SUPEROXIDE AND PHOTOSYNTHETIC REDUCTION OF OXYGEN

J.F. ALLEN

Botany School
University of Oxford
South Parks Road
Oxford OX1 3RA, U.K.

I. INTRODUCTION

In plant-type photosynthesis sunlight is able to bring about the transfer of reducing equivalents from water to an oxidant, with a consequent liberation of molecular oxygen. This process involves formation of NADPH (by reduction of the oxidant NADP) and of ATP (by phosphorylation of ADP). NADPH and ATP are respectively oxidised and hydrolysed in the subsequent pathway of CO_2-assimilation, a process which itself has no direct requirement for light.

Oxygen evolution is by no means a necessary condition for the conversion of radiant energy into chemical potential energy. In the most abundant form of photosynthesis in the biosphere, water is the electron donor and so oxygen is produced. The alternatives represented by anaerobic, bacterial photosynthesis and by *Halobacterium* photophosphorylation are interesting biochemically and as evolutionary relics, but they today make a negligible contribution to global primary production.

While evolution of oxygen is the prominent feature of at least that form of photosynthesis found in plants, light-dependent reduction of oxygen may also occur in certain circumstances. In the absence of other electron acceptors, isolated chloroplasts may actually consume oxygen in the light. Photosynthetic oxygen uptake by chloroplasts is termed the "Mehler reaction", and hydrogen peroxide is its first stable product.

The relatively recent identification of the superoxide anion as the initial product of photosynthetic oxygen reduction may be a valuable one for certain areas of photosynthesis research, because chloroplast oxygen uptake is widely used as a measure of photosynthetic electron transport *in vitro*. The theme of the present chapter is that several discrete categories of Mehler reaction can now be described, each in terms of the way in which superoxide functions as an intermediate.

II. REDUCTION AND CONSUMPTION OF OXYGEN BY ISOLATED CHLOROPLASTS

A. *The Mehler Reaction: Oxygen as a Hill Oxidant*

In the "Hill reaction" (Hill, 1939) oxygen is produced by illuminated chloroplasts when electrons are transferred from water to an electron acceptor which consequently may be termed a "Hill oxidant" (X in equation 1)

$$H_2O \longrightarrow 2H + \tfrac{1}{2}O_2$$

$$2H + X \longrightarrow XH_2$$

$$\text{Overall: } H_2O + X \longrightarrow \tfrac{1}{2}O_2 + XH_2 \qquad (1)$$

Artificial Hill oxidants include ferric oxalate, potassium ferricyanide and indophenol dyes. The natural electron acceptor, NADP, is reduced by chloroplasts only in the presence of the soluble electron carrier ferredoxin. NADP/NADPH has a low redox potential (E'_o = -320 mV) but NADPH is not autoxidisable; when NADP is reduced energy is stored in a relatively stable form. This energy is consumed in the conversion of diphosphoglycerate to triose phosphate in the "dark" pathway of CO_2-fixation.

If NADP is absent from the chloroplast preparation, electrons may be transferred to oxygen instead. Mehler (1951a) identified hydrogen peroxide as a product of this reaction by using catalase in its peroxidative capacity in order to show that ethanol could be converted by illuminated chloroplasts to acetaldehyde. In this situation oxygen is consumed (equation 2), and net oxygen consumption has the same stoichiometric relationship to electron transport as oxygen evolution has when some other electron acceptor is used.

$$H_2O \longrightarrow 2H + \tfrac{1}{2}O_2$$

$$O_2 + 2H \longrightarrow H_2O_2$$

$$\text{Overall: } H_2O + \tfrac{1}{2}O_2 \longrightarrow H_2O_2 \qquad (2)$$

That oxygen is simultaneously consumed and evolved in this reaction was confirmed, in experiments using ^{18}O-oxygen as a tracer, by Mehler and Brown (1952) and by Brown and Good (1955). Dismutation of the hydrogen peroxide by catalase results in zero net oxygen exchange. In measurements of chloroplast oxygen uptake it is now customary to add sodium azide as an inhibitor of endogenous catalase.

Good and Hill (1955) showed that the rate of oxygen uptake in the Mehler reaction could be increased by addition of various autoxidisable electron acceptors such as flavin derivatives, dyes (Janus green and litmus) and the bipyridyl compounds methyl viologen and benzyl viologen. In the presence of saturating concentrations of methyl viologen, rates of oxygen uptake of several hundreds of micromoles per milligram of chlorophyll per hour can be observed; these represent rates of electron transport as high or higher than those which must occur *in vivo*. ATP synthesis is, of course, coupled to photosynthetic electron transport, and "pseudocyclic" is the term used to describe photophosphorylation that is driven by non-cyclic electron transport with oxygen as the terminal electron acceptor (Arnon *et al.*, 1961; Arnon *et al.*, 1967).

Good and Hill (1955) also confirmed earlier observations that chloroplast oxygen uptake could be stimulated by manganese ions (Mn^{2+}) (Gerretsen, 1950) and by ascorbate (Mehler, 1951b). Net oxidation of such reagents was

observed, while this was not the case for the flavins, quinones or viologens which merely serve catalytically as electron carriers between the chloroplast and oxygen. Mehler (1951b) nevertheless ascribed to manganese ions a catalytic role similar to that of quinones, while to account for ascorbate's effect he suggested a reduction by ascorbate of the more oxidised product of the photolysis of water (HO·). The terminal steps of oxygen evolution would then be replaced by oxidation of ascorbate and net oxygen uptake would thereby be enhanced. The same proposition is contained within the more recent statement by Bohme and Trebst (1969) and by Ben-Hayyim and Avron (1970a), that ascorbate can by itself competitively replace water as an electron donor to photosystem II.

Non-cyclic electron transport in plant photosynthesis involves two light reactions which operate in series (Hill and Bendall, 1960). Fig.1 depicts an outline of the "Z-scheme" for photosynthetic electron transport, and includes some of the oxygen-reducing and other reactions which are discussed in this chapter. The Z-scheme is produced by plotting the sequence of electron transfer reactions on a scale of the standard redox potential of its components.

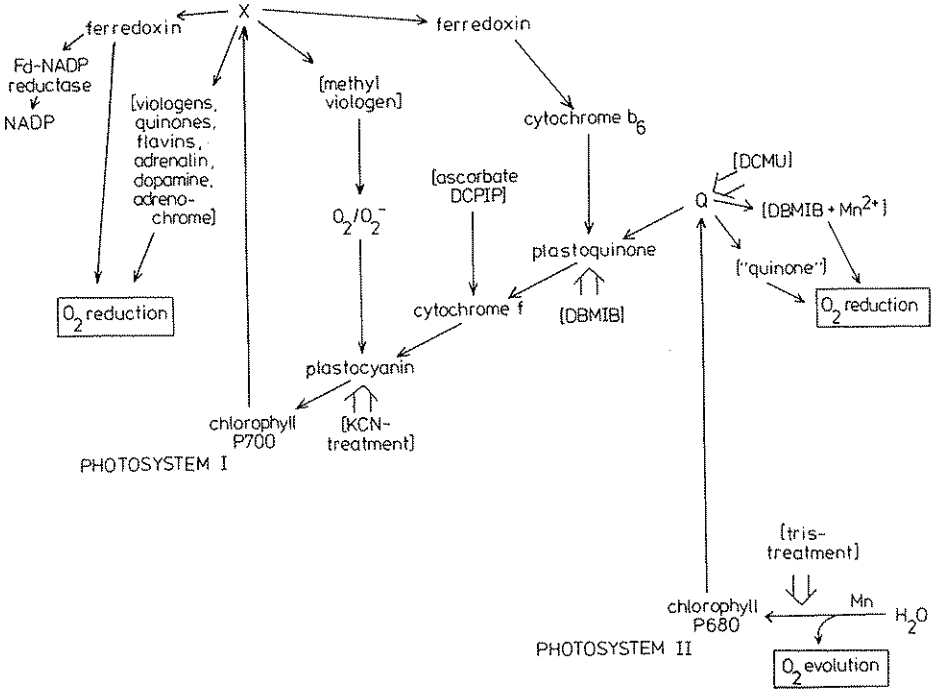

Fig.1. *Photosynthetic electron transport and associated reactions that involve oxygen. Artificial treatments or additions are in brackets.*

If the oxygen-evolving mechanism is inactivated by some treatment such as incubation of the chloroplasts with tris buffer, then ascorbate will indeed serve as an alternative electron donor (Yamashita and Butler, 1968), though without such treatment it now seems that ascorbate will not by itself compete with water. Like ascorbate, Mn^{2+} has been thought to be able to displace water as a donor to photosystem II (Ben-Hayyim and Avron, 1970b; Walker *et al.*, 1970).

With the replacement of manometric methods of measuring oxygen exchange

by polarographic ones, oxygen uptake has become widely used in studies of photosynthetic electron transport. If electron transport in photosystem II is inhibited (e.g. by Tris-treatment or by addition of DCMU) then oxygen evolution can no longer be observed, though electron transport which is associated with photosystem I may still easily be measured as oxygen uptake, provided electrons are supplied by a reductant such as ascorbate together with catalytic amounts of a dye such as DCPIP (Vernon and Zaugg, 1960). Despite the obvious utility of the Mehler reaction and its modifications, care must be taken in interpretation of the oxygen electrode traces obtained. Some experimental conditions (e.g. catalase activity) which must be taken into account have been described by Whitehouse *et al.* (1971), and, specifically for the photosystem I reaction, by Allen and Hall (1974). The fact that ferredoxin is an effective mediator of the Mehler reaction (Telfer *et al.*, 1970) raises the possibility that what is apparently a straightforward Hill reaction with NADP as electron acceptor may actually involve oxygen uptake as well (Allen, 1975b).

B. *Chloroplast Oxygen Uptake With Methyl Viologen as the Mediator*

1. *Stimulation by Ascorbate and Manganese.* If methyl viologen (MV) is used as the carrier of electrons between the chloroplast and oxygen, a special case of the Mehler reaction described by equation 2 can be written as follows:

$$H_2O + 2MV^{2+} \longrightarrow 2MV^{+} + \tfrac{1}{2}O_2 + 2H^{+} \qquad (3)$$

$$2MV^{+} + O_2 + 2H^{+} \longrightarrow 2MV^{2+} + H_2O_2 \qquad (4)$$

$$\text{Overall: } H_2O + \tfrac{1}{2}O_2 \longrightarrow H_2O_2 \qquad (2)$$

Both light reactions (see Fig.1) are involved in the electron transfer represented by equation 3.

If ascorbate (AH_2) were to replace water as the electron donor (equation 5), the stoichiometry of the overall reaction would be altered from that described by equation 2 to that described by equation 6.

$$AH_2 + 2MV^{2+} \longrightarrow 2MV^{+} A + 2H^{+} \qquad (5)$$

$$2MV^{+} + O_2 + 2H^{+} \longrightarrow 2MV^{2+} + H_2O_2 \qquad (4)$$

$$\text{Overall: } AH_2 + O_2 \longrightarrow A + H_2O_2 \qquad (6)$$

For any given rate of electron transport, ascorbate would increase the rate of oxygen uptake by no more than a factor of two. Bohme and Trebst (1969) used anthraquinone as the mediator, and found that the rate of oxygen uptake was more than doubled on addition of ascorbate. To account for this observation they suggested that ascorbate, in acting as an electron donor to photosystem II, by-passed a phosphorylation site which was situated between water and photosystem II, and which was also limiting to the overall rate of electron transport. The fact that the rate of photophosphorylation was apparently unaffected by ascorbate is consistent with this explanation provided that it is assumed that a second phosphorylation site exists and is not by-passed in the same way. DCMU-sensitivity and susceptibility to a "red-drop" effect (Ben-Hayyim and Avron, 1970a) made it necessary to assume that the ascorbate-stimulated rate of oxygen uptake was driven by electron transport involving both photosystems. The same mechanism was invoked for similar

effects of manganese ions (Mn^{2+}) on chloroplast oxygen uptake mediated by diquat (Ben-Hayyim and Avron, 1970b). One difficulty with this explanation was that NADP reduction was unaffected by ascorbate, while ascorbate oxidation was much slower with NADP as the electron acceptor than it was with oxygen as the electron acceptor (Bohme and Trebst, 1969). A second problem was that ascorbate was able to increase the rate of methyl viologen-mediated oxygen uptake by more than a factor of two even in uncoupled chloroplasts, where, by definition, electron transport cannot be limited in rate by phosphorylation reactions (Allen and Hall, 1973).

Though NADP reduction itself was unaffected by ascorbate, net oxygen evolution with NADP (with added ferredoxin) as the electron acceptor was, under certain conditions, inhibited by ascorbate (Ben-Hayyim and Avron, 1970a). This effect was interpreted as additional evidence for ascorbate's ability to suppress oxidation of water and itself become the electron donor to photosystem II.

2. *The Effect of Superoxide Dismutase.* An alternative solution to the problem of ascorbate's effect on chloroplast oxygen uptake was proposed by Epel and Neumann (1972). Following Elstner *et al.* (1970), Epel and Neumann suggested that superoxide was the initial product of autoxidation of methyl viologen, and that the reduction of oxygen to hydrogen peroxide (equation 4) could be regarded as an overall reaction which involved both univalent reduction of oxygen (equation 7) and dismutation of superoxide (equation 8). The methyl viologen-mediated Mehler reaction may then be written:

$$H_2O + 2MV^{2+} \longrightarrow 2MV^{+} + \tfrac{1}{2}O_2 + 2H^{+} \qquad (3)$$

$$2MV^{+} + 2O_2 \longrightarrow 2MV^{2+} + 2O_2^{-} \qquad (7)$$

$$2O_2^{-} + 2H^{+} \longrightarrow O_2 + H_2O_2 \qquad (8)$$

$$\text{Overall: } H_2O + \tfrac{1}{2}O_2 \qquad H_2O_2 \qquad (2)$$

Ascorbate's effect in stimulating oxygen uptake may then be attributed to a replacement of the dismutation step (equation 8) with oxidation of ascorbate by superoxide (equation 9). The overall reaction then becomes that shown in equation 10, with three times as much oxygen being consumed as is the case in the absence of ascorbate (equation 2).

$$H_2O + 2MV^{2+} \longrightarrow 2MV^{+} + \tfrac{1}{2}O_2 + 2H^{+} \qquad (3)$$

$$2MV^{+} + 2O_2 \longrightarrow 2MV^{2+} + 2O_2^{-} \qquad (7)$$

$$AH_2 + 2O_2^{-} + 2H^{+} \longrightarrow 2A + 2H_2O_2 \qquad (9)$$

$$\text{Overall: } H_2O + AH_2 + 1\tfrac{1}{2}O_2 \longrightarrow A + 2H_2O_2 \qquad (10)$$

Accordingly the effect of ascorbate is merely a consequence of its reaction with superoxide, and the electron transport reactions which lead to reduction of methyl viologen (equation 3) are quite unaffected. Thus the scheme is consistent with the evidence for the involvement of both photosystems in ascorbate-stimulated oxygen uptake, and with the unchanged rate of phosphorylation.

A three-fold increase in methyl viologen-mediated oxygen uptake in broken, washed chloroplasts is accompanied by an equivalent effect of ascorbate in

which the ADP/O ratio in the same system is decreased (Allen and Hall, 1973).

Epel and Neumann (1972) also pointed out that their explanation of ascorbate's effect on oxygen uptake could accommodate an explanation of inhibition by ascorbate of net oxygen evolution with NADP as the electron acceptor. If a ferredoxin-mediated, oxygen-consuming component of this reaction were susceptible to stimulation by ascorbate, then ascorbate's reaction with superoxide would cause an inhibition of net oxygen evolution without affecting electron transport to NADP.

The significant advantage that Epel and Neumann's (1972) hypothesis (that ascorbate stimulates oxygen uptake because of its reaction with superoxide) has over its alternative (that ascorbate replaces water as the electron donor to photosystem II) is that the former hypothesis is the one more vulnerable to experimental test. A disproof of Epel and Neumann's hypothesis (though not of its alternative) would be the persistence of ascorbate-stimulation of oxygen uptake in a situation where the ascorbate-superoxide reaction (equation 9) could not occur. In fact the addition of superoxide dismutase, which accelerates the dismutation reaction (equation 8), not only prevents ascorbate from stimulating chloroplast oxygen uptake; it also reverses the effect of ascorbate which has previously been added (Epel and Neumann, 1973; Allen and Hall, 1973). Superoxide dismutase is known to be antagonistic to the effect of ascorbate in all the cases that were quoted previously as evidence for ascorbate's suppression of oxygen evolution associated with Photosystem II. Ascorbate's stimulatory effect on oxygen uptake (Epel and Neumann, 1973), its inhibitory effect on the ADP/O ratio (Allen and Hall, 1973), and its inhibitory effect on NADP-dependent net oxygen evolution (Epel and Neumann, 1973; Allen, 1975c) are all reversed in a cyanide-sensitive fashion by addition of superoxide dismutase to the appropriate reaction.

Epel and Neumann (1973) used a chloroplast-free model system in which methyl viologen was reduced enzymatically by ferredoxin-NADP reductase in the presence of NADPH. By adding limiting amounts of NADPH they were able to show directly that the stoichiometry of oxygen uptake was changed by ascorbate in the way predicted by their scheme; from $NADPH/O_2 = 1$ (without ascorbate) to $NADPH/O_2 = \frac{1}{2}$ (with ascorbate). Epel and Neumann (1973) also demonstrated that Mn^{2+} and dithiothreitol will each substitute for ascorbate in causing a superoxide dismutase-sensitive enhancement of oxygen uptake in the chloroplast-free system.

Dithiothreitol was subsequently shown by Marchant (1974) to accelerate oxygen uptake in chloroplasts, albeit at roughly fifty-fold higher concentrations than ascorbate. Cysteine, another reductant for superoxide, is similar to dithiothreitol in this respect (Allen, 1975c). Marchant (1974) also obtained results relating to ascorbate's effect on the ADP/O ratio of oxygen-consuming chloroplasts. Marchant's data gave some statistically rigorous support to the supposition that ascorbate, dithiothreitol (at least at lower concentrations) and superoxide dismutase have no effect on either electron transport or photophosphorylation *per se*, and that their effects on the ADP/O ratio are merely consequences of their effects on the stoichiometry of oxygen uptake in the Mehler reaction.

Another observation by Allen and Hall (1973), Epel and Neumann (1973) and Marchant (1974) was that in order to achieve a given degree of inhibition of ascorbate's effect on oxygen uptake, a greater amount of superoxide dismutase had to be added to broken, washed chloroplasts than had to be added to intact chloroplasts. This clearly represents evidence for chloroplast-associated

superoxide dismutase activity. From the magnitude of this effect Allen and Hall (1973) estimated that such an activity in spinach chloroplasts would correspond to about five hundred superoxide dismutase units per milligram of chlorophyll.

Any reagent that will reduce superoxide to peroxide should be a suitable substitute for ascorbate in such experiments. Thus Mn^{2+} and glutathione also produce enhanced chloroplast oxygen uptake which can then be inhibited by superoxide dismutase. With cysteine or glutathione the concentration-dependence of the effect resembles that with dithiothreitol. In broken chloroplasts, which present fewer problems of permeability, the principal factors involved should be the rate constant of the reaction of the reductant with superoxide and any residual or membrane-associated superoxide dismutase activity. Spinach superoxide dismutase, which can be present as a contaminant of ferredoxin-NADP reductase preparations, may also have to be taken into account in the chloroplast-free model system (Allen, 1975c).

The identity of the autoxidisable electron acceptor is to a certain extent immaterial, provided that it mediates only univalent oxygen reduction as outlined above. A list of such mediators can now include the bipyridyl derivatives methyl viologen (dimethyl), benzyl viologen (dibenzyl) and diquat (ethylene); FMN and adrenochrome (Allen, 1975c); and anthraquinone and phenazine methosulphate (Epel and Neumann, 1973). Autoxidisable compounds which seem to mediate a different type of Mehler reaction include adrenalin (section IIC of the present chapter) and ferredoxin (seection IID).

3. *Superoxide, Ascorbate and Stoichiometries of Oxygen Uptake.* It is clear that in situations where oxygen exchange is to be taken as an absolute measure of photosynthetic electron transport, the number of oxygen molecules evolved or consumed per electron pair transferred must be known. In normal Hill (equation 1) or Mehler (equation 2) reactions $O_2/2e^- = \frac{1}{2}$, while for the methyl viologen-mediated Mehler reaction in the presence of ascorbate (according to equation 10) $O_2/2e^- = 1\frac{1}{2}$.

Electron transport in photosystem I can also be measured as methyl viologen-mediated oxygen uptake, provided that a suitable electron donor such as DCPIP is present. The site at which electrons are donated lies between the two photosystems, as shown in Fig.1. In practice DCPIP is used in catalytic amounts, being reduced by a compound which consequently provides the pool from which electrons are drawn by photosystem I; this compound is invariably ascorbate. The relationship of oxygen uptake to electron transport in such a system has widely been assumed to differ from that of the complete non-cyclic chain only in that oxygen is not evolved where ascorbate instead of water is the ultimate source of electrons (Izawa, 1968; Strotmann and von Gosseln, 1972; Ort and Izawa, 1973; Gould and Izawa, 1973a). The overall reaction would, in this view, be that of equation 6, the value $O_2/2e^- = 1$ would therefore hold, and in measuring the stoichiometry of photophosphorylation the relationship $P/2e^- = P/2O$ would be expected to apply.

If, however, the generation by methyl viologen of superoxide is recognised (equation 7), the overall reaction becomes that of equation 11 (below), as a consequence of the oxidation of ascorbate by superoxide (equation 9).

$$AH_2 + 2MV^{2+} \longrightarrow 2MV^{+} + A + 2H^{+} \qquad (5)$$

$$2MV^{+} + 2O_2 \longrightarrow 2MV^{2+} + 2O_2^{-} \qquad (7)$$

$$AH_2 + 2O_2^- + 2H^+ \longrightarrow A + 2H_2O_2 \qquad (9)$$

$$\text{Overall: } 2AH_2 + 2O_2 \longrightarrow 2A + 2H_2O_2 \qquad (11)$$

An important implication is that the ratio $O_2/2e^-$ for the photosystem I reaction (equation 11) has a value of two, and so the adoption of equation 6 and its $O_2/2e^-$ value of one leads to a corresponding overestimate of the rate of electron transport in this sytem, and hence to an underestimate of the stoichiometry of photosystem I phosphorylation. Equation 11 implies the relationship $P/2e^- = P/40$ rather than $P/2e^- = P/20$.

Support for this interpretation is the observation that superoxide dismutase is inhibitory to oxygen uptake by photosystem I (Epel and Neumann, 1973; Allen and Hall, 1974; Ort and Izawa, 1974). Allen and Hall found that with saturating concentrations of superoxide dismutase the rate of oxygen uptake was half that of the control, while Ort and Izawa obtained a significantly smaller percentage inhibition. Addition of catalase also halves the rate of oxygen uptake, both in the presence and in the absence of superoxide dismutase (Allen and Hall, 1974). Oxygen uptake in the presence of both superoxide dismutase and catalase therefore proceeds at only a quarter of the rate that is observed with neither enzyme present.

Fig.2 depicts a more general scheme for oxygen uptake in the Mehler reaction, and the accompanying Table I shows the stoichiometry of oxygen uptake that is associated with each set of conditions. Five possible values for the $O_2/2e^-$ ratio are given. For measurable O_2 uptake that results from electron transport through both photosystems the $O_2/2e^-$ ratio has a value of $\frac{1}{2}$, or, in the presence of ascorbate but of neither superoxide dismutase nor catalase, of $1\frac{1}{2}$. For oxygen uptake driven only by photosystem I, the $O_2/2e^-$ ratio has three possible values; 1 with either superoxide dismutase or catalase, 2 with neither enzyme, and $\frac{1}{2}$ with both. Less than saturating concentrations of ascorbate, superoxide dismutase or catalase (or of the enzymes' inhibitors) will, of course, produce $O_2/2e^-$ ratios which are intermediate between any two of the five values in Fig.2.

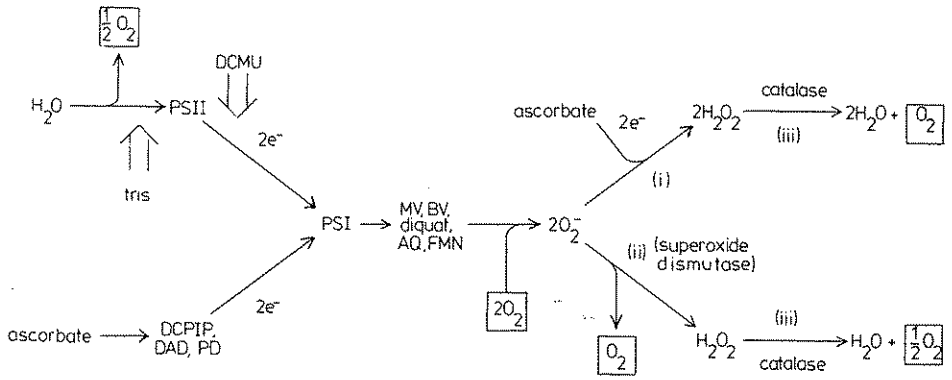

Fig.2. (with an accompanying Table). *Oxygen-evolving and oxygen-consuming reactions that determine the relationship of chloroplast oxygen uptake to photosynthetic electron transport: effects of ascorbate, superoxide dismutase, and catalase.*

TABLE I

	Enzyme Additions	Reactions Involved	$O_2/2e^-$	$P/2e^-$ Equals
Water as electron donor; PSI and PSII				
without ascorbate	None	(ii)	½	*P/O*
	SOD			
	catalase	(ii) + (iii)	0	-
	SOD + catalase			
with ascorbate	None	(i)	1½	*P/30*
	SOD	(ii)	½	*P/O*
	catalase	(i) + (iii)	½	*P/O*
	SOD + catalase	(ii) + (iii)	0	-
Ascorbate as electron donor; PSI only				
	None	(i)	2	*P/40*
	SOD	(ii)	1	*P/20*
	catalase	(i) + (iii)	1	*P/20*
	SOD + catalase	(ii) + (iii)	½	*P/O*

It follows from this interpretation that the effects of ascorbate, superoxide dismutase, and catalase are effects only on the fate of the oxygen which is reduced initially to superoxide. Electron transport, when measured as NADP reduction, is indeed unaffected by these treatments (Allen and Hall, 1974).

4. *Further Evidence for Superoxide as an Intermediate.* Elstner *et al.* (1970) were the first to suggest that the superoxide radical is the initial product of photosynthetic oxygen reduction and that photosynthetic ascorbate oxidation can therefore result from ascorbate's reaction with superoxide. Elstner and Kramer (1973) agreed with Epel and Neumann's (1972) explanation of ascorbate's effect on the chloroplast oxygen uptake resulting from autoxidation of low-potential electron acceptors such as methyl viologen or anthraquinone. At the same time they proposed a different scheme to account for ascorbate-stimulated phosphorylation *per se* in the absence of artificial cofactors.

Evidence that superoxide is the initial product of autoxidation of diquat has been provided by Stancliffe and Pirie (1971). In a study of the reaction of methyl viologen (paraquat) with oxygen, Farrington *et al.* (1973)

identified superoxide as the initial product, and suggested that diffusion of superoxide from its site of formation in the chloroplast could result in peroxidation of membrane lipids in the chloroplast envelope and tonoplast; these are sites which are particularly vulnerable to the herbicidal action of paraquat.

By following the EPR signal of the tiron semiquinone Greenstock and Miller (1975) have shown that tiron can be used as a specific probe for superoxide in chloroplast reactions. With this technique, Miller and Macdowall (1975) showed that chloroplast superoxide production was considerably enhanced by addition of methyl viologen. Both ascorbate and adrenalin competitively obscured formation of the tiron semiquinone. A similar effect of superoxide dismutase was observed only when photosystem I sub-chloroplast particles were pre-incubated with the enzyme. This led Miller and Macdowall (1975) to conclude that tiron gains access to the site of superoxide production in undamaged thylakoids whereas superoxide dismutase does not. Evidence for enhancement by methyl viologen of chloroplast superoxide production has also been obtained in an EPR spin-trapping study by Harbour and Bolton (1975).

Asada and Kiso have identified production of superoxide in chloroplasts by a number of more commonly encountered techniques. Initiation of sulphite oxidation that was sensitive to both DCMU and superoxide dismutase was found to occur in chloroplasts even without the addition of autoxidisable cofactors (Asada and Kiso, 1973a); similar results were also obtained for oxidation of adrenalin (Asada and Kiso, 1973b). Asada *et al.* (1974) demonstrated a superoxide dismutase-sensitive reduction of cytochrome *c* by chloroplasts. In the presence of cytochrome *c*, hydrogen peroxide production was absent, indicating that chloroplast oxygen reduction is entirely univalent under these conditions. Though superoxide production was apparently stimulated by methyl viologen, benzyl viologen, diquat and triquat, a significant "endogenous" level of both adrenalin oxidation and cytochrome *c* reduction led Asada *et al.* (1974) to conclude that a membrane-bound chloroplast component, possibly the primary electron acceptor of photosystem I, is able to bring about univalent reduction of oxygen.

C. *Chloroplast Oxygen Uptake Mediated by Adrenalin or Dopamine and by Adrenochrome*

1. *Inhibition by Superoxide Dismutase and by Ascorbate: Two-Step Oxygen Reduction.* In the methyl viologen-mediated Mehler reaction superoxide dismutase has no effect on oxygen uptake. Elstner and Heupel (1974a) described a photosynthetic chloroplast oxygen uptake which was sensitive to inhibition by superoxide dismutase. They were able to demonstrate this effect both on the ferredoxin-mediated reaction and by using dopamine as the autoxidisable cofactor. That increasing the rate of superoxide dismutase (by adding superoxide dismutase) inhibits the overall reaction suggests that superoxide normally participates in some reaction other than dismutation in oxygen uptake mediated by ferredoxin or by dopamine. Elstner and Heupel (1974a) proposed that superoxide is reduced to peroxide by one (reduced) state of the autoxidising cofactor, while a second (intermediate) state is the one responsible for generation of superoxide. A third (oxidised) state then accepts from the photosynthetic chain two electrons per molecule, hence completing the cycle. For the reactions mediated by dopamine or adrenalin, this scheme is endorsed by the present author, and evidence for it will be reviewed in this section. The reaction in the presence of ferredoxin is thought by

Elstner and Heupel (1974a and 1974b; Elstner *et al.*, 1975) to be mediated by a putative chloroplast component with properties similar to dopamine's, while in the present chapter the ferredoxin-mediated reaction will be assumed to proceed by a different mechanism, and hence to merit a section (IID) of its own.

Among the compounds that were tested by Elstner and Heupel (1974a) as cofactors for a Mehler reaction, adrenalin and dopamine gave the highest rates of oxygen uptake. Either cofactor, at catalytic concentrations, gives a light-dependent, DCMU-sensitive and catalase-sensitive chloroplast oxygen uptake, which is directly proportional to chloroplast concentration at saturating light intensity (Allen, 1975c). To this extent each of these compounds resembles any other mediator of the Mehler reaction. They nevertheless have a number of peculiar properties, one of which is the aforementioned sensitivity to inhibition by superoxide dismutase.

Using the terminology of Misra and Fridovich (1972), a two step cycle (equations 12 and 13) for the chain-oxidation of adrenalin can be used to explain the sensitivity to superoxide dismutase of adrenalin-mediated chloroplast oxygen uptake. An initial oxidation of adrenalin (RH_3^-) by superoxide (equation 12) is followed by univalent reduction of oxygen by RH_3 (equation 13). Similar steps are likely to be involved in the oxidation of dopamine (Heikkila and Cohen, 1973). RH_2 may then function as a Hill oxidant (equation 14) to regenerate adrenalin, and the overall reaction (equation 2) would be the Mehler reaction as it has previously been defined.

$$O_2^- + RH_3^- + 2H^+ \longrightarrow RH_3 + H_2O_2 \qquad (12)$$

$$RH_3 + O_2 \longrightarrow RH_2 + O_2^- + H^+ \qquad (13)$$

$$H_2O + RH_2 \longrightarrow RH_3 + \tfrac{1}{2}O_2 + H^+ \qquad (14)$$

$$\text{Overall: } H_2O + \tfrac{1}{2}O_2 \longrightarrow H_2O_2 \qquad (2)$$

The observed inhibition by superoxide dismutase of overall oxygen uptake can be understood to be a result merely of competitive replacement of the reaction of equation 12 by the reaction of superoxide dismutation. This would prevent regeneration of the electron acceptor (RH_2), and both oxygen uptake and electron transport would cease.

Initiation of the cycle would require production of superoxide in order for the first reaction (equation 12) to take place; this could be achieved by a slow univalent reduction of oxygen by endogenous chloroplast components such as the membrane-bound iron-sulphur proteins of photosystem I (Asada *et al.*, 1974). The time taken for accumulation of the intermediates RH_3 and RH_2 would represent the lag-phase which Elstner and Heupel (1974a) reported.

Both the overall rate of oxygen uptake and the degree of sensitivity to superoxide dismutase are also dependent on pH. The rate of oxygen uptake has a maximum value in the region pH 8.0 to pH 8.5, while sensitivity to inhibition by superoxide dismutase is most marked at pH 7.5, and decreases with increasing pH (Allen, 1975c). At higher pH the oxygen uptake, though perhaps initiated by formation of superoxide in the chloroplast, becomes largely a consequence of the chain-oxidation of adrenalin to adrenochrome, a process which has been shown to have a pH-dependence consistent with this interpretation (Misra and Fridovich, 1972). In this view the cycle would break down at higher pH because of replacement or regeneration of adrenalin

(equation 14) by the following reactions:

$$RH_2 + O_2^- + H^+ \longrightarrow RH + H_2O_2 \qquad (15)$$

$$RH + O_2 \longrightarrow R + O_2^- + H^+ \qquad (16)$$

Both dopamine-mediated (Elstner and Heupel, 1974a) and adrenalin-mediated (Allen, 1975c) Mehler reactions may be either stimulated by ascorbate or inhibited by it. Inhibition occurs if ascorbate is added to the chloroplasts before the mediator, and is likely to be a result of the ascorbate-superoxide reaction (equation 9) preventing initial oxidation of adrenalin (equation 12) and, as a consequence, accumulation of intermediates of the cycle. Like superoxide dismutase, ascorbate would then competitively inhibit both oxygen uptake and electron transport. Stimulation occurs if ascorbate is added after the mediator and during the linear phase of the sigmoidal oxygen uptake trace. Here saturating concentrations of RH_3 and RH_2 would already have accumulated, and disappearance of superoxide caused by its reaction with ascorbate would not, at first, interrupt the cycle. It could, however, cause a transient increase in the rate of oxygen uptake by preventing oxygen evolution from a residual spontaneous dismutation of superoxide. In any event the stimulation is transient, and the rate subsequently declines to less than that observed before addition of ascorbate.

2. *Adrenochrome-Mediated Single-Step Oxygen Reduction.* Of the five redox states of adrenalin that are thought to be involved in the chain-reaction of adrenalin oxidation (Misra and Fridovich, 1972), only three (RH_3^-, RH_3 and RH_2) are included in the foregoing explanation of adrenalin's mediation of a Mehler reaction. In support of this explanation, Allen (1975c) showed that the end-product of adrenalin oxidation, adrenochrome (R), mediates chloroplast oxygen uptake but does so by the single-step mechanism described in section IIB.

Adrenochrome at catalytic concentrations produces a light-dependent chloroplast oxygen uptake which is completely inhibited by DCMU and by catalase. The oxygen uptake appears promptly on addition of the mediator, and any lag-phase is shorter than the response-time (a few seconds) of the oxygen electrode. Oxygen uptake is unaffected by addition of superoxide dismutase, but acceleration of oxygen uptake resulting from addition of ascorbate is prevented. The reaction is clearly analogous to the methyl viologen-mediated one and is therefore likely to involve one-electron reduction of adrenochrome (R) by a component of the chloroplast, and one-electron reduction of oxygen by RH. The superoxide produced will either dismute or be reduced by ascorbate.

D. *Ferredoxin-Mediated Chloroplast Oxygen Uptake*

1. *Superoxide Production on Autoxidation of Ferredoxin.* In an investigation of the reaction of reduced iron-sulphur proteins with oxygen, Orme-Johnson and Beinert (1969) found that autoxidation of ferredoxin from *Clostridium pasteurianum* led to the production of an EPR-detectable concentration of superoxide, while autoxidation of spinach ferredoxin did not. Misra and Fridovich (1971) reported that both clostridial and spinach ferredoxins, when reduced enzymatically with NADPH and spinach ferredoxin-NADP reductase, bring about an aerobic oxidation of adrenalin which is sensitive to inhibition by superoxide dismutase. Nakamura and Kimura (1972) reported a similar result

for spinach ferredoxin. Allen (1975c) produced confirmatory evidence (oxidation of adrenalin and reduction of nitroblue tetrazolium) that superoxide can be produced by autoxidation of spinach ferredoxin. In all experiments involving enzymatic reduction of ferredoxin, ferredoxin-NADP reductase by itself seems either not to reduce oxygen or to do so very slowly.

Leaving aside for the moment the experiment of Orme-Johnson and Beinert (1969), there seems to exist good evidence that ferredoxins reduce oxygen univalently. Misra and Fridovich (1971) found, however, that adrenochrome production was relatively slower than NADPH oxidation, and concluded that a variable proportion of total oxygen reduction by both bacterial and plant-type ferredoxins proceeded by a two-electron transfer. For spinach ferredoxin they showed that the "percent univalent flux" increases with oxygen concentration, and was greater at pH 7.8 than at pH 6.8. Asada *et al.* (1974) found that while methyl viologen stimulated production of superoxide by illuminated chloroplasts, spinach ferredoxin did not. Since ferredoxin is known to stimulate chloroplast oxygen uptake, it might be concluded that a ferredoxin-mediated Mehler reaction, unlike the methyl viologen-mediated one, proceeds by some mechanism which involves at least some divalent reduction of oxygen.

2. *Inhibition by Superoxide Dismutase: Two-Step Oxygen Reduction by Ferredoxin.* Although hydrogen peroxide is its product, and it has the usual overall stoichiometry

$$H_2O + \tfrac{1}{2}O_2 \longrightarrow H_2O_2 \qquad (2)$$

(Telfer *et al.*, 1970), the ferredoxin-mediated Mehler reaction is indeed unusual in a number of respects. The rate of oxygen uptake is not, for example, appreciably increased by addition of ascorbate (Allen, 1975a and 1975c). It may, in fact, be inhibited by it (Elstner and Heupel, 1974a and 1974b).

A more significant observation is that chloroplast oxygen uptake in the presence of spinach ferredoxin is inhibited by superoxide dismutase on its own (Elstner and Heupel, 1974a; Allen, 1975a). Such an inhibition, observed with either adrenalin or dopamine as the mediator, results from a necessary involvement of superoxide in catalysis of oxygen reduction by three valence states of the mediator. If ferredoxin itself is the component which reduces oxygen in the ferredoxin-mediated chloroplast oxygen uptake, then the adrenalin-type mechanism cannot apply, since, under physiological conditions plant-type ferredoxins have only two valence states (Tagawa and Arnon, 1968). How, then, can superoxide dismutase cause an inhibition? Elstner and Heupel's (1974a and 1974b) solution to this problem is the proposal that ferredoxin itself does not reduce oxygen directly, but instead transfers electrons to an "oxygen reducing factor" which confers sensitivity to ascorbate and to superoxide dismutase on the overall reaction. The oxygen reducing factor is then assumed to have three valence states, and to participate in an oxygen-reducing cycle which, like that involving adrenalin or dopamine, has superoxide as an essential intermediate.

Allen (1975a) has proposed an alternative explanation to account for inhibition by superoxide dismutase of the ferredoxin-mediated Mehler reaction. This explanation rests on the proposal that ferredoxin itself reduces oxygen in the Mehler reaction, and that it does so by a "two-step" mechanism in which reduced ferredoxin reduces both oxygen to superoxide (equation 17) and superoxide to peroxide (equation 18). Re-reduction of ferredoxin by non-

cyclic photosynthetic electron transport (equation 19) gives the familiar overall reaction (equation 2) which is the one required by the catalase-sensitivity of net oxygen uptake by chloroplasts in the presence of ferredoxin.

$$Fd_{red} + O_2 \longrightarrow Fd_{ox} + O_2^- \quad (17)$$

$$Fd_{red} + O_2^- + 2H^+ \longrightarrow Fe_{ox} + H_2O_2 \quad (18)$$

$$H_2O + 2Fd_{ox} \longrightarrow 2Fd_{red} + \tfrac{1}{2}O_2 + 2H^+ \quad (19)$$

$$\text{Overall: } H_2O + \tfrac{1}{2}O_2 \longrightarrow H_2O_2 \quad (2)$$

Addition of superoxide dismutase would then eliminate one route of ferredoxin oxidation, by replacing the reaction of equation 18 with superoxide dismutation (equation 8).

$$2Fd_{red} + 2O_2 \longrightarrow 2Fd_{ox} + 2O_2^- \quad (17)$$

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (8)$$

$$H_2O + 2Fd_{ox} \longrightarrow 2Fd_{red} + \tfrac{1}{2}O_2 + 2H^+ \quad (19)$$

$$\text{Overall: } H_2O + \tfrac{1}{2}O_2 \longrightarrow H_2O_2 \quad (2)$$

If this were to occur when ferredoxin concentration limits the rate of the overall reaction, then the effect of superoxide dismutase would be to inhibit the rate of regeneration of oxidised ferredoxin, and hence competitively to inhibit both oxygen uptake and photosynthetic electron transport.

The introduction of superoxide dismutase would not prevent regeneration of oxidised ferredoxin by the first step (equation 17), and so addition of superoxide dismutase would neither totally inhibit autoxidation of ferredoxin (as it would with adrenalin) nor change the stoichiometry of oxygen uptake to electron transport (as it would with methyl viologen in the presence of ascorbate). The overall stoichiometry (equation 2) would remain unchanged, while the overall process would be subject to a partial feedback inhibition caused by a decrease in the steady-state concentration of oxidised ferredoxin.

The hypothesis requires reduced ferredoxin to have a greater affinity for superoxide than it has for oxygen. The relative rates of the reactions described by equations 17 and 18 will, of course, depend on oxygen concentration. At lower oxygen concentrations superoxide as an intermediate would have a lower steady-state concentration than it would at higher oxygen concentrations. This could help to explain the observations of Misra and Fridovich (1971) that univalent oxygen reduction, as measured by adrenalin oxidation, constitutes a greater proportion of total oxygen reduction at higher oxygen concentrations. An ability of the putative reaction of ferredoxin with superoxide (equation 18) to compete with a superoxide-detecting reaction such as adrenalin oxidation would give the appearance of direct reduction of oxygen to hydrogen peroxide. This may well account for the "divalent flux" of Misra and Fridovich, and for ferredoxin's apparent failure to enhance superoxide production by illuminated chloroplasts (Asada *et al.*, 1974). A comparatively low steady-state concentration of superoxide could also explain the absence of EPR-detectable superoxide production by plant ferredoxin in the experiment of Orme-Johnson and Beinert (1969). Mehler reactions mediated by any of a variety of both plant and bacterial ferredoxins show sensitivity

to inhibition by superoxide dismutase (Allen, 1975c), though at equal concentrations (10 μM) the bacterial proteins seemed to mediate significantly (approximately two-fold) faster reactions than did the plant-types. Despite a common mechanism of oxygen reduction, relative affinities for oxygen may in part explain the generation of higher concentrations of superoxide by clostridial ferredoxin than by spinach ferredoxin (Orme-Johnson and Beinert, 1969; Misra and Fridovich, 1971).

The two-step hypothesis makes no specific prediction about the effect of ascorbate on chloroplast oxygen uptake, since replacement of the ferredoxin-superoxide reaction (equation 18) by the ascorbate-superoxide reaction (equation 9), should result in inhibition of electron transport itself as well as an increase in the stoichiometry of oxygen uptake to electron transport.

3. *Evidence For Two-Step Oxygen Reduction.* Allen (1975a) obtained a double reciprocal plot of the rate of chloroplast oxygen uptake against ferredoxin concentration, in which the effect of superoxide dismutase appeared to increase the ferredoxin concentration required for half saturation from 30 μM to 50 μM, while V_{max} was unchanged at about 70 μmoles (O_2 (mg chl)$^{-1}$h^{-1}. Superoxide dismutase may therefore compete with ferredoxin for an intermediate (superoxide) of the overall reaction. A similar plot of the rate of ascorbate-stimulated oxygen uptake against methyl viologen concentration shows superoxide dismutase to be a non-competitive inhibitor. The "K_m" was unchanged at 3 μM methyl viologen, and the rate of oxygen uptake at infinite methyl viologen concentration would be decreased by superoxide dismutase from 670 to 280 μmoles (mg chl)$^{-1}$h^{-1}.

In the chloroplast-free model system, in which ferredoxin is reduced by NADPH via ferredoxin-NADP reductase, there is a one-to-one molar ratio between oxygen consumption and NADP oxidation, regardless of the presence of superoxide dismutase or ascorbate, while the rate of both NADPH oxidation and oxygen uptake is slower in the presence of superoxide dismutase than in its absence (Allen, 1975c). Elstner and Heupel (1974b) found no effect of superoxide dismutase in this system. They concluded that a chloroplast membrane-bound factor is required for such inhibition to occur. Spinach superoxide dismutase present as a contaminant of ferredoxin-NADP reductase (Shin, 1971) will also produce this result (Allen, 1975c). In any event the pattern of inhibition of overall reaction rate but not of oxygen uptake relative to NADPH oxidation contrasts markedly with that obtained in a similar experiment performed with methyl viologen. Here NADPH oxidation is unaffected by ascorbate and by superoxide dismutase. In the presence of ascorbate however, superoxide dismutase inhibits both the rate of oxygen uptake and the number of oxygen molecules reduced per NADPH molecule oxidised (Epel and Neumann, 1973; Allen, 1975c).

On addition of ferredoxin to chloroplasts or to the chloroplast-free model system, oxygen uptake appears promptly (Allen, 1975c), suggesting that an adrenalin-like mediator is not involved.

The inhibition by superoxide dismutase of ferredoxin-mediated oxygen uptake also occurs in chloroplasts depleted of ferredoxin-NADP reductase (Allen, 1975c), indicating that this enzyme plays no part in the reaction.

Another line of evidence against the assumption that a factor other than ferredoxin plays some part in superoxide dismutase's inhibition of a ferredoxin-mediated Mehler reaction is that the autoxidation of chemically-reduced

Spirulina ferredoxin was shown to be inhibited by superoxide dismutase (Allen, 1975a). When superoxide dismutase was present from the start, the rate of ferredoxin autoxidation was stimulated by addition of cyanide.

E. *Alternative Schemes*

Elstner *et al.* (1970) suggested that the superoxide radical could be considered an active intermediate in ascorbate oxidation by chloroplasts. They found that photosynthetic oxidation of hydroxylamine, ascorbate or glycollate required the presence of an autoxidisable acceptor (anthraquinone or triquat), and occurred only in the presence of cyanide. The requirement for cyanide can now be viewed as the need to inhibit a chloroplast-associated superoxide dismutase (Elstner *et al.*, 1975), though in the earlier paper (Elstner *et al.*, 1970) a "cyanoperoxidase" was invoked as a catalyst of these reactions.

Elstner and Kramer (1973) found no effects of ascorbate or superoxide dismutase on ATP synthesis by chloroplasts in the presence of a low potential, autoxidisable electron acceptor. Their conclusions about the mechanism of ascorbate-stimulation of oxygen uptake in such circumstances agree with those of Epel and Neumann (1973); oxidation of ascorbate was found to be sensitive to inhibition by superoxide dismutase. Elstner and Kramer (1973) also found that low "endogenous" rates of phosphorylation (i.e. those occurring in the absence of added electron acceptors or cofactors) were, however, stimulated by addition of ascorbate, and that the stimulation was sensitive to inhibition by superoxide dismutase. They observed no net oxidation of ascorbate accompanying the stimulated rate of phosphorylation, and concluded that a catalytic cycle which involved three valence states of ascorbate was responsible for the stimulation. According to this scheme, ascorbate is oxidised by superoxide to monodehydroascorbate, which in turn becomes oxidized by donating electrons to the photosynthetic chain. Dehydroascorbate is then assumed to accept electrons from photosystem I, thus completing the cycle. Apart from the *ad hoc* character of the assumptions which the scheme involves, its chief weakness as a candidate for a physiological process is that ferredoxin is likely to be a more effective electron acceptor than dehydroascorbate.

If the "ascorbate-cycle" of Elstner and Kramer (1973) were to function *in vivo*, the necessary concentration of ascorbate within the chloroplast would be expected to inhibit electron flow to oxygen via "oxygen reducing factor" (ORF), another process held to occur *in vivo* by Elstner and Heupel (1974a, 1974b). Here "observed rates of oxygen reduction by chloroplast lamellar systems in the presence of ferredoxin are not due to an autoxidation of reduced ferredoxin but to the catalysis by a membrane-bound factor (ORF_{bound})" (Elstner and Heupel, 1974b). Stated in this way it is contradictory to the proposal that ferredoxin itself is the autoxidising component of the ferredoxin-mediated Mehler reaction (Telfer *et al.*, 1970; Allen, 1975a, and section IID of the present chapter). ORF is assumed by its proponents to have three valence states which participate in a catalytic cycle of the type outlined by them for dopamine (Elstner and Heupel, 1974a), and discussed in section IIC of this chapter. ORF is thereby thought to confer sensitivity on ferredoxin-mediated oxygen uptake to inhibition by superoxide dismutase and ascorbate. The two-step mechanism of ferredoxin autoxidation already discussed (Allen, 1975a) explains these observations and is both more economical and vulnerable to experimental test.

The ORF which Elstner and Heupel (1974b) obtained in the supernatant from a heat-treated sugar beet or spinach chloroplast preparation is clearly active in mediating superoxide dismutase-sensitive chloroplast oxygen uptake and glyoxylate decarboxylation. The small volumes in which the chloroplasts were washed prior to heat-treatment may not entirely exclude the possibility of cytoplasmic contamination. Heat-activation of the glyoxylate-decarboxylating properties of the factor is another possibility. In other respects the factor resembles the incompletely characterized "cytochrome reducing substance" of Fujita and Myers (1971).

A low-molecular weight factor which catalyses photosynthetic production of both hydrogen peroxide and superoxide has been isolated from *Euglena gracilis* by Elstner and Heupel (1976). In spinach chloroplasts this compound causes an oxygen uptake which is stimulated by addition of ferredoxin. In a similar system with *Euglena* chloroplasts or detergent-treated spinach chloroplasts, ferredoxin has an inhibitory effect on oxygen uptake (Elstner *et al.*, 1976). A role *in vivo* for Elstner and Heupel's catalyst of univalent oxygen reduction has yet to be described.

F. *Oxygen Uptake and Photosystem II*

Addition of DBMIB, an inhibitor at the plastoquinone site (Bohme *et al.*, 1971), to chloroplasts makes possible a separation of two sites of electron transport and coupled phosphorylation (see Fig.1); one site is associated with each of the two photosystems (see Trebst, 1974). Photophosphorylation normally occurs only when plastoquinone is able to function as the hydrogen carrier of a proton-translocating loop, though in the presence of DBMIB an artificial, hydrophobic hydrogen carrier (such as PMS or DCPIP) may replace phastoquinone and so restore phosphorylation.

Gould and Izawa (1973b) reported that in the absence of ferricyanide DBMIB may also mediate a Mehler reaction. The chloroplast oxygen uptake resulted in formation of hydrogen peroxide, as indicated by release of oxygen on subsequent addition of catalase. Insensitivity of the oxygen uptake to treatment of the chloroplasts with cyanide or polylysine (both of which inhibit electron flow through plastocyanin) led Gould and Izawa (1973b) to conclude that photosystem I was not involved. The stoichiometry of photosystem II phosphorylation which they obtained with the DBMIB-mediated Mehler reaction was equivalent to a $P/2e^-$ ratio of about 0.35.

The role of superoxide in photosystem II oxygen uptake has not yet been established, but a significant stimulation of the oxygen uptake by Mn^{2+} has been reported (Miles, 1976). However, superoxide dismutase inhibits only the initial phase of a biphasic oxygen consumption which follows addition of $MnCl_2$ (Allen, unpublished results). It is possible that Mn^{2+}/Mn^{3+} catalyses an overal dismutation of superoxide (Lumsden and Hall, 1975), though slow post-illumination evolution of oxygen (which is prevented by prior addition of catalase) suggests strongly that net oxidation of Mn^{2+} has occurred during oxygen uptake by photosystem II. This oxygen evolution probably results from oxidation of hydrogen peroxide by Mn^{3+}; a similar dark evolution of oxygen was reported by Walker *et al.* (1970) to be associated with the now more clearly understood effect of manganese on FMN-mediated chloroplast oxygen uptake.

Trebst *et al.* (1976) have recently introduced a cofactor of DBMIB-insensitive and KCN-insensitive chloroplast oxygen uptake; 2,3-dimethyl-5,6-methylenedioxy-p-benzoquinone. This compound ("quinone" in Fig.1) functions

as a Mehler reaction cofactor both in the absence of DBMIB, and in the presence of DBMIB as a concentration (1 μM) which is inhibitory to conventional non-cyclic electron flow but which is also too small for DBMIB itself to function as the cofactor.

In all examples of chloroplast oxygen uptake which have been assigned to photosystem II alone, the precise mechanism of oxygen reduction remains to be established. Caution must therefore be exercised in interpreting such oxygen uptake as an absolute measure of electron transport in photosystem II.

III. CONCLUSION

There is as yet no compelling evidence that reduction of oxygen plays an essential role in photosynthesis. The Mehler reaction which occurs *in vitro* in broken, washed chloroplasts in the presence of an autoxidisable electron acceptor is perhaps more fully understood as a result of identification of superoxide as an intermediate, though any genuinely biological advance represented by the work discussed here must rest on whatever help it gives to an understanding of photosynthesis *in vivo*. The next step in this direction is likely to take the form of a demonstration that hydrogen peroxide or superoxide either does or does not participate in the "complete" photosynthetic process as it occurs in an intact, CO_2-fixing chloroplast. Evidence for hydrogen peroxide production during CO_2-fixation has already been provided for chloroplasts by Egneus *et al.* (1975) and Kaiser (1976) and, for algal suspensions, by Patterson and Myers (1973) and Radmer and Kok (1976). Enhancement by superoxide dismutase of CO_2-fixation by a chloroplast preparation has been reported (Ziegler and Libera, 1975), but the authors' interpretation of the effect is that broken chloroplasts in the preparation produce superoxide which, if not dismuted catalytically, impairs in some way the CO_2-fixing activity of the unbroken chloroplasts remaining.

The envelope of an intact chloroplast represents simultaneously a necessary condition for complete chloroplast photosynthesis and a barrier to the relatively crude experimental manipulation that is carried out on broken chloroplasts and on chloroplast particles. To some extent this problem is resolved by the reconstituted chloroplast system of Walker and Lilley (1974), in which soluble components of the chloroplast stroma are added back to the chloroplast lamellae. Generation of hydrogen peroxide or superoxide in the reconstituted system should be relatively easy to identify.

Even if photosynthetic reduction of oxygen were to occur only in unusual physiological circumstances, such as during the onset of poised cyclic phosphorylation or during pseudo-cyclic phosphorylation when demands for ATP are exceptionally high, a purely univalent reduction of oxygen would certainly be detrimental to chloroplast function. Thus reduced ferredoxin's relatively high affinity for oxygen and the mechanism of its reaction with oxygen are both quite likely to be of some adaptive significance.

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ABBREVIATIONS

AQ	:	anthraquinone
BV	:	benzyl viologen
DAD	:	diaminodurene
DBMIB	:	2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone
DCMU	:	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCPIP	:	2,6-dichlorophenolindophenol
FMN	:	flavin mononucleotide
MV	:	methyl viologen
PD	:	p-phenylenediamine
PMS	:	phenazine methosulphate
PS	:	photosystem