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A CONVENIENT ELECTROCHEMICAL PREPARATION OF REDUCED METHYL VIOLOGEN AND A KINETIC STUDY OF THE REACTION WITH OXYGEN USING AN ANAEROBIC STOPPED-FLOW APPARATUS

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SUMMARY

1. Single reduced methyl viologen ($MV^{\cdot+}$) acts as an electron donor in a number of enzyme systems. The large changes in extinction coefficient upon oxidation (λ_{max} 600 nm; $MV^{\cdot+}$, $\varepsilon = 1.3 \cdot 10^4\ M^{-1} \cdot cm^{-1}$; oxidised form of methyl viologen (MV^{2+}), $\varepsilon = 0.0$) make it ideally suited to kinetic studies of electron transfer reactions using stopped-flow and standard spectrophotometric techniques.

2. A convenient electrochemical preparation of large amounts of $MV^{\cdot+}$ has been developed.

3. A commercial stopped-flow apparatus was modified in order to obtain a high degree of anaerobicity.

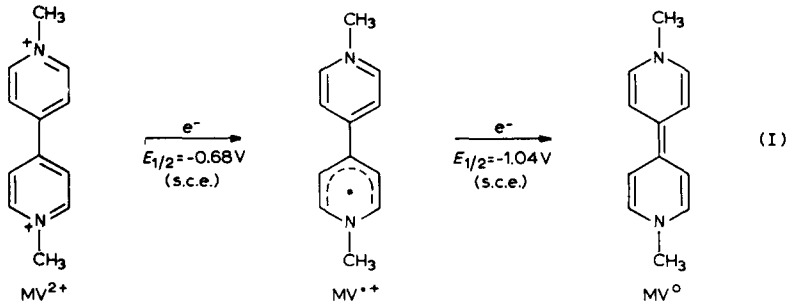
4. The reaction of $MV^{\cdot+}$ with O_2 produced H_2O_2 ($k > 5 \cdot 10^6\ M^{-1} \cdot s^{-1}$, pH 7.5, 25 °C). H_2O_2 subsequently reacted with excess $MV^{\cdot+}$ ($k = 2.3 \cdot 10^3\ M^{-1} \cdot s^{-1}$, pH 7.5, 25 °C) to produce water. The kinetics of this reaction were complex and have only been interpreted over a limited range of concentrations.

5. The results support the theory that the herbicidal action of methyl viologen (Paraquat, Gramoxone) is due to H_2O_2 (or radicals derived from H_2O_2) induced damage of plant cell membrane.

INTRODUCTION

Methyl viologen, 1,1'-dimethyl-4,4'-bipyridinium dichloride has frequently been used as an electron donor or acceptor in biological systems. Its first redox potential (Eqn 1) [1] is close to that of the electron transfer proteins, the flavodoxins and ferredoxins from various sources [2]. This potential and a structure capable of electron transfer to proteins enables methyl viologen to substitute for the natural electron donor–acceptor proteins with high efficiency in nitrogenase [3], nitrate reductase [4], and a number of other enzyme assays. In these systems methyl viologen has only

Abbreviations: MV^{2+}, $MV^{\cdot+}$, MV^0 are the oxidised, reduced and double reduced forms of methyl viologen, respectively.



been used to mediate electron transfer between two proteins, between dithionite ion and a protein or as a terminal electron acceptor. It has not previously been used as a primary source of electrons. Benzyl viologen, reduced at a palladized asbestos catalyst, has been used as a primary electron donor to nitrate reductase [5]. However, this method of reduction is non-specific and the time-dependent production of various levels of double reduced viologens is undesirable. This paper describes a convenient electrochemical method for the preparation of large volumes (approx. 100 ml) of single reduced methyl viologen ($MV^{\cdot+}$). The ability to prepare, handle and monitor spectrophotometrically $MV^{\cdot+}$ is potentially of great use in the study of biological electron transfer reactions. A number of anaerobic techniques are also described, including a modified commercially available stopped-flow apparatus. These have been developed in order to study the pre-steady-state kinetics of the nitrogenase from *Klebsiella pneumoniae*. The kinetics of the reaction of O_2 with $MV^{\cdot+}$ are also reported. This reaction was of interest for two reasons. Firstly, the complete removal of O_2 from protein solutions (without the use of added dithionite ion) is extremely difficult. Hence there is a risk that the reaction of O_2 with $MV^{\cdot+}$ could be attributed to a redox reaction involving a protein. Secondly, the herbicidal action of methyl viologen (Paraquat, Gramoxone) is thought to involve the reaction of $MV^{\cdot+}$ with O_2 to produce H_2O_2, which subsequently causes plant death by peroxide degradation of plant cell membranes [6].

MATERIALS AND METHODS

Methyl viologen, chloride salt (B.D.H. Chemicals Ltd, Poole, Dorset, Great Britain) was recrystallised three times from cold methanol by the addition of acetone (Found: C, 24.47; H, 6.04; N, 9.55; Cl, 24.47%. $C_{12}H_{14}N_2Cl_2 \cdot 2H_2O$ requires: C, 24.20; H, 6.14; N, 9.54; Cl, 24.2%).

Methyl viologen was reduced at a mercury cathode in a two compartment cell with a platinum foil anode and a calomel reference electrode (Electronic Instruments Ltd, Chertsey, Surrey, Great Britain). A saturated KCl salt bridge was used to connect the calomel electrode to the cathodic cell compartment. A controlled potential was provided by a potentiostat (Type A 1680, Southern Analytical Ltd, Camberley, Surrey, Great Britain). The cathodic compartment was kept anaerobic by flushing with Ar gas (Puragon, Air Products Ltd, Hythe, Southampton, Great Britain). A cathodic operating potential of −800 mV relative to the calomel electrode was used.

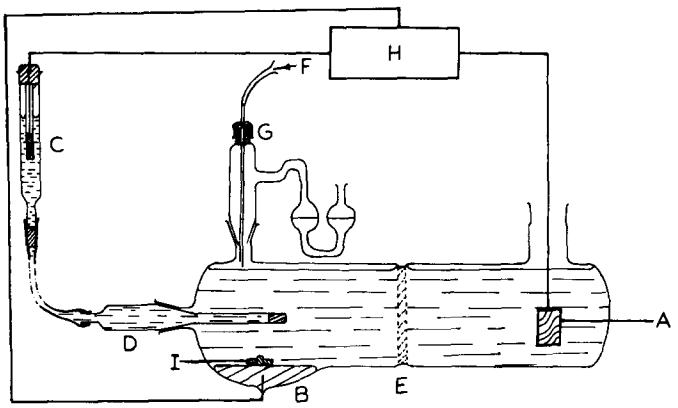


Fig. 1. Electrolysis cell for the preparation of $MV^{\cdot +}$. A, platinum foil anode; B, mercury pool cathode; C, calomel reference electrode; D, saturated KCl bridge; E, No. 4 glass sinter; F, Ar gas stream; G, Suba-Seal rubber closure; H, Potentiostat; I, magnetic stirrer.

Under these conditions it was calculated that 0.3% double reduced methyl viologen (MV°) was produced (Eqn 1). The reduction of MV^{2+} (120 ml, $2 \cdot 10^{-4}$ M, 0.025 M Tris–HCl buffer, pH 7.4, 0.1 M NaCl, 25 °C) was typically complete after 3 h during which time the cell current declined from 2.0 mA to less than 0.2 mA. The anodic compartment contained a buffer and salt medium identical to that in the cathodic compartment. The time for reduction depended to some extent (about 1 h) on the initial O_2 content of the MV^{2+} solution. The Ar pressure was kept as low as possible to minimise diffusion losses across the glass sinter, yet high enough to maintain anaerobicity in the cathodic compartment. No attempt was made to measure the diffusion losses or the oxidation losses due to diffusion of oxidizing species from the anodic compartment. However, since levels of reduction >80% were obtained without special precautions, these losses cannot have been very great. Samples of $MV^{\cdot +}$ were withdrawn using an all glass syringe and a stainless steel needle inserted through a Suba-Seal rubber closure (Griffin and George, Wembley, Middlesex, Great Britain). Plastic syringes were unsatisfactory due to O_2 absorbed on to the syringe walls. The transfer of $MV^{\cdot +}$ from an all glass syringe into a standard 1-cm path length spectrophotometric cell was achieved using a vacuum line on which the cell had been deoxygenated at reduced pressure (0.01 mm Hg) and then filled under an atmosphere of Ar. Spectrophotometric cells fitted with Suba-Seal rubber closures or teflon stoppers were unsatisfactory due to O_2 diffusion. Lightly greased ground glass stoppers produced no O_2 leakage over a period of hours.

An American Instrument Company stopped-flow apparatus incorporating a linear-log photometer and a Tektronix 549 oscilloscope was used in the kinetic studies. A number of modifications to the commercially available apparatus were necessary in order to obtain a high degree of anaerobicity. The combined observation and mixing chamber was fitted with a jacket made of black acrylic plastic, through which a solution of sodium dithionite (approx. 0.1 M) in Tris–HCl buffer (approx. 1.0 M) pH 7.4, was circulated via a closed thermostatted loop. The teflon tubes connecting the drive and stopping syringes to the mixing chamber were sheathed in vinyl tubing through which N_2 gas was flushed. The Kel-f drive syringe pistons were modified so that a

stream of N_2 gas flowed down a hole in the centre of the pistons and emerged from four holes (90° to each other) drilled just behind the rubber 'O' ring. This gas stream reduced both O_2 leakage past the 'O' ring and O_2 absorption on to the cylinder walls when the pistons were in the depressed position. When not in use, the apparatus was kept filled with sodium dithionite (0.1 M) in Tris–HCl buffer (1.0 M) at pH 7.4. With these modifications, the loss of $MV^{\cdot+}$ due to O_2 diffusion over a period of 30 min corresponded to an O_2 concentration of less than 0.5 μM. This was at least a 100-fold improvement on the unmodified apparatus.

Solutions were deoxygenated on a vacuum line and stored under Ar. Residual O_2 in these solutions as determined by the oxidation of $MV^{\cdot+}$ in the stopped-flow apparatus was approx. 1 μM. Full details of the procedures used for the deoxygenation of solutions and for anaerobic gel filtration of proteins will be given in a separate publication.

Tris(hydroxymethyl)aminomethane (Sigma (London) Chemical Co., Kingston-on-Thames, KT2 7BH, Surrey) and H_2O_2 (Aristar, 100 vol, B.D.H. Chemicals Ltd., Poole, Dorset) were used without further purification. H_2O_2 solutions were standardised by titration with $KMnO_4$. A rapid vacuum deoxygenation of a H_2O_2 (0.1 M) stock solution, followed by a large dilution (100–1000 times) into deoxygenated buffer gave solutions containing initially approx. 1 μM O_2. However, these solutions decomposed over a period of 15 min to give in the case of the highest H_2O_2 concentrations up to the equivalent of 10 μM O_2. These O_2 levels were calculated from the amplitude of the rapid phase of $MV^{\cdot+}$ oxidation (see Results) which was either absent or small for freshly diluted H_2O_2 solutions but which increased with time. The decomposition product was most likely O_2 since (a) H_2O_2 is known to decompose to O_2 and (b) the slow phase of the reaction of a large excess $MV^{\cdot+}$ with partially decomposed H_2O_2 solutions was identical with that of freshly prepared solutions. This indicated that the decomposition product was rapidly reduced to H_2O_2 in the first phase. Standard oxygen solutions were made up by the dilution of air saturated buffer ($[O_2] = 2.5 \cdot 10^{-4}$ M) [7] with deoxygenated buffer in a syringe with no gas space above the liquid. Spectra were recorded on a Unicam SP 1800 spectrophotometer fitted with a thermostatted cell holder. The extent of dimerisation and applicability of Beer 's Law for $MV^{\cdot+}$ were determined in 10- and 1-mm quartz cells fitted with Suba–Seal rubber closures. Standard MV^{2+} solutions were partially deoxygenated by bubbling Ar through them for 10 min while thermostatting in the cell holder. Sodium dithionite (0.1 ml, 0.1 M) was syringed in through the rubber closure. The absorbance at 600 nm was quickly measured. After 5 min approx. 5% bleaching was observed due to formation of MV°. A large excess of dithionite ion was employed in order to maintain O_2-free conditions.

RESULTS

Applicability of Beer's Law and the determination of the dimerisation constant

The observed extinction coefficients for $MV^{\cdot+}$ over a wide range of concentrations are given in Table I.

Beer's Law was obeyed under these conditions of ionic strength and solvent only up to approx. 10^{-4} M. If the deviation from Beer's Law is assumed to be due to a monomer–dimer equilibrium then

TABLE I

DEPENDENCE OF THE OBSERVED EXTINCTION COEFFICIENT AT 600 nm FOR $MV^{\cdot+}$ ON THE TOTAL CONCENTRATION OF $MV^{\cdot+}$ AND CALCULATED VALUES OF THE DIMERISATION CONSTANT IN 0.025 M Tris–HCl, pH 7.5, 0.1 M NaCl AT 25 °C

$[MV^{\cdot+}]$ (M) $\times 10^4$	ε_{obs} ($M^{-1} \cdot cm^{-1}$) $\times 10^{-4}$	K_D (M) $\times 10^3$
0.252	1.28	—
0.510	1.30	—
1.27	1.21	—
2.52	1.08	1.4
5.10	0.99	1.6
10.3	0.89	1.7
20.6	0.77	1.7

$$K_D = \frac{[MV^{\cdot+}]^2}{[MV_2^{2+}]} \tag{2}$$

where K_D is the dissociation constant of the dimer. The observed extinction coefficient ε is given by

$$\varepsilon = \varepsilon_m x + \tfrac{1}{2}\varepsilon_D (1-x) \tag{3}$$

where ε_m and ε_D are the molar extinctions of the monomer and dimer, respectively, and x is fraction of $MV^{\cdot+}$ in the monomeric form. K_D and x are related by

$$K_D = \frac{2Cx^2}{(1-x)} \tag{4}$$

where C is the total concentration of $MV^{\cdot+}$, i.e.

$$C = [MV^{\cdot+}] + 2[MV_2^{2+}] \tag{5}$$

ε_m and ε_D are obtained by extrapolating ε to $C = 0$ and ∞, respectively. A value of $\varepsilon_m = 1.30 \cdot 10^4\ M^{-1} \cdot cm^{-1}$, which was also that obtained by spectrophotometric titration of electrochemically produced $MV^{\cdot+}$ by $[Fe(CN)_6]^{3-}$, and a value for ε_D of $6 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ at 600 nm, gave $K_D = 1.6 \pm 0.2 \cdot 10^{-3}$ M in 0.1 M salt.

Stoichiometry and kinetics of the reaction of $MV^{\cdot+}$ with O_2

The stoichiometry of the reaction of $MV^{\cdot+}$ with O_2 was determined by measuring the absorbance change at 600 nm which occurred on mixing standard solutions in the stopped-flow apparatus. The concentrations used were the same as those given in Table II for the kinetic data. One mole of O_2 reacted with four moles of $MV^{\cdot+}$ in a biphasic manner. The first phase, in which two equivalents of $MV^{\cdot+}$ reacted, occurred within the mixing time of the stopped-flow apparatus (2 ms). This gives a lower limit for the second-order rate constant, $k > 5 \cdot 10^6\ M^{-1} \cdot s^{-1}$ for the reaction of $MV^{\cdot+}$ with O_2. The second phase of the reaction, in which a further two equivalents of $MV^{\cdot+}$ reacted occurred with half-lives in the range 0.5–2.0 s. The kinetics of this second slow phase were investigated over a range of concentrations of $MV^{\cdot+}$ and O_2 (Table II). Each rate constant is the mean of at least three determinations. The devia-

TABLE II

RATE CONSTANTS FOR THE SLOW PHASE OF THE REACTION OF $MV^{\cdot+}$ WITH OXYGEN

$[MV^{\cdot+}]$ is the concentration after completion of the rapid phase in 0.025 M Tris–HCl, 0.1 M NaCl at 25 °C.

pH	$[MV^{\cdot+}]$ (M) $\times 10^5$	$[O_2]$ (M) $\times 10^6$	k ($M^{-1} \cdot s^{-1}$) $\times 10^{-3}$
7.5	16.9	6.2	2.2
7.5	15.2	9.2	2.2
7.5	7.3	6.0	2.3
7.5	6.7	12.5	2.8
8.5	9.4	8.3	6.3
8.5	7.6	8.6	7.5
9.0	17.2	8.6	6.0
9.0	10.4	12.6	6.8
9.0*	9.6	11.7	7.5

* 0.1 M Tris–HCl, 0.1 M NaCl.

tions of the individual rate constants from the mean were within approx. 10%. The range of concentrations was limited by the desire to avoid high concentrations of $MV^{\cdot+}$ ($> 2 \cdot 10^{-4}$ M) at which appreciable concentrations of dimers are present. Additionally, because of the rapid phase, $MV^{\cdot+}$ had to be in at least a 2-fold excess over the O_2 concentration. Stopped-flow absorbance–time curves for the runs under pseudo-first-order conditions ($[MV^{\cdot+}]_0 : 2[O_2]_0 > 8:1$) were analysed by matching them with electronically generated exponential curves of known time constant, τ. Good fits to a single exponential were obtained and second-order rate constants were calculated from

$$k = \frac{1}{\tau[MV^{\cdot+}]_0} \tag{6}$$

For the runs under second-order conditions (Fig. 2) the integrated rate equation

$$kt = \frac{2.3}{[MV^{\cdot+}]_0 - 2[X]_0} \log \frac{2[X]_0}{[MV^{\cdot+}]_0} \frac{[MV^{+}]_t}{2[X]_t} \tag{7}$$

was used. $[MV^{\cdot+}]_0$ is the concentration of $MV^{\cdot+}$ at the beginning of the second phase of the reaction, $[X]_0$ is the corresponding concentration of the intermediate formed from O_2 in the first phase. X is almost certainly H_2O_2, since the amplitude of the rapid phase was consistent with a two electron reduction product of O_2 being formed. Additionally when O_2 was replaced by H_2O_2, no rapid phase was present and a single process was observed with kinetics identical to those recorded for the second phase of the reaction with O_2 (Table III). Second-order plots of log $[MV]_t/2[X]_t$ against time were linear to $> 80\%$ reaction and yielded second-order rate constants in agreement with those obtained under pseudo-first-order conditions (Table II). However, the reaction of $MV^{\cdot+}$ with H_2O_2 did not obey this rate law under all conditions. When an excess of H_2O_2 was used, anomalous stopped-flow traces were obtained (Fig. 2). An attempt was made to analyse these curves assuming two consecutive, one equivalent

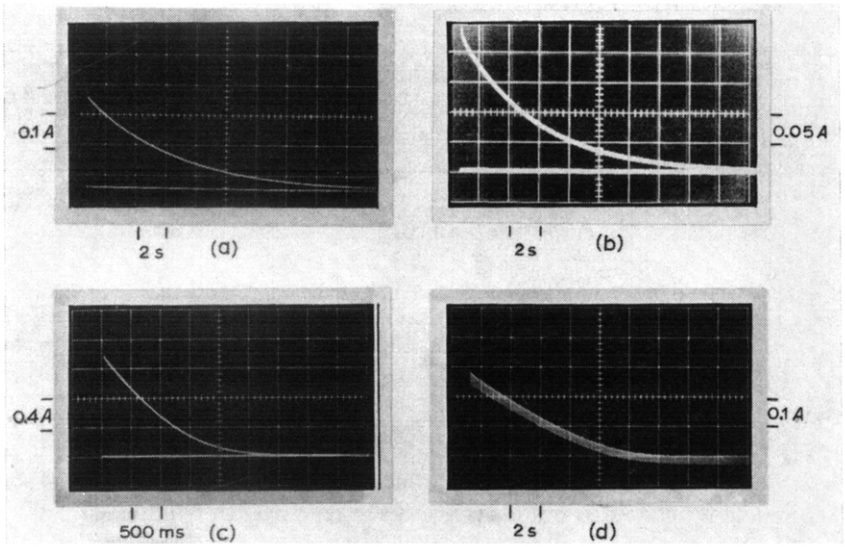


Fig. 2. Stopped-flow oscillograms for the second phase of the reaction of $MV^{\cdot+}$ with O_2 and for the reaction of $MV^{\cdot+}$ with H_2O_2 at 25 °C, 0.1 M NaCl, 0.025 M Tris–HCl. (a) $[MV^{\cdot+}]$, $6.7 \cdot 10^{-5}$ M; $[O_2]$ $1.25 \cdot 10^{-5}$ M (pH 7.5). (b) $[MV^{\cdot+}]$, $7.8 \cdot 10^{-5}$ M; $[H_2O_2]$, $9.6 \cdot 10^{-6}$ M (pH 7.5). (c) $[MV^{\cdot+}]$, $1.0 \cdot 10^{-4}$ M; $[H_2O_2]$, $4.8 \cdot 10^{-4}$ M (pH 7.5). (d) $[MV^{\cdot+}]$, $8.1 \cdot 10^{-5}$ M; $[O_2]$, $1.1 \cdot 10^{-5}$ M (pH 9.5).

electron transfers to H_2O_2. An analogue computer was programmed for a competitive-consecutive second-order reaction. The ratio of the two rate constants has varied from 1:10 to 10:1, with the initial concentrations of reactants as used in the stopped-flow experiments with excess peroxide. The simulated curves in no way resembled those obtained experimentally. Anomalous curves were also obtained for the reaction of O_2 with an excess of $MV^{\cdot+}$ at pH 9.5 (Fig. 2). No further analysis of these data was attempted. At pH 9.0, neither increasing the Tris–HCl concentration from 0.025 to 0.1 M nor increasing the incident light (slits changed from 0.2 or 2.0 mm) significantly affected the rate. $MV^{\cdot+}$ after reaction with O_2, was fully re-reduced by dithionite ion. However, oxidation of $1 \cdot 10^{-4}$ M $MV^{\cdot+}$ by $1 \cdot 10^{-3}$ M H_2O_2 caused a 15% loss in absorbance at 600 nm, after re-reduction with dithionite ion.

TABLE III

RATE CONSTANTS FOR THE REACTION OF $MV^{\cdot+}$ WITH H_2O_2 IN 0.025 M Tris–HCl, 0.1 M NaCl AT 25 °C

pH	$[MV^{\cdot+}]$ (M) $\times 10^5$	$[H_2O_2]$ (M) $\times 10^6$	k ($M^{-1} \cdot s^{-1}$) $\times 10^{-3}$
7.5	14.0	9.2	2.4
7.5	7.8	9.6	2.7

DISCUSSION

The molar extinction coefficient determined for $MV^{\cdot+}$ at 600 nm, $\varepsilon = 1.3 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$, is significantly higher than the average value of $1.18 \pm 0.02 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ determined by Trudinger [8]. However, no elemental analysis data were given by Trudinger and he did not allow for the two water molecules of crystallisation (Trudinger, P. A., personal communication). If a correction is applied to his data, a value of $1.34 \pm 0.02 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ is obtained.

The dimerisation constant, $K_D = 1.6 \pm 0.2 \cdot 10^{-3}$ M determined in a 0.1 M NaCl medium compares with a value of $2.6 \cdot 10^{-3}$ M obtained by Schwarz [9] in 1 M salt. Blandamer et al. [10] have used electron spin resonance to measure the relative concentrations of monomer and dimer and have detected higher order 'cluster' complexes at concentrations $> 10^{-3}$ M. No significant dependence of K_D on concentration was observed in this study.

Sweetzer [11] showed that at pH 10.0 O_2 is reduced by $MV^{\cdot+}$ to water, but at pH 6.5 only to H_2O_2. The stoichiometry determined in this study indicates that from pH 7.5–9.0 complete reduction to water occurs. Sweetzer's system contained EDTA, proflavin and phosphate buffer (necessary for the photoreduction of the MV^{2+}), and it is likely that one of these reagents competes successfully at pH 6.5, but unsuccessfully at pH 10 with $MV^{\cdot+}$ for H_2O_2. Oxidation products of EDTA must also have been present, since EDTA was used as the source of electrons in the photoreduction. These oxidative products could also have been competitors for H_2O_2. Bellin et al. [12] have reported that H_2O_2 is not produced as an intermediate in the air oxidation of $MV^{\cdot+}$. They argue that the oxidation of $MV^{\cdot+}$ by air is reversible [11] and that syringing H_2O_2 into photochemically produced $MV^{\cdot+}$ causes decomposition to a form that cannot be re-reduced. They then conclude that H_2O_2 is not produced in the air oxidation and therefore that the phytotoxicity of MV^{2+} is not due to H_2O_2-induced degradation of plant cell membranes [13]. In this study only a 15% loss of $MV^{\cdot+}$ was observed on re-reduction with dithionite ion after $MV^{\cdot+}$ had been oxidised with a large excess of H_2O_2. Bellin et al. [12] do not report the conditions under which they observed complete decomposition of $MV^{\cdot+}$ by H_2O_2. If they added H_2O_2 to their photoreducing system, it is possible that either the $MV^{\cdot+}$ was degraded by products of the reaction of H_2O_2 with the other components of the system (proflavin, EDTA, phosphate) or even that the photoreducing system and not the $MV^{\cdot+}$ was completely degraded. The slow reaction of $MV^{\cdot+}$ with H_2O_2 and the ability of the components

TABLE IV

RATE CONSTANTS FOR THE REACTION OF OXYGEN WITH ORGANIC-FREE RADICALS

Radical	Solvent	Temp. (°C)	Rate constant ($M^{-1} \cdot s^{-1}$)	Ref.
$(OH)C_6H_5^{\cdot}$	Water	23	$5 \cdot 10^8$	14
$C_5H_9^{\cdot}$	Cyclopentane	−40	$3.9 \cdot 10^6$	15
$C_6H_{11}^{\cdot}$	Cyclohexane	25	$4.3 \cdot 10^7$	15
$MV^{\cdot+}$	Water	25	$> 5 \cdot 10^6$	This work

of the photoreducing system to compete successfully at least at low pH, makes this explanation feasible.

The lower limit of $k > 5 \cdot 10^6\ M^{-1} \cdot s^{-1}$ for the reaction of O_2 with $MV^{\cdot +}$ is not surprising. A few absolute rates for the reaction of organic-free radicals with O_2 have been measured using the pulse radiolysis technique (Table IV). All the rates are above, or would be at room temperature, the lower limit obtained in this work. Kuwana and Winograd [16] have measured the rate of the electron exchange reaction between MV^{2+} and MV^0, $k > 3 \cdot 10^9\ M^{-1} \cdot s^{-1}$ using reflection spectroscopy at optically transparent electrodes. This rate is probably diffusion controlled. It is also likely that superoxide ion, O_2^-, reacts with $MV^{\cdot +}$ with $k > 5 \cdot 10^6\ M^{-1} \cdot s^{-1}$. However, the possibility that H_2O_2 is produced by disproportionation of O_2^- can not be discounted (Eqns 8, 9 and 10) [17].

$$HO_2^{\cdot} \rightleftharpoons H^+ + O_2^{\cdot} \quad pK_a = 4.8 \tag{8}$$

$$HO_2^{\cdot} + HO_2^{\cdot} \longrightarrow H_2O_2 + O_2 \quad k = 7.6 \cdot 10^5\ M^{-1} \cdot s^{-1} \tag{9}$$

$$HO_2^{\cdot} + O_2^{\cdot} \xrightarrow{H_2O} H_2O_2 + O_2 + OH^- \quad k = 8.5 \cdot 10^7\ M^{-1} \cdot s^{-1} \tag{10}$$

Although these reactions are too slow $k_{obs} = 1.4 \cdot 10^5\ M^{-1} \cdot s^{-1}$ at pH 7.6 (calculated from data in ref. 17), to account for the production of H_2O_2 within the mixing time of the stopped-flow apparatus, it is known to be greatly accelarated by trace impurities of metal ions. Since no special precautions were taken to avoid metal ion impurities (i.e. stainless steel syringe needles were used) this reaction could account for the rapid formation of H_2O_2.

The kinetics of the reaction of $MV^{\cdot +}$ with H_2O_2 were identical with those observed for the second phase of the reaction with O_2. This kinetic equivalence is good evidence for H_2O_2 production in the air oxidation of $MV^{\cdot +}$. The kinetics over a limited concentration range were first order in both $MV^{\cdot +}$ and H_2O_2. The linearity of these plots under second-order conditions indicates a slow-fast sequence for two successive, one equivalent electron transfer reactions, Eqns 11 and 12, where the intermediate X_2 is either identical with X_1 or rapidly formed from X_1.

$$MV^{\cdot +} + H_2O_2 \xrightarrow{slow} X_1 + MV^{2+} \tag{11}$$

$$MV^{\cdot +} + X_2 \xrightarrow[fast]{} 2\,H_2O + MV^{2+} \tag{12}$$

This mechanism is obviously a gross simplification since attempts to study the reaction with excess H_2O_2 or at high pH resulted in anomalous stopped-flow traces. This is not surprising since radical disproportionation, chain and competing redox and substitution reactions are well known features of the reactions of H_2O_2 with organic-free radicals. However, these kinetic studies do indicate the time range over which the reactions of $MV^{\cdot +}$ with O_2 and subsequently with H_2O_2 occur over the concentration and pH range likely to be encountered in experiments involving proteins with low levels of O_2 contamination.

The large differences in apparent rate constants for the reactions of O_2 ($k > 5 \cdot 10^6\ M^{-1} \cdot s^{-1}$) and H_2O_2 ($k = 2.2 \cdot 10^3\ M^{-1} \cdot s^{-1}$, pH 7.5) with $MV^{\cdot +}$ means that in the presence of excess O_2, H_2O_2 is the major product, with very little reduction to water. Davenport [18] reported the 'instantaneous' formation of H_2O_2 in illuminated chloroplasts containing MV^{2+}. This led to the suggestion that the herbicidal

activity of the viologens is due to H_2O_2 (or radicals derived from H_2O_2) induced degradation of the chloroplast membrane [13, 19]. Black and Myers [20], who proposed that methyl viologen destroys reduced pyridine nucleotides in all cells in the light or dark, have argued that there is sufficient catalase in chloroplasts to break down any H_2O_2 formed from the $MV^{\cdot+}$–O_2 reaction. This seems to be unlikely for two reasons. The high rate of reaction of O_2 with $MV^{\cdot+}$ ($k > 5 \cdot 10^6\ M^{-1} \cdot s^{-1}$, pH 7.5) is probably faster than the rate of reaction of H_2O_2 with catalase, $k_1 = 6 \cdot 10^6\ M^{-1} \cdot s^{-1}$, pH 7.0 [21]. Hence unless catalase is present in large excess over $MV^{\cdot+}$, the steady-state level of H_2O_2 will remain high. In addition Gregory [22] found low catalase activity associated with spinach chloroplasts. Most of the catalase is present in extra chloroplastic microbodies such as the peroxisomes and glyoxysomes [23, 24]. This supports the view of Dodge [19] that if H_2O_2 is broken down in chloroplasts, then it would be by peroxidase action.

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