Fig. 1 shows the response to added $MnCl_2$ in a reaction mixture in which O_2 uptake had already acceler-

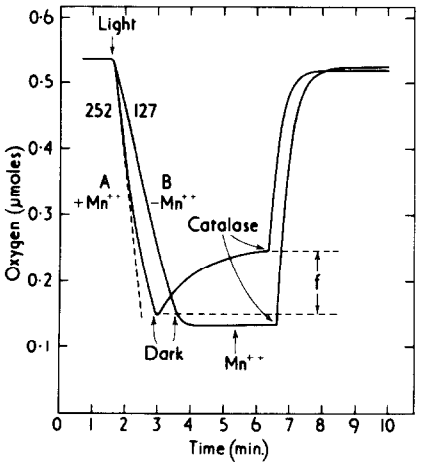

Fig. 1. Effect of $MnCl_2$ (2 μmoles) on the O_2 uptake of isolated chloroplasts in the light and its subsequent effect on O_2 evolution in darkness. Additional O_2 was released from accumulated H_2O_2 by excess catalase added as indicated. Initial rates of O_2 uptake are given in μmoles/mg chlorophyll/hr. $MnCl_2$ was omitted from reaction mixture B. For further details of reaction mixture and method of O_2 measurement in this and other figures, see text.

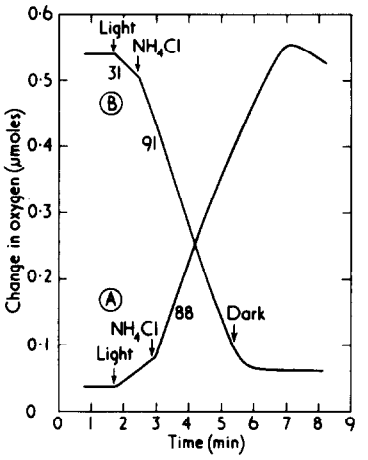

Fig. 2. A comparison of the rate of O_2 evolution in the Hill Reaction (A) (ferricyanide as the electron acceptor) with the net rate of O_2 uptake in an FMN mediated Mehler Reaction (B). Electron transport was uncoupled with NH_4Cl (40 μmoles) as indicated. Rates are expresses as μmoles O_2/mg chlorophyll/hr.

ated by the addition of FMN and NH_4Cl (both FMN and NH_4Cl are required for maximal rates whether or not manganese is also present). With FMN (or FAD) the rate of oxygen uptake equals the rate of evolution in the presence of ferricyanide, [8] and there is a corresponding response to NH_4Cl (fig. 2). The addtion of $MnCl_2$ then brings about a further doubling of rate but the initial velocity is not maintained (fig. 1), presumably because of an increasingly rapid simultaneous oxygen evolution which becomes apparent when masking O_2 uptake ceases in the dark. It should be emphasized that the causal agent promoting this dark evolution of O_2 appears to be formed from $MnCl_2$ in the light. Neither pre-illumination (fig. 1), nor addition of $MnCl_2$ following pre-illumination (fig. 1) is sufficient, in itself, to promote oxygen evolution. Manganese chelated with EDTA is ineffective (fig. 2).

The subsequent addition of excess catalase (sufficient to overcome the inhibition by cyanide) is informative. In the absence of manganese the oxygen "recovery" observed on the addition of catalase is almost complete, i.e. it is close to that which could be predicted if reaction 2 occurs in the light and is followed by reaction 3 on the addition of catalase. This recovery is not changed if manganese is added in the dark some time prior to the addition of catalase (fig. 1B). However, if manganese is present from the outset the quantity of oxygen recovered depends upon when the catalase is added. If it is added at the moment illumination is stopped, the oxygen recovered (fig. 3A) is not far short of that taken up in the preceding light period, indicating that manganese accelerates both oxygen uptake and H_2O_2 accumulation. If the addition of catalse is delayed until the non-catalatic O_2 evolution is complete (fig. 3B) the extent of recovery is increased. It now exceeds the evolution which follows immediate addition of catalase by almost exactly one half of the non-catalatic evolution. This relationship has been observed consistently, with chloroplasts from both spinach and peas. It indicates (a) that the non-catalatic evolution depends upon the presence of H_2O_2 and (b) that it is a consequence of oxidation (reaction 4) rather than dismutation (reaction 3).

$$H_2O_2 \rightarrow 2H^+ + 2e^- + O_2 \qquad (4)$$

Thus if n molecules of H_2O_2 were to undergo dismutation (reaction 3) $n/2$ molecules of oxygen would be recovered (fig. 3, curve A). If, however, a fraction f

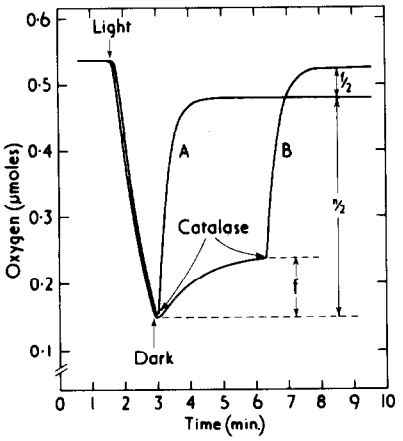

Fig. 3. O_2 evolution in the dark following pre-illumination of chloroplasts in the presence of $MnCl_2$. Curve A, dismutation of accumulated H_2O_2 by immediate addition of excess catalase. Curve B, non-catalatic oxidation of H_2O_2 (f) by Mn^{3+} followed, on completion, by catalatic dismutation. For further explanation of *f* and *n*, see text.

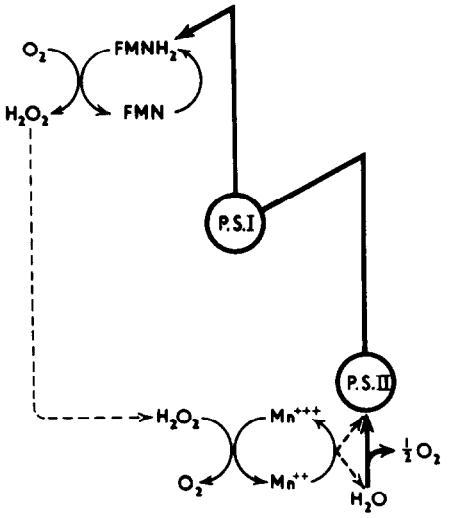

Fig. 4. Proposed scheme showing the oxidation of Mn^{2+} by PS II in the light and the subsequent reduction of Mn^{3+} by H_2O_2 produced in an FMN mediated Mehler Reaction.

were to undergo oxidation (reaction 4) thus yielding *f* molecules of oxygen, the total O_2 recovered would be $n/2 - f/2 + f$. That is, there would be excess O_2 equal to $f/2$ (fig. 3B). In fig. 3 the values of *n* and *f* are 0.66 and 0.09 μmoles respectively.

The non-catalatic O_2 evolution is inhibited by the addition of a small quantity of ferrous sulphate, but not by DCMU (dichloromethyl urea) or heat treatment. The preceding O_2 uptake is stopped completely by 10^{-6} M DCMU, and largely inhibited (> 80%) by preheating chloroplasts at 50°C for 90 sec (the Hill and Mehler reactions, *per se*, are > 90% inhibited by the same treatment).

FMN may be replaced by FAD or methyl viologen and a small non-catalatic evolution occurs in the absence of these additives. Photo-oxidation of FMN [9] in the absence of chloroplasts is negligible in red light (see Methods).

4. Conclusions

Our interpretation is as follows. The transfer of electrons, from water to oxygen, by illuminated chloroplasts is accelerated by manganese. This overall reaction sequence is stimulated by NH_4Cl and FMN (or methyl viologen) and inhibited by DCMU or mild heat treatment. For these reasons, it is not a simple, non-biological chlorophyll-sensitised or flavin-sensitised [9] photo-oxidation of Mn^{2+} nor does the stoichiometry permit the conclusion that the increased electron transport initiated by Mn^{2+} is entirely a consequence if its net oxidation or peroxidation [10]. Direct oxidation of Mn^{2+} by peroxyl radical cannot be ruled out but there seems to be no compelling reason why this should be assumed when reduced FMN (or methyl viologen) could readily donate electrons to peroxyl and be in turn reduced by photosystem I. In short our results are consistent with Bachofen's proposal [11, 12] that Mn^{2+} is oxidised to Mn^{3+} by photosystem II and that the electrons are passed via photosystem I and to O_2 (fig. 4). Part of the hydrogen peroxide thus formed can then be *oxidised* by manganic ion, giving rise to O_2 evolution in the dark.

Acknowledgements

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Since preparing this manuscript our attention has been drawn (M.Avron, personal communication) to work on the oxidation of manganous ion which is to be published in Biochimica Biophysica Acta.

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