Biochimica et Biophysica Acta, 387 (1975) 176–187

BBA 46891

THE TIRON FREE RADICAL AS A SENSITIVE INDICATOR OF CHLOROPLASTIC PHOTOAUTOXIDATION*

R. W. MILLER and F. D. H. MACDOWALL

Chemistry and Biology Research Institute, Agriculture Canada, Ottawa, Ontario (Canada)

(Received October 1st, 1974)

SUMMARY

Wheat chloroplasts photochemically reduced molecular oxygen, as a Hill oxidant in the Mehler reaction, to superoxide anion which then oxidized added 1,2-dihydroxybenzene-3,5-disulfonate to its semiquinone, a comparatively stable free radical at pH 7. The last mentioned reaction was rapid in aqueous solution, but the rate of formation of 1,2-dihydroxybenzene-3,5-disulfonate semiquinone by the chloroplast system was calculated as a $T_{\frac{1}{2}}$ of 0.6 s. The Mehler reaction, or more specifically the univalent reduction of oxygen by Photosystem I, was rate-limiting so that the 1,2-dihydroxybenzene-3,5-disulfonate semiquinone was a useful spin probe for superoxide anion production at room temperature. The ESR signal of 1,2-dihydroxybenzene-3,5-disulfonate semiquinone was proportional to its steady state concentration and decayed in the dark with a $T_{\frac{1}{2}}$ of 5–6 s. This oxygen-dependent signal was enhanced by mediation of chloroplastic oxygen reduction through methyl viologen. The superoxide anion scavengers ascorbate and L-epinephrine competitively obscured 1,2-dihydroxybenzene-3,5-disulfonate semiquinone formation, but added superoxide dismutase was not as effective in this role. Partial inhibition by superoxide dismutase was achieved only by preincubation of Photosystem I enriched particles with ten times the endogenous concentration of superoxide dismutase. This and the persistence of a small amount of a 1,2-dihydroxybenzene-3,5-disulfonate (Tiron) oxidizing species in the dark supports the concept of Tiron accessibility but not the superoxide dismutase accessibility of superoxide anion bound in its formative enzyme complex. Benzoquinone and naphthoquinone disulfonate also reacted with superoxide anion, and supported both the Hill reaction and the Mehler reaction as final oxidants of both water and superoxide anion.

Abbreviations: Tiron, 1,2-dihydroxybenzene-3,5-disulfonate; $(TH)^{\cdot}$, Tiron semiquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea. $O_2^{\cdot-}$, superoxide anion.

* Chemistry and Biology Research Institute contribution No. 832.

INTRODUCTION

It is well known that univalently reduced oxygen or superoxide anion readily reduces quinones. The rates of single electron transfer to quinones have been reported to be greater than $10^8\ M^{-1} \cdot s^{-1}$ in aqueous solutions of 2.3 μM superoxide anion and 50 mM quinones [1]. Moreover, rates of electron transfer increase with increasing redox potential of the acceptor [1]. Superoxide anion also has been shown to produce semiquinones by extracting a single electron from catechols [2] and hydroquinone [1]. The hydroquinone reaction is known to be rapid with $k_2 > 10^7\ M^{-1} \cdot s^{-1}$ [1]. Previously it was reported that the substituted catechol, 1,2-dihydroxybenzene-3,5-disulfonate, could be used as an indicator for the steady state production of oxygen free radicals either bound to an electron donor (such as $FMNH_2$) or free in solution. The rate of formation of the Tiron semiquinone free radical was determined by ESR spectroscopy. This method was applied to both photochemical and enzymatic systems which generate superoxide anion [2].

Mehler [3, 4] showed that isolated, illuminated chloroplasts not only reduce added quinones but also photochemically catalyze subsequent oxygen uptake. The latter process is now known to involve the photochemical reduction of molecular oxygen by Photosystem I of chloroplasts with the production of univalently reduced oxygen which then either oxidizes ascorbic acid or other added reductants to produce hydrogen peroxide [5–8] or dismutates into hydrogen peroxide and oxygen [8]. The rate of transfer of reducing equivalents from Photosystem I to oxygen is enhanced by electron carriers such as viologen dyes [8–10]. In this report ascorbate, quinones and Tiron are shown to compete for the superoxide anion produced endogenously by chloroplastic System I in the presence of oxygen.

Catalysis of the dismutation of $O_2^{\cdot-}$ may occur in chloroplasts provided that this species is released from the site of formation and can diffuse freely to chloroplast superoxide dismutase. Although several dismutases are present in chloroplasts [7], their reactivity in intact organelles may be limited by the accessibility of enzyme and substrate [2].

MATERIALS AND METHODS

Chloroplasts were extracted from recently fully expanded leaf blades of Kharkov wheat (*Triticum aestivum*, L.), after controlled growth as previously described [11] with a light intensity of $6 \cdot 10^4\ ergs \cdot cm^{-2} \cdot s^{-1}$. About 15 g of fresh lamina were sliced and ground in 100 ml of extraction medium which contained 0.4 M sucrose, 0.02 M tricine buffer, pH 8, 5 mM $MgCl_2$, 1 % bovine serum albumin, 5 mM mercaptoethanol, 1 mM EDTA and 0.02 M NaCl. Debris was removed by centrifuging the muslin- and nylon cloth filtrate at $120 \times g$ for 3 min, and whole chloroplasts were pelleted at centrifugal forces of $6000 \times g$ for 5 min. The sediment was washed with a solution containing the first three components of the above extraction medium and divided into two aliquots. One aliquot containing some thylakoid particles but mainly whole chloroplasts (about 20 % of which had intact outer envelopes, Class I), was resuspended in 0.5 M sucrose, quick frozen in 1 ml portions and stored at −80 °C. The other aliquot was lysed and extracted by resuspending and standing one half hour in distilled water, then it was centrifuged and stored as above as a thylakoid

suspension. Chlorophyll was estimated by Bruinsma's modification of Arnon's procedure [12]. Subchloroplast fragments were prepared by Anderson and Boardman's digitonin method [13] except that the final fractions were suspended and stored as above. The pellet which sedimented at 144 000 × *g* was used as a preparation depleted in Photosystem II activity and designated as Photosystem I enriched particles. Depending on the digitonin concentration which was used (0.4 or 0.5 % w/v), chlorophyll *a* content of these particles varied from 4.5 mg to 6.5 mg per mg chlorophyll *b*. The 0.4 % digitonin particles retained some Photosystem II activity and were of more use in this work.

Oxygen uptake was measured with a Clark electrode at 25 °C in a thermostated vessel. The vessel was illuminated with a tungsten lamp which produced an intensity of about $2 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} within the reaction chamber. Electron spin resonance spectra were recorded with a Varian E-3 spectrometer. Thin (0.2 mm) quartz, aqueous sample cells were illuminated in the slotted spectrometer cavity with white light ($2 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}) or monochromatic light (narrow band pass interference filter, 717 nm). Variations in intensity were obtained by moving the non-collimated source away from the cavity and measuring the light intensity at the edge of the sample cell. Rates of semiquinone production were monitored by holding the magnetic field at a constant value and recording the height of the first derivative ESR signal as a function of time. ESR samples were made anaerobic by repeated evacuation of thylakoid preparations in a Thunberg cuvette followed by flushing with nitrogen. The ESR cells were also flushed with O_2-free nitrogen before filling with the anaerobic reaction mixture. Light intensity was measured with a Kettering radiometer (Yellow Springs Instrument Co., Ltd., Yellow Springs, Ohio).

Superoxide dismutase activities of the whole and fragmented chloroplasts were measured by a previously described procedure which employed *p*-nitrobluetetrazolium as an electron acceptor [14].

RESULTS

ESR signals with Photosystem I enriched particles and chloroplasts

Illumination of isolated Photosystem I enriched particles in the absence of Tiron produced the expected signal of P_{700} (Fig. 1, curve 1). Large modulation amplitudes (10 G) were employed in recording the chlorophyll spectrum. The signal was allowed to decay for 10 min in the dark and then Tiron was added. A lower modulation amplitude (1.0 G), chosen to resolve the hyperfine spectrum of Tiron, then revealed a small semiquinone signal (curve 3). This indicated the persistance of an oxidizing species in the dark. At this modulation amplitude, paramagnetism due to P_{700} produced only a small broad signal underlying the semiquinone signal so that the semiquinone signal appears with an essentially straight base line.

When the Tiron-containing sample was illuminated with white light, a much stronger semiquinone spectrum (Fig. 1, curve 4) was obtained. This spectrum represents the steady state concentration of the Tiron free radical. Some P_{700} remained oxidized in the presence of Tiron as evidenced by the large signal obtained in the light when the modulation amplitude was increased to 10 G (Fig. 1, curve 2). The Tiron signal was overmodulated here and appeared as small shoulders on the chlorophyll resonance.

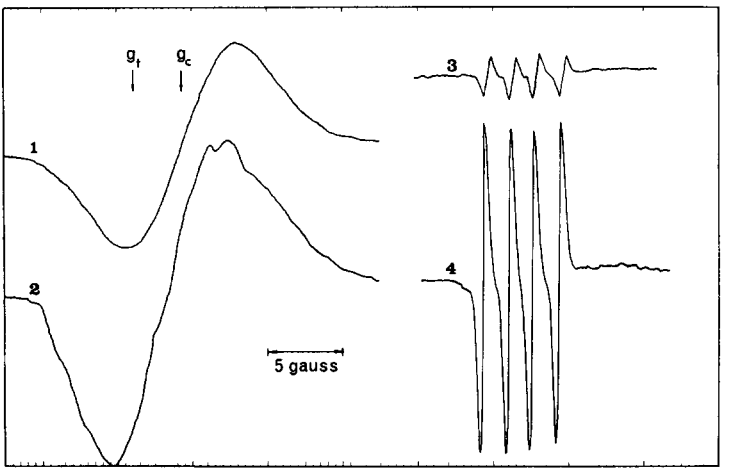

Fig. 1. Oxidation of Tiron by Photosystem I particles. Photosystem I particles (0.345 mg chlorophyll *a*/ml) were prepared in 0.5 M sucrose containing no $MgCl_2$. Tiron (4 mM) was added after curve 1 had been recorded and the final chlorophyll *a* concentration was 0.340 mg/ml. Samples were introduced into ESR cells and light and dark spectra were recorded under the following conditions: All spectra were obtained at 20 mW microwave power; microwave frequency, 9.44 GHz; recorder time constant, 1.0 s; field scanning rate, 16 min full scale; spectrometer gain, $2.5 \cdot 10^5$. Modulation amplitudes were: curves 1 and 2, 10 G; curves 3 and 4, 1.0 G. Irradiation intensity (white light) was $2 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} for curves 1, 2 and 4 while curve 3 was recorded in the dark. Field strengths at which the *g* values for $(TH)^{\cdot}$ (g_t) and for the P_{700} cation radical (g_c) occur are indicated above curve 1.

Illumination of the whole chloroplast preparations in the same reaction mixtures produced results qualitatively similar to those illustrated for Photosystem I particles in Fig. 1. With chloroplasts and thylakoids a composite of ESR signals from Systems I and II was obtained in white light in the absence of Tiron at high modulation amplitudes. Filtered light (717 nm) induced mainly the P_{700} signal. However, in the presence of Tiron and at 1 G modulation amplitude the appearance of the light-dependent $(TH)^{\cdot}$ signal was similar to that shown in Fig. 1 with either white or filtered light.

Filtered light and DCMU (10^{-5} M) partially blocked P_{700} reduction as evidenced by an increase in the decay time of the P_{700} signal from $T_{\frac{1}{2}} < 0.6$ s to $T_{\frac{1}{2}} = 18$ s. These treatments did not inhibit Tiron oxidation during the first cycle of illumination after 30 min storage of samples in the dark. However, on subsequent cessation of illumination for only 1 min followed by reillumination, the magnitude of the Tiron signal increase (but not the initial rate of formation) was reduced greatly due to incomplete reduction and recycling of P_{700} under these conditions.

The two hyperfine splitting constants for the Tiron semiquinone were 1.72 G and 3.32 G corresponding to interaction between the two non-equivalent ring protons of Tiron and the unpaired electronic spin of the free radical. The nearly even spacing of the hyperfine lines of the spectrum (Fig. 1, curve 4) reflects the fact that one coupling interaction is about twice as strong as the other. The *g* values of the $(TH)^{\cdot}$ and P_{700} signals were displaced by about 2.5 G as indicated in Fig. 1. This difference in *g* values allows independent observations of the kinetics of formation and decay of the P_{700} cation radical and $(TH)^{\cdot}$.

Rate of formation and decay of P_{700} ESR signals

The ESR signals due to the P_{700} cation radical appeared instantly on illumination of thylakoids and whole chloroplast preparations. On cessation of illumination, the signal decayed in less than 0.5 s. The presence of Tiron did not alter this result. In the Photosystem I enriched particles, the signal appeared very rapidly on illumination but decayed more slowly in the dark. The $T_{\frac{1}{2}}$ for decay was determined to be 2 s with or without Tiron.

Kinetics of $(TH)^{\cdot}$ formation in whole chloroplasts

The kinetic course of the formation and decay of the $(TH)^{\cdot}$ signals in whole chloroplasts is illustrated in Fig. 2. Curve 1 shows that a comparatively small Tiron signal rapidly appeared on illumination. The observed half-time ($T_{\frac{1}{2}}$) for this reaction was about 0.6 s, a value close to the experimental resolution. At a single level of illumination it was not possible to distinguish the rate of formation of the free radical signal from that of the P_{700} signal. The steady state signal height represents a condition where the rates of disproportionation of the radical and oxidation of the semiquinone by O_2 balance the rate of electron transfer from Tiron to superoxide anion.

Decay of the signal was much slower than its formation, the half-time being 5–6 s. However, decay to the original base line did not occur as elaborated below. The rate of decay was suggestive of chemical disproportionation as it was close to

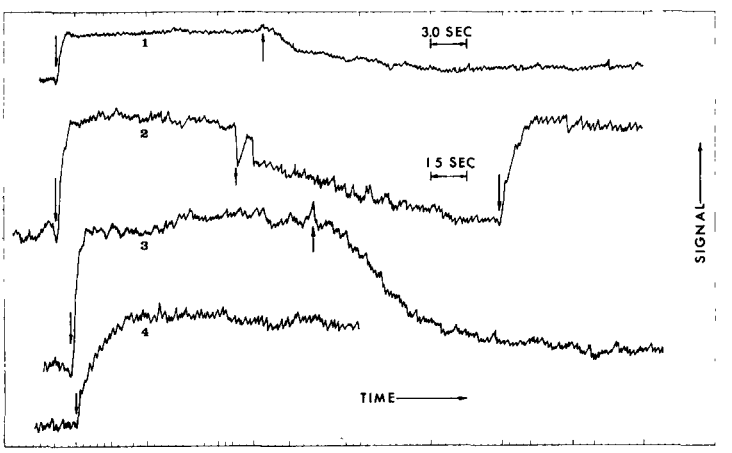

Fig. 2. Kinetics of Tiron oxidation in whole chloroplasts. Isolated chloroplasts were suspended in 0.5 M sucrose containing 0.05 M potassium phosphate buffer at pH 7. Chlorophyll content was 2.4 mg/ml. Tiron, 4 mM, was incorporated into the suspension which was aspirated into quartz-aqueous sample cells. The field producing the maximum positive-going portion of the high field signal of the Tiron free radical was located and the field scan locked at this value. Methylviologen (14 μ M)and methylviologen plus KCN (7 mM) were added before recording curves 2 and 3, respectively. The illuminating lamp was switched on at points indicated by the downward pointing vertical arrows while upward pointing arrows indicate cessation of illumination. A sample was incubated at 25 °C for 18 h before reaerating and recording curve 4. The time scale indicated near the top of the figure applies only to curve 1. The other time scale applies to curves 2–4. Spectroscopic parameters are summarized below: All curves; microwave power, 20 mW; modulation amplitude, 1.0 G; spectrometer gain, $2.5 \cdot 10^5$; microwave frequency, 9.450 GHz. Pen travel rate (x-axis): curve 1, 1.0 min full scale; curves 2–4, 0.5 min full scale. Recorder time constant, curve 1, 0.3 s; curves 2–4, 0.1 s.

TABLE I

DEPENDENCE OF RATE OF FORMATION AND STEADY STATE CONCENTRATION OF TIRON SEMIQUINONE ON LIGHT INTENSITY

Whole chloroplasts (1.0 mg chlorophyll per ml) were suspended in 0.05 M sodium phosphate at pH 7 containing methylviologen, 12.5 μM, and Tiron, 12.5 mM. The sample was reaerated after each determination. Values given are averages of two duplicate runs.

Light intensity* (ergs · $cm^{-2} \cdot s^{-1}$ $\times 10^5$)	Maximum signal height** (arbitrary units)	Half time of reaction (s)
1.4	72	0.77
1.0	68	1.20
0.41	66	2.25
0.30	50	2.70
0.12	42	4.65
0.076	34	6.00
0.080	39	5.20
0.050	37	8.55

* Intensity at ESR cell face in cavity measured with Kettering radiometer (Yellow Springs Instrument Co.).

** ESR spectrometer parameters were as specified for the data of Fig. 3.

that which was previously reported for an FMN photocatalyzed system [2]. The addition of the autoxidizable mediator, methylviologen (14 μM), more than doubled the magnitude of the signal (curve 2, Fig. 2) but did not affect the rate of decay when the illumination was switched off. After incomplete decay of the semiquinone signal, the excitation could be repeated a number of times until dissolved oxygen in the sample was exhausted.

Inhibition of copper containing superoxide dismutases by pretreatment of chloroplasts with 7 mM KCN at pH 7 did not noticeably affect the rate of formation of $(TH)^{\cdot}$. However, as can be seen by comparing curves 2 and 3 of Fig. 2, this treatment did enhance the amount of $(TH)^{\cdot}$ formed by almost 20 %. The amount of KCN used was insufficient to inhibit electron transport through plastocyanin at pH 7 [15]. Aging of the chloroplasts in the dark at 25 °C for 18 h markedly decreased the rate of formation of $(TH)^{\cdot}$ on illumination to a $T_{\frac{1}{2}}$ of about 1.5 s, a value within the resolution capability of the system (Fig. 2, curve 4). The signal decay rate was unaffected by aging.

In view of the rapid rate (relative to the time resolution of the instrument) of $(TH)^{\cdot}$ formation on illumination of the Tiron and whole chloroplast mixtures in the presence of methylviologen, the reaction was retarded by decreasing the illuminating light intensity. Table I illustrates the effect of intensity on $T_{\frac{1}{2}}$ for the appearance of the semiquinone signal and on the magnitude of the steady state signal. Since the rectangular hyperbolic relationship between the velocity of the Hill reaction and the light intensity can be used to compute the maximum velocity of the rate limiting dark reaction [16], the $T_{\frac{1}{2}}$ was measured at eight intensities of light from $0.05 \cdot 10^5$ to $1.4 \cdot 10^5$ ergs · $cm^{-2} \cdot s^{-1}$ which spanned the initial portion of the hyperbola and the value of $T_{\frac{1}{2}}$ for the maximum velocity was computed as 0.56 s.

Kinetics of $(TH)^{\cdot}$ formation in thylakoids

Curve segments 1 and 2 of Fig. 3 show that in the thylakoid preparations there was a biphasic, light dependent production of the Tiron semiquinone. The signal did not attain a final level and remain constant but continued to increase in a second slow phase. If the illuminating lamp was left on and the signal height recorded on a much slower time scale (curve segment 3), the signal eventually disappeared completely due to exhaustion of O_2. Under anaerobic conditions, i.e. N_2 saturation of the sample, no appreciable amount (less than half Fig. 1, curve 3) of Tiron signal could be induced by light. Reaeration of the sample restored the signal to the original magnitude.

With thylakoid preparations, added ascorbate (0.35 mM) eliminated detectable

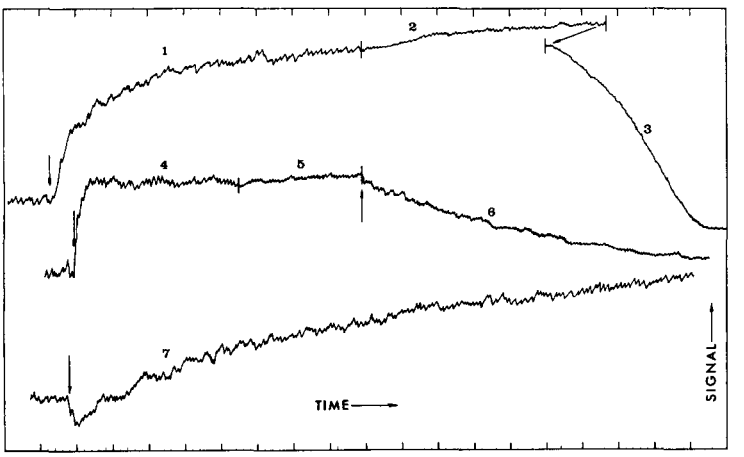

Fig. 3. Effects of preparation, light, oxygen concentration and superoxide dismutase on the rate of $(TH)^{\cdot}$ production. The conditions for recording the curve segments are summarized below.

Curve segment no.	Sample	Chlorophyll concentration (mg/ml)	Pen travel, full scale (min)	Time constant (s)
1	Thylakoids	0.82	0.5	0.1
2	Thylakoids	0.82	2.0	0.3
3	Thylakoids	0.82	30.0	1.0
4	Photosystem I particles	0.34	0.5	0.1
5	Photosystem I particles	0.34	4.0	1.0
6	Photosystem I particles	0.34	4.0	1.0
7	Photosystem I particles plus dismutase	0.34	0.5	0.1

All curve segments were recorded at 20 mW microwave power; 1.0 G, modulation amplitude and microwave frequency, 9.450 GHz; temperature, 25 °C. The illumination commenced at points indicated by the downward pointing vertical arrows and ceased at the upward pointing arrows. For curve segment 3, illumination remained on until oxygen was exhausted. The sample which was used for recording curve 7 was preincubated for 1 h at 25 °C with 200 μg of pure superoxide dismutase from bovine erythrocytes. Other samples contained only endogenous superoxide dismutase.

(TH)· formation and produced a monodehydroascorbate radical signal. Added cytochrome *c* (0.25 mM) did not compete with Tiron as a trap for $O_2^{\cdot-}$ contrary to its action in an $FMNH_2$-producing photochemical system [2]. L-Epinephrine (5 mM), completely obscured Tiron semiquinone formation.

Partial inhibition of (TH)· formation by superoxide dismutase in Photosystem I enriched particles

The rate of (TH)· appearance on illumination of Photosystem I enriched particles is illustrated by curve segment 4 of Fig. 3. The radical is formed rapidly ($T_{\frac{1}{2}} < 0.5$ s) without a slow phase as long as the chlorophyll *a*/*b* ratio is less than 5. In 0.5 % digitonin Photosystem I preparations the P_{700} signal formed in light but did not decay in the dark. Consequently Tiron oxidation could not be followed in these preparations.

When 0.4 % digitonin particles were preincubated for 1 h at room temperature with 200 μg of pure bovine superoxide dismutase, the $T_{\frac{1}{2}}$ of formation of (TH). increased to almost 15 s (curve segment 7, Fig. 3). This effect was not observed with whole chloroplasts or with Photosystem I particles incubated without superoxide dismutase. No change was observed in the rate of formation of the P_{700} signal due to this pretreatment. The exogenous dismutase apparently required appreciable time to approach the site of generation of $O_2^{\cdot-}$ which is the oxidant of Tiron. Endogenous superoxide dismutase activity was determined to be 50 and 20 enzyme units per mg of chlorophyll in preparations of whole chloroplasts and thylakoids, respectively. This activity may be compared to 1000 enzyme units per mg of purified bovine erythrocyte superoxide dismutase and to 590 units of added superoxide dismutase per mg chlorophyll in the preincubation experiments under the assay conditions which were used [14].

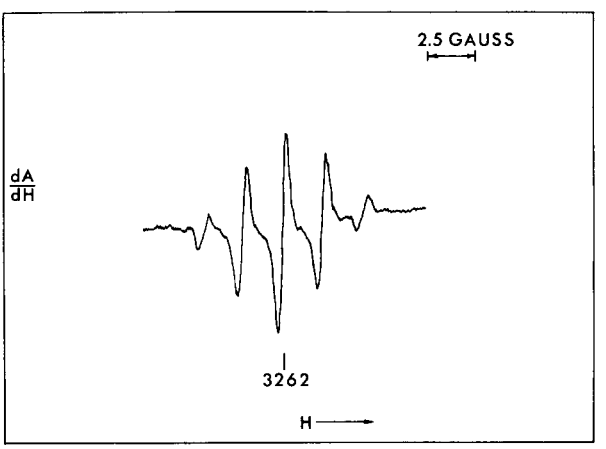

Fig. 4. ESR spectrum of benzosemiquinone. The sample contained chloroplast thylakoid preparation, 0.82 mg/ml chlorophyll *a*, suspended in 0.5 M sucrose. Benzoquinone, 4 mM, was added under anaerobic conditions and the sample was introduced in to the spectrometer in the dark. Initial spectra were recorded (not shown) and then the sample was illuminated and the spectrum was recorded as in the figure, and finally aerated and re-illuminated. Spectroscopic parameters were: temperature, 25 °C; spectrometer gain, $2.5 \cdot 10^5$; modulation amplitude, 1.0 G; time constant, 1.0 s; field scan rate, 16 min full scale; microwave power, 20 mW; microwave frequency, 9.450 GHz.

Reduction of quinones by illuminated thylakoids

Since quinones are reduced univalently by $O_2^{\cdot-}$, a comparison of this reaction with the oxidation of Tiron was made. Thylakoid membranes were treated anaerobically with benzoquinone or naphthoquinone disulfonate in the dark and illuminated. A small, dark semiquinone signal increased 4-fold on illumination. The steady state signal obtained with benzoquinone in the light is illustrated by Fig. 4. As the magnitude of this signal was not influenced by aeration, the added quinone was functioning as a Hill oxidant besides reacting with $O_2^{\cdot-}$ under aerobic conditions.

Effect of Tiron and benzoquinone on oxygen stoichiometry

The wheat thylakoid preparations were tested for endogenous Mehler reactivity as shown by Fig. 5, curve 1. In white light, O_2 was consumed at a rate of 10 μM per min while in the dark there was no net O_2 uptake. Tiron (3.3 mM) doubled the light-dependent rate of O_2 uptake (curve 2) due to its reduction of $O_2^{\cdot-}$ to H_2O_2 in opposition to the dismutation of $O_2^{\cdot-}$ which could yield 0.5 mol of O_2 per mol of $O_2^{\cdot-}$ formed. Benzoquinone (0.7 mM), as expected, acted as an electron acceptor from chloroplasts [4] and also oxidized superoxide anion to oxygen [1]. Hence in the presence of this reagent only oxygen evolution was observed (curve 3). The oxygen concentration increased at twice the rate of net oxygen consumption by the control reaction mixture because oxygen was being evolved as in a typical Hill reaction while the molecular oxygen that functioned as a Hill oxidant was being recycled. The relatively low concentration of benzoquinone did not support a stimulated Mehler reaction [4] subsequent to reduction of the quinone.

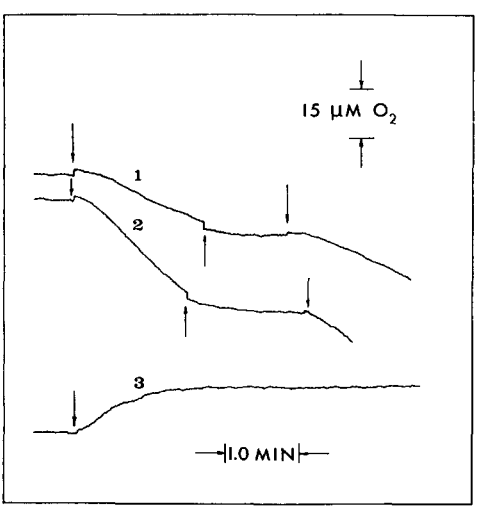

Fig. 5. Influence of Tiron or benzoquinone on oxygen consumption and evolution by thylakoid preparations. Chlorophyll concentration was 0.4 mg/ml in the 1.4 ml volume Clark electrode reaction chamber thermostated at 25 °C. Illumination with a 60 W white light was begun at points indicated by the vertical downward pointing arrows. Cessation of illumination is indicated by the upward pointing arrows. The reaction mixture for curve 1 contained 0.05 M potassium phosphate buffer at pH 7.0 and 0.5 M sucrose. For curve 2, Tiron, 4 mM, was added while for curve 3, benzoquinone, 4 mM, was added prior to illumination. All reaction mixtures contained ethanol, 200 mM, and bovine liver catalase, 2 mg/ml.

Catalase and ethanol were added to the reaction mixture for these experiments in order to completely remove hydrogen peroxide formed from the endogenous dismutation of O_2^- by Tiron.

DISCUSSION

An ascorbate-induced change in the stoichiometry of oxygen uptake in chloroplastic photoautoxidation has been previously reported [6, 7] to be due to the reduction of O_2^-. This chemical reaction competes with the dismutation of O_2^- by endogenous superoxide dismutase and hence prevents O_2 recycling. Ascorbate free radicals are formed and this process could be monitored by ESR spectroscopy. Other free radical traps might also be employed in this role. However, probe specificity of the reagent for reaction with O_2^- as well as stability of the product would be important in determining the usefulness of such a system for quantitative measurements of rates and amounts of O_2^- production.

Since protonated O_2^- is a weak acid, proton uptake from the suspending medium should occur during autoxidation of the System I photoreductant. Fowler and Kok measured this proton uptake with a glass electrode and found it to be a slow process in the absence of methylviologen. Addition of the dye increased the rate of H^+ uptake after flash excitation of spinach chloroplasts.

The reduction of oxygen by Photosystem I can be distinguished in 2 ways from the primary photoact in chloroplasts with the Tiron probe system. First, the difference in g values and hyperfine patterns of P_{700} and $(TH)^{\cdot}$ allow independent monitoring of the ESR signals. In addition, the rates of the reactions are vastly different. The saturating light-produced initial rate (half time) for Tiron oxidation in chloroplasts was estimated to be about 0.6 s, a value which reflects the fact that the formation of superoxide free radicals is far slower than either the photooxidation of P_{700} or the oxidation of Tiron by O_2^-. Hence the relatively slow rate of production of Tiron radicals monitors the rate of univalent oxygen reduction by Photosystem I and this rate can be time-resolved from the much faster photoact. Photoautoxidation and P_{700} kinetics could also be resolved in undamaged chloroplasts, for example, by using flash photolysis in conjunction with a suitably programmed ESR spectrometer.

In aged or damaged chloroplasts, or in the presence of superoxide dismutase, the slower rate of Tiron oxidation can be monitored directly at high light intensities. The preincubation of Photosystem I particles with relatively high concentrations of exogenous superoxide dismutase is required probably to bring the enzyme close to the topographical site of formation of superoxide within the catalytic particle structure. Apparently this site is normally effectively separated from the enzyme since neither endogenous nor exogenous superoxide dismutase affect the rate of production of O_2^- (as monitored with Tiron) in whole chloroplasts. Even large amounts of exogenous superoxide dismutase or cytochrome c did not completely eliminate O_2^- in our experiments.

On the other hand, L-epinephrine and L-ascorbate exhibited free accessibility to the site of O_2 reduction. Both of these chemical scavengers of O_2^- might have also reacted with $(TH)^{\cdot}$, reducing it to the catechol form and thus obscuring its formation.

The detection of small amounts of O_2^- in darkened, aerobic chloroplasts points to the high sensitivity of the Tiron method and the persistence of low concen-

trations of an autoxidizable species under these conditions. Even less Tiron signal was obtained under illumination in anaerobic conditions although the complete removal of O_2 is probably not possible in a system such as our thylakoid preparations. The persistence of a Tiron oxidizing species in darkened chloroplasts points to some form of bound superoxide anion which cannot freely diffuse and achieve dismutation in the reaction mixtures. The apparent separation of the site of $O_2^{\cdot-}$ formation from endogenous and exogenous superoxide dismutase supports this. Moreover, in Photosystem I particles exogenous superoxide dismutase requires considerable time at room temperature to approach this site closely enough to react with free superoxide which might be in equilibrium with the bound form.

A postulated process for univalent oxygen reduction by chloroplasts is shown in reactions I–VI. Q and TH^- represent benzoquinone and Tiron respectively, while X is the endogenous reductant (probably a reduced ferredoxin) which is formed on illumination of Photosystem I [18].

$$X^+ + P^* \rightarrow P_{700} + X \quad \text{I}$$

$$X + O_2 \rightleftharpoons [X^{\cdot-}O_2] \quad \text{II}$$

$$H^+ + TH^- + [X \cdot O_2] \rightarrow HO_2^- + (TH)^{\cdot} + X^+ \quad \text{III}$$

$$[X \cdot O_2] \rightleftharpoons O_2^{\cdot-} + X^+ \quad \text{IV}$$

$$H^+ + TH^- + O_2^{\cdot-} \rightarrow HO_2^{\cdot-} + (TH)^{\cdot} \quad \text{V}$$

$$H^+ + Q + O_2^{\cdot-} \rightarrow HO_2^{\cdot-} + (QH)^{\cdot} \quad \text{VI}$$

The formation of a bound superoxide complex, $[X \cdot O_2]$, in equilibrium with free $O_2^{\cdot-}$ could explain the behaviour of Photosystem I particles towards exogenous superoxide dismutase. The enzyme in high concentration might slowly diffuse to a position near the complex and then by reacting with free $O_2^{\cdot-}$, shift the equilibrium towards dissociation. Hence in aged or damaged material, but principally in thylakoids or smaller fragments, diffusion of superoxide dismutase to the complex site might occur more readily than in whole chloroplasts where added superoxide dismutase had no effect on Tiron oxidation. In thylakoid preparations, a portion of the particles may be damaged and may account for the slow phase (due to a superoxide dismutase accessible site) of Tiron oxidation which is superimposed on the rapid phase in the biphasic oxidation of Tiron illustrated in Fig. 3. Tiron, quinones, ascorbate and epinephrine apparently can react with $[X \cdot O_2]$ as well as with $O_2^{\cdot-}$.

The source of the electrons transferred to oxygen in our Photosystem I enriched particles was endogenous. The oxidation of Tiron by the P_{700} cation radical is not probable because of the O_2 requirement for $(TH)^{\cdot}$ formation and also because P_{700} remains oxidized in the presence of excess Tiron. The fact that the P_{700} cation radical signal of Photosystem I decays completely in 2 s indicates the presence of a functional system for recycling P_{700}. However, this result was only obtained in Photosystem I particles which retained a considerable amount of Photosystem II activity. Although Photosystem II components were depleted in the 0.4 % digitonin particles it is probable that water provides the electrons through residual Photosystem II activity. In whole chloroplasts and thylakoids it is certain that Photosystem II activity provides these reducing equivalents since DCMU inhibited and neither Tiron nor $(TH)^{\cdot}$ appear to rapidly reduce oxidized P_{700}.

ACKNOWLEDGEMENT

The authors wish to thank N. Huner and A. Martin for technical assistance in preparation of the manuscript.

REFERENCES

1 Rao, P. S. and Hayon, E. (1973) Biochem. Biophys. Res. Commun. 51, 468–473
2 Miller, R. W. and Rapp, U. (1973) J. Chem. 248, 6084–6090
3 Mehler, A. H. (1951) Arch. Biochem. Biophys. 33, 65–77
4 Mehler, A. H. (1951) Arch. Biochem. Biophys. 34, 339–351
5 Elstner, E. F. and Kramer, R. (1973) Biochem. Biophys. Acta 314, 340–353
6 Epel, B. L. and Newmann, J. (1973) Biochim. Biophys. Acta 325, 520–529
7 Allen, J. F. and Hall, D. O. (1973) Biochim. Biophys. Res. Commun. 52, 856–862
8 Lumsden, J. and Hall, D. O. (1974) Biochem. Biophys. Res. Commun. 58, 35–41
9 Witt, H. T., Müller, A. and Rumberg, B. (1963) Nature 197, 987–991
10 Ke, B., Hansen, R. E. and Beinert, H. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2941–2945
11 Macdowall, F. D. H. (1974) Can. J. Bot. 52, 151–157
12 Bruinsma, J. (1961) Biochim. Biophys. Acta 52, 576–578
13 Boardman, N. K. (1971) Methods in Enzymology XXIII (San Pietro, A., ed.), 268–276, Academic Press, New York
14 Rapp, U., Adams, W. C. and Miller, R. W. (1973) Can. J. Biochem. 51, 158–171
15 Ouitrakul, R. and Izawa, S. (1973) Biochim. Biophys. Acta 305, 105–118
16 Rieske, J. S., Lumry, R. and Spikes, J. D. (1959) Plant Physiol. 34, 293–300
17 Fowler, C. F. and Kok, B. (1974) Biochim. Biophys. Acta 357, 299–307
18 Malkin, R. and Bearden, A. J. (1971) Proc. Natl. Acad. Sci. U.S. 68, 16–19