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FREE RADICAL PRODUCTION FROM THE AEROBIC OXIDATION OF REDUCED PYRIDINE NUCLEOTIDES CATALYSED BY PHENAZINE DERIVATIVES

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Free radical production from the reaction of reduced pyridine nucleotides with phenazine derivatives in aerobic media at pH 7.5 has been studied by ESR spectroscopy and the ESR technique of spin trapping. With the spin trapping agent, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), the oxidation of NADH and NADPH catalysed by phenazine methosulphate, phenazine ethosulphate and 1-methoxyphenazine methosulphate gave exclusively the hydroxyl radical spin adduct of DMPO, 2-hydroxy-5,5-dimethylpyrrolidino-1-oxyl (DMPO-OH). DMPO-OH production was inhibited from these systems by catalase and sodium benzoate whereas superoxide dismutase gave a small increase in the rate of DMPO-OH production. NADH gives a higher rate of DMPO-OH production than NADPH with initial rates of DMPO-OH production in the order 1-methoxyphenazine methosulphate > phenazine ethosulphate > phenazine methosulphate. However, for an oxygen-limited system, the maximum DMPO-OH concentration attained varied in the order 1-methoxyphenazine methosulphate > phenazine methosulphate > phenazine ethosulphate. DMPO-OH production occurred in both the aerobic and anaerobic phases of the reaction with these phenazine derivatives. A similar system with pyocyanine gave DMPO-OH and the superoxide spin adduct of DMPO, 2-hydroperoxy-5,5-dimethylpyrrolidino-1-oxyl (DMPO-OOH). Addition of superoxide dismutase to this system stimulated the rate of DMPO-OH production and inhibited DMPO-OOH production. Addition of catalase and sodium benzoate decreased the production of DMPO-OH only. No DMPO-OH production was observed in the anaerobic phase of the reaction. The auto-oxidation of fully reduced phenazine methosulphate, 5,10-methylhydrophenazine methosulphate, produced the phenazine methosulphate radical cation $PMSH^{+\cdot}$ and DMPO-OH in the presence of DMPO. A mechanism for the auto-oxidation of reduced phenazine derivatives is proposed where superoxide production occurs in discrete steps in the auto-oxidation of fully reduced pyocyanine whereas for the auto-oxidation of fully reduced phenazine methosulphate, phenazine ethosulphate and 1-methyoxyphenazine methosulphate, the production of superoxide appears masked by the rapid further reduction to hydrogen peroxide.

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Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DMPO-OH, 2-hydroxy-5,5-dimethylpyrrolidino-1-oxyl; DMPO-OOH, 2-hydroperoxy-5,5-dimethylpyrrolidino-1-oxyl; DETAPAC, diethylenetriaminepentaacetic acid; PMSH, 5,10-methylhydrophenazine methosulphate (fully reduced phenazine methosulphate).

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Introduction

5-Methylphenazonium methosulphate, otherwise known as phenazine methosulphate, has been used in many dehydrogenase assays [1,2] as an electron carrier between the dehydrogenase and tetrazolium dyes. Phenazine methosulphate

oxidises NADH and NADPH; the cellular response to this is the stimulation of glycolysis and the hexose monophosphate shunt [2,3]. Phenazine methosulphate is cytotoxic [4], an effect which appears to be oxygen dependent.

The auto-oxidation of fully reduced phenazine methosulphate, 5,10-methylhydrophenazine methosulphate (PMSH), has been shown to produce hydrogen peroxide [6], superoxide free radicals [7,8] and is thought to involve the production of the phenazine methosulphate radical cation ($PMSH^+$) [5]. Using NADH to reduce phenazine methosulphate, an NADH/phenazine methosulphate/O_2 system has been shown to hydroxylate a variety of aromatic compounds [9,11]. Aromatic hydroxylation by this system gives the same isomeric mixture of hydroxybenzoic acids as observed for radiolytically generated hydroxyl radical-promoted hydroxylation [11].

The mechanism of the non-enzymatic oxidation of reduced pyridine nucleotides by phenazine derivatives has been investigated by following NADH and oxygen consumption, superoxide production, and the involvement of other paramagnetic reactive intermediates by ESR spectroscopy. Since phenazine methosulphate is known to be photochemically labile [12], the reactions of NADH/O_2 with phenazine ethosulphate, a slightly less photochemically labile phenazine methosulphate analogue [13], 1-methoxyphenazine methosulphate, a photochemically stable analogue of phenazine methosulphate [12], a pyocyanine, the major photochemical decomposition product from phenazine methosulphate [14], were also studied.

Materials and Methods

Phenazine derivatives. 5-Methylphenazonium methosulphate and 5-ethylphenazonium ethosulphate were purchased from Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset, U.K. 1-Methoxyphenazine was prepared by condensing oxidised 3-methoxycatechol with *o*-phenylenediamine [14]. The product was alkylated with dimethyl sulphate to yield 1-methoxy-5-methylphenazonium methosulphate. Pyocyanine was prepared by the photochemical oxidation of phenazine methosulphate in aqueous solution [14]. Freshly prepared solutions of phenazine methosulphate and phenazine ethosulphate, protected from the light, were used in all experiments.

Other reagents. The spin trapping agent DMPO was prepared and purified by the methods of Bonnett et al. [15]. Reduced pyridine nucleotides, NADH and NADPH, were supplied by Sigma. Bovine superoxide dismutase was a generous gift from Dr. J.V. Bannister (Inorganic Chemistry Laboratory, University of Oxford) and had an activity of 3300 U/mg. Catalase was supplied by Sigma as the thymol-free powder with an activity of 25 000 U/mg. Sodium benzoate was supplied by Sigma. The chelating ligands, DETAPAC and desferrioxamine mesylate, were supplied by Aldrich Chemical Co. Ltd. (Gillingham, Dorset, U.K.) and Ciba (Summit, NJ, U.S.A.).

ESR. ESR spectra were recorded using a Varian E104 X-band ESR spectrometer with a Varian E900-3 data acquisition system. The time course of spin adduct production was followed by setting an applied magnetic field for the top of a peak in the first-derivative ESR spectrum of the spin adduct and following the signal intensity with time. Spin adduct concentrations were computed from a calibrated double integral.

Oxygen consumption. The consumption of oxygen during the oxidation of NADH and NADPH in the presence of phenazine methosulphate and pyocyanine, respectively, was monitored using a Clark-type oxygen electrode (YSI 5331, Yellow Spring Instrument Co., OH, U.S.A.).

Anaerobic electrochemical reduction of phenazine methosulphate. Bulk reduction of phenazine methosulphate was performed in the dark using a

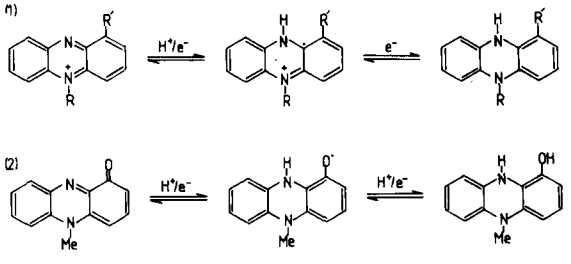

Fig. 1. Predominant redox forms of phenazonium derivatives in aqueous solution, pH 7.5. (1) Phenazine methosulphate: R = Me, R′ = H; phenazine ethosulphate: R = Et, R′ = H; 1-methoxyphenazine methosulphate: R = Me, R′ = OMe. (2) Pyocyanine.

glass cell of approx. 10 cm^3 volume incorporating a 10 cm^2 gold grid working electrode, a platinum gauze counter electrode behind a glass sinter and a saturated calomel electrode as reference, connected via a Luggin capillary. The aqueous electrolyte solution, containing 0.5 mM phenazine methosulphate (oxidised form), 100 mM KCl and 10 mM potassium phosphate, pH 7.5, was stirred magnetically with the working electrode poised at −300 mV (vs. saturated calomel electrode), under argon. The potential was applied with a PAR (Princeton, NJ, U.S.A.) Model 173 potentiostat/galvanostat, monitoring the amount of charge passed on a PAR Model 179 digital coulometer. The ESR spectrum of the phenazine methosulphate radical cation, $PMSH^+$, was recorded under argon from a sample after the passage of 1 $F \cdot mol^{-1}$.

Results

Phenazine methosulphate

In aerobic aqueous solution at pH 7.5, the reaction of 1 mM NADH with 50 μM phenazine methosulphate in the presence of 100 mM DMPO gave the ESR spectrum reported in Fig. 2a. This ESR spectrum is characterised by the parameters $g = 2.005$, $a_N = a_H = 14.9$ G and is identical to that previously attributed to the hydroxyl radical spin adduct of DMPO, [16]. The time course of DMPO-OH production (Fig. 2b, curve i) shows a gradual increase in DMPO-OH concentration over the initial 8 min of reaction time. A subsequent slow decrease in DMPO-OH concentration is observed. Monitoring the oxygen concentration in this system (Fig. 2b, curve ii), it can be seen that after 3 min of reaction time the system becomes almost anaerobic. Thus, DMPO-OH is being produced both in the aerobic and anaerobic phases of the reaction. NADPH gives a much slower rate of DMPO-OH production (Fig. 2b, curve iii). The slow decrease in DMPO-OH late in the reaction may be due to the slow reduction of DMPO-OH by reduced phenazine methosulphate when the concentrations of other suitable oxidants, e.g., oxygen and hydrogen peroxide, have decreased. Addition of sodium benzoate to the NADH/phenazine methosulphate/O_2 system in the presence of DMPO progressively decreased the

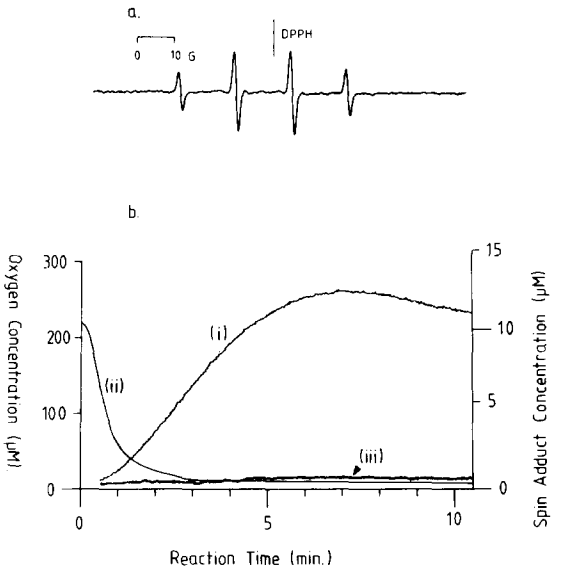

Fig. 2. Spin-trapped intermediates from the NADH/phenazine methosulphate/O_2 system. (a) ESR spectrum of reaction mixture: 1 mM NADH, 50 μM phenazine methosulphate, 1 mM DETAPAC, 100 mM DMPO in 50 mM potassium phosphate, pH 7.5: with spectrometer settings – field set 3385 G, field scan 100 G, modulation frequency 100 kHz, modulation amplitude 1.0 G, receiver gain $2.5 \cdot 10^3$, time constant 0.128 s, scan time 60 s, number of scans 4, microwave frequency 9.478 GHz, microwave power 10 mW. (b) Spin adduct production and oxygen consumption: (i) DMPO-OH production with NADH, (ii) oxygen concentration with NADH and (iii) DMPO-OH production with NADPH. Reaction mixtures are as for Fig. 1a except (iii) where NADPH was added in place of NADH. The spin adduct production curves begin at $t = 30$ s. DPPH, diphenylpicrylhydrazyl.

DMPO-OH production (Fig. 3, a–d); sodium benzoate, which is hydroxylated by the NADH/phenazine methosulphate/O_2 system [11], competes with DMPO for hydroxyl radicals and thereby decreases DMPO-OH formation.

The effect of superoxide dismutase and catalase on DMPO-OH production from the NADH/phenazine methosulphate/O_2 system is reported in Fig. 4. Production of DMPO-OH from 1 mM NADH, 50 μM phenazine methosulphate in aerobic 50 mM potassium phosphate, pH 7.5, with 100 mM DMPO, is shown in curve b. On addition of 0.1 mg/ml superoxide dismutase to this system, the rate of DMPO-OH production is slightly stimulated (curve a) whereas on addition of 250 and 500 U/ml catalase (curves c and d, respec-

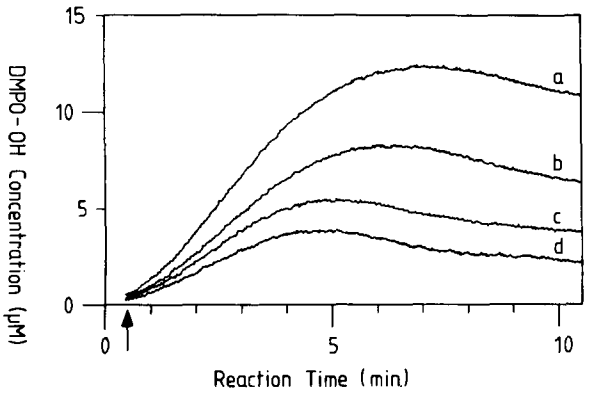

Fig. 3. The effect of sodium benzoate on DMPO-OH production from the NADH/phenazine methosulphate/O_2 system. Reaction mixtures contain: 1 mM NADH, 50 μM phenazine methosulphate, 1 mM DETAPAC and 100 mM DMPO in 50 mM potassium phosphate, pH 7.5, with (a) 0 (control), (b) 10 mM, (c) 20 mM, (d) 50 mM sodium benzoate. ↑ denotes the start of the spin adduct time course observation.

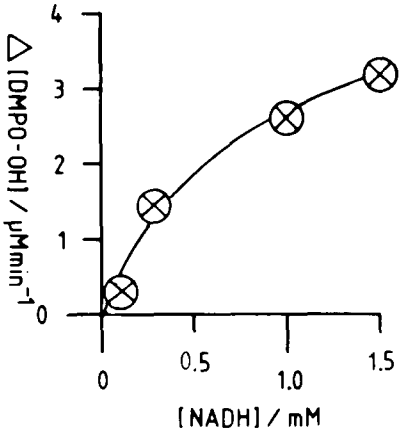

Fig. 5. The effect of NADH concentration on the initial rate of DMPO-OH production from the NADH/PMS/O_2 system. Reaction mixtures contained 50 μM phenazine methosulphate, 1 mM DETAPAC, 100 mM DMPO in 50 mM potassium phosphate, pH 7.5, with 1.5, 1.0, 0.27 and 0.1 mM NADH.

tively), the rate of DMPO-OH production is decreased. This suggests that the hydroxyl radical precursor is hydrogen peroxide and the reduction of hydrogen peroxide to hydroxyl radicals is not superoxide dependent.

The effect of varying the NADH and phenazine methosulphate concentrations on the initial rate of DMPO-OH production from the NADH/phenazine methosulphate/O_2 system is shown in Figs. 5 and 6, respectively. The initial rate of DMPO-OH production tends toward a maximum at both high concentrations of NADH and phenazine methosulphate.

An aliquot of electrochemically fully reduced phenazine methosulphate, PMSH, added to an

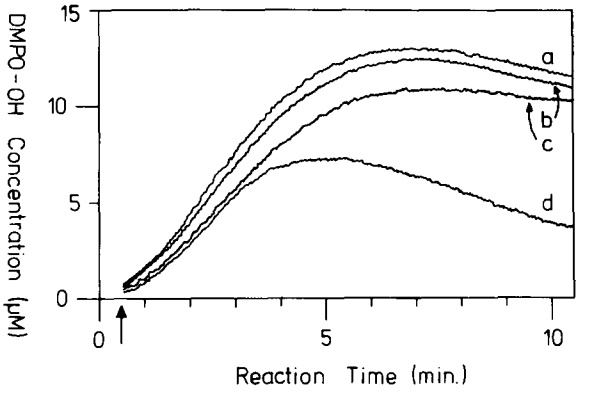

Fig. 4. The effect of superoxide dismutase and catalase on DMPO-OH production by the phenazine methosulphate/NADH/O_2 system. Reaction mixtures contain: 1 mM NADH, 50 μM phenazine methosulphate, 1 mM DETAPAC and 100 mM DMPO in 50 mM potassium phosphate, pH 7.5, with (a) +0.1 mg/ml superoxide dismutase, (b) no additions (control), (c) +250 U/ml catalase, (d) +500 U/ml catalase. ↑ denotes the start of the spin adduct time course observation.

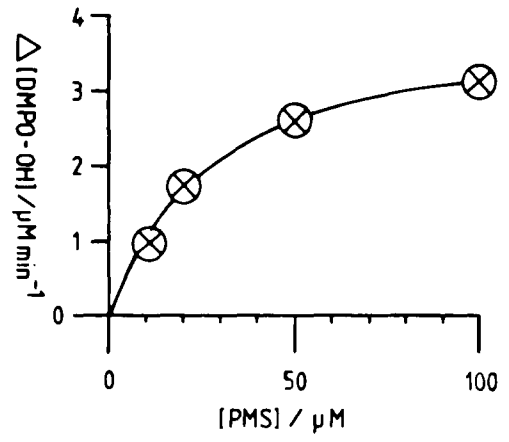

Fig. 6. The effect of phenazine methosulphate concentration on the initial rate of DMPO-OH production from the NADH/phenazine methosulphate/O_2 system. Reaction mixtures contain 1 mM NADH, 1 mM DETAPAC and 100 mM DMPO in 50 mM potassium phosphate, pH 7.5, with 100, 50, 20 and 10 μM phenazine methosulphate.

aerobic solution of 100 mM DMPO in 50 mM potassium phosphate, pH 7.5, gave the ESR spectra reported in Fig. 7a and c. The ESR spectrum in Fig. 7a is that recorded from $t = 30$ to 270 s reaction time and that in Fig. 7c was recorded from the same PMSH system from $t = 300$ to 540 s. Fig. 7a shows a component four-line spectrum with hyperfine intensity ratios of 1 : 2 : 2 : 1 and $a_N = a_H = 14.9$ G, characteristic of DMPO-OH, together with an underlying ESR spectrum with partly resolved hyperfine components centred at $g = 2.0034$. The underlying complex component is the same as that observed by the univalent reduction of phenazine methosulphate in aqueous solution (Fig. 7b), which has previously been assigned to the phenazine methosulphate radical cation, $PMSH^{+\cdot}$ [17]. The $PMSH^{+}$ component is transient in aerobic media and has become undetectable by the next 4 min scan (Fig. 7c). This demonstrates both hydroxyl radical and $PMSH^{+\cdot}$ production during the auto-oxidation of PMSH.

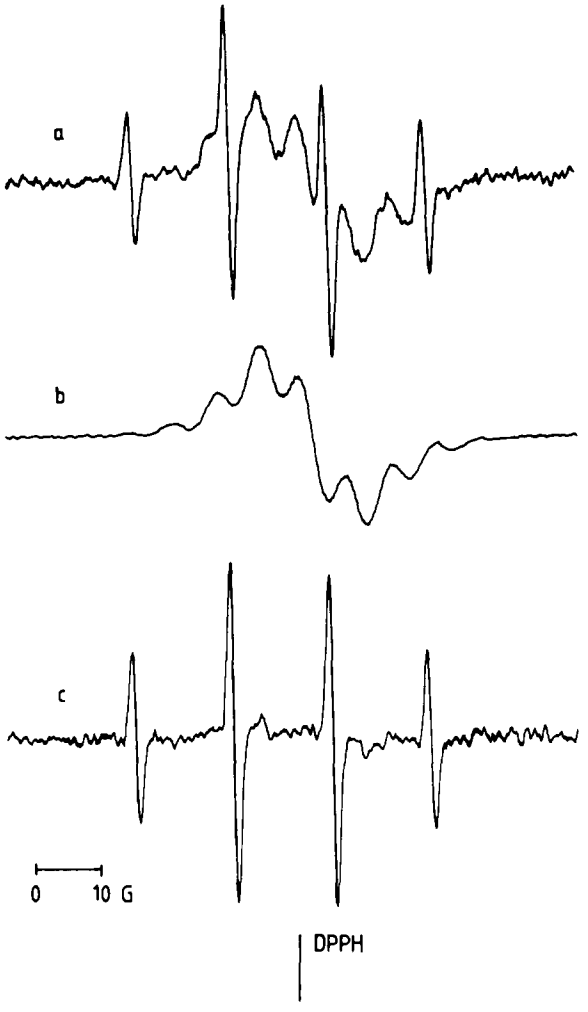

Fig. 7. Free radical production from the aerobic oxidation of PMSH. Reaction mixtures contain: (a) and (c) 0.1 mM PMSH, 100 mM DMPO and 1 mM DETAPAC in 50 mM potassium phosphate, pH 7.5. (b) 0.5 mM phenazine methosulphate after electrochemical reduction (1 $F \cdot mol^{-1}$) under argon, 100 mM KCl and 10 mM potassium phosphate, pH 7.5. Instrumental details as for Fig. 2 except receiver gain: (a and c), $2.5 \cdot 10^4$; (b), $1.25 \cdot 10^3$.

Phenazine ethosulphate and 1-methoxyphenazine methosulphate

Phenazine methosulphate and 1-methoxyphenazine methosulphate gave results similar to those of phenazine methosulphate in the $NADH/O_2$ system. Only DMPO-OH production was observed in the presence of DMPO, again indicating hydroxyl radical production. Inspection of the time courses of DMPO-OH production for these systems (Fig. 8) shows that the initial rates of DMPO-OH production are in the order 1-methoxyphenazine methosulphate > phenazine ethosulphate > phenazine methosulphate and DMPO-OH attains a maximum concentration (after all

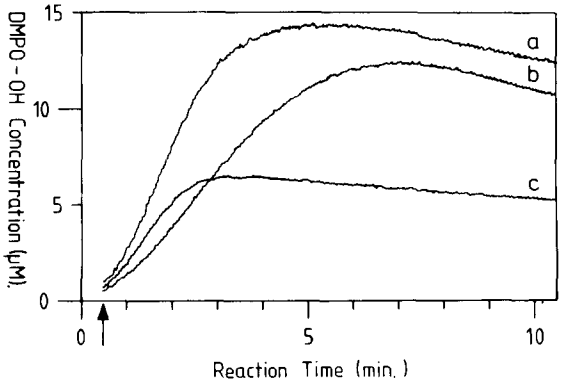

Fig. 8. DMPO-OH production from phenazine methosulphate, phenazine ethosulphate and 1-methoxyphenazine methosulphate with NADH in aqueous aerobic solution at pH 7.5. Reaction mixtures contain: 1 mM NADH, 100 mM DMPO and 1 mM DETAPAC in 50 mM potassium phosphate, pH 7.5, with (a) +50 μM 1-methoxyphenazine methosulphate, (b) +50 μM phenazine methosulphate, and (c) +50 μM phenazine ethosulphate. ↑ denotes the start of the spin adduct time course observation.

available oxygen and hydrogen peroxide has been reduced): the order of the maximum DMPO-OH concentrations is 1-methoxyphenazine methosulphate > phenazine methosulphate > phenazine ethosulphate.

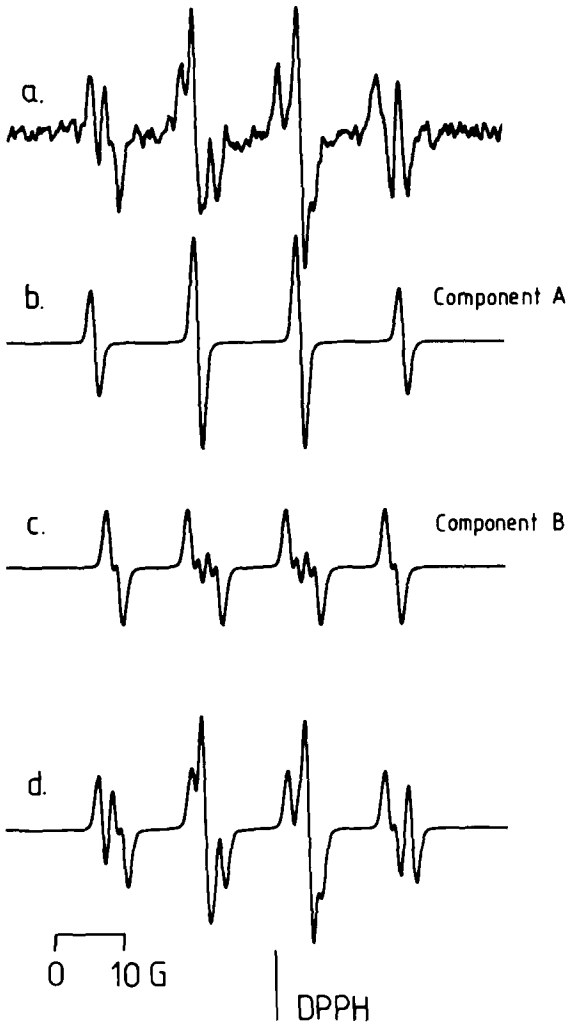

Fig. 9. Spin-trapped intermediates from the NADPH/pyocyanine/O_2 system. (a) Experimental spectrum – reaction mixture: 1 mM NADPH, 50 μM pyocyanine, 100 mM DMPO, 1 mM DETAPAC in 50 mM potassium phosphate, pH 7.5. Instrumental details as for Fig. 2 except receiver gain $1.25 \cdot 10^4$. (b) Simulation of DMPO-OH component: $g = 2.0050$, $a_N = a_H = 14.9$ G. (c) Simulation of DMPO-OOH component: $g = 2.0061$, $a_N = 14.3$ G, $a_H^{\beta} = 11.7$ G, $a_H^{\gamma} = 1.25$ G. (d) Addition spectrum of b + c.

Pyocyanine

Addition of 50 μM pyocyanine to 1 mM NADH in 50 mM aerated poatssium phosphate, pH 7.5, in the presence of 100 mM DMPO, gave the ESR spectrum reported in Fig. 9a. This ESR spectrum can be assigned to two spin adducts: component A (simulated in Fig. 9b), characterised by ESR parameters $g = 2.0050$, $a_N = a_H = 14.9$ G; component B (simulated in Fig. 9c), characterised by ESR spectral parameters $g = 2.0061$, $a_N = 14.3$ G, $a_H^{\beta} = 11.7$ G and $a_H^{\gamma} = 1.25$ G: simulations of components A and B are added together in Fig. 9d, which is in good agreement with the experimental spectrum (Fig. 9a). Component A has identical ESR parameters to those ascribed to DMPO-OH. Component B has ESR parameters identical to

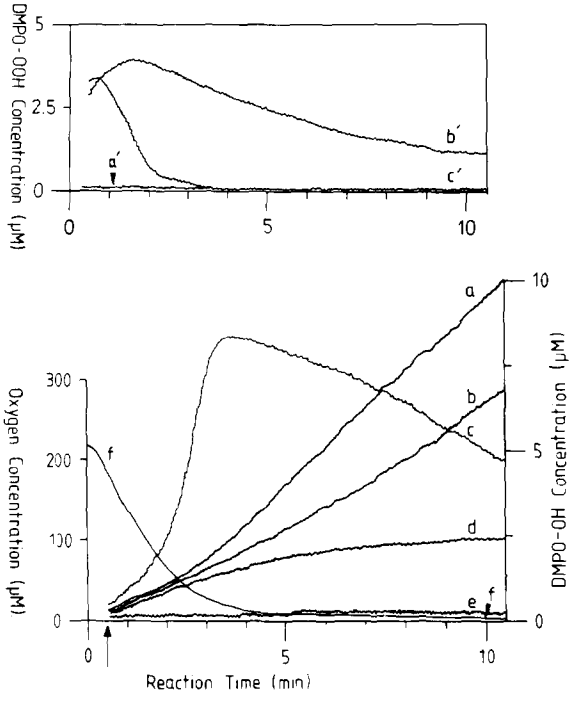

Fig. 10. Spin adduct production from the NAD(P)H/Pyocyanine/O_2 system. Reaction mixture all contain 100 mM DMPO, 1 mM DETAPAC and 50 μM pyocyanine in 50 mM potassium phosphate buffer, pH 7.5. DMPO-OOH production: (a')+1 mM NADH and 0.1 mg/ml superoxide dismutase, (b')+1 mM NADH and (c')+1 mM NADPH. DMPO-OH production: (a)+1 mM NADH and 0.1 mg/ml superoxide dismutase, (b) +1 mM NADH, (c) +1 mM NADPH, (d) +1 mM NADH and 20 mM sodium benzoate, (d) +1 mM NADH and 250 U/ml catalase. Oxygen concentration: (f)+1 mM NADPH. ↑ denotes the start of the spin adduct time course observation.

those assigned to the superoxide spin adduct of DMPO, DMPO-OOH [16]. The NADH/pyocyanine/O_2 system, in the presence of DMPO, produces detectable amounts of both the superoxide and hydroxyl radical spin adducts of DMPO (cf. the analogous phenazine methosulphate system). A time course of DMPO-OH and DMPO-OOH production with oxygen consumption data is given in Fig. 10: curve f shows oxygen concentration data, curve c DMPO-OH production and curve c' DMPO-OOH production. The NADPH/pyocyanine/O_2 system becomes anaerobic after approx. 4 min of reaction time. After this time, no more DMPO-OH is produced, i.e., in the pyocyanine system, there appears to be little reduction of hydrogen peroxide to hydroxyl radicals in the anaerobic phase of the reaction (cf. phenazine methosulphate system). Moreover, in the pyocyanine system, spin adduct production is faster with NADPH than with NADH (Fig. 10, curves c, c' and curves b, b'). Addition of 0.1 mg/ml superoxide dismutase to the NADH/pyocyanine/O_2 system inhibits the formation of DMPO-OOH and stimulates the production of DMPO-OH (curves a, a' and curves b, b'). Whereas with addition of catalase and sodium benzoate, the production of DMPO-OH is decreased only – curves d and e, respectively.

Discussion

The oxidation of reduced pyridine nucleotides catalysed by phenazine derivatives in aerobic media

As a mediator of electron transfer, phenazine methosulphate is usually regarded as a hydride acceptor [1,2]; it is implicit in the NADH/phenazine methosulphate/O_2 hydroxylating system that the auto-oxidation of PMSH, presumably formed from the reaction of phenazine methosulphate with NADH, generates the hydroxylating species. The observation that the auto-oxidation of electrochemically reduced phenazine methosulphate (to PMSH) generates the same hydroxylating species as the NADH/phenazine methosulphate/O_2 system, i.e., the hydroxyl radical, supports this.

The role of superoxide. The NADH/phenazine methosulphate/O_2 system and the auto-oxidation of electrochemically produced PMSH, in the presence of 100 mM DMPO, gave no detectable superoxide spin adduct (DMPO-OOH). In contrast to this, the NADH/pyocyanine/O_2 system gave a detectable superoxide spin adduct production. The relative kinetics of the two successive one-electron transfer steps (1 and 2 – see below) involved in the reduction of oxygen to hydrogen peroxide by fully reduced phenazine derivatives, and the decay of the transient free radical encounter $PMSH^{+\cdot}$-O_2^{-}, will describe the apparent role of superoxide in the auto-oxidation of fully reduced phenazine derivatives.

$$PMSH + O_2 \underset{r_{-1}}{\overset{r_1}{\rightleftharpoons}} (PMSH^{+\cdot} + O_2^{-\cdot}) \overset{H^+}{\underset{r_2}{\rightarrow}} PMS^+ + H_2O_2$$
$$\Big\Downarrow r_3$$
$$PMSH^{+\cdot} + O_2^{-\cdot}$$

The experimental observations are, a priori, dependent on the efficiency of the superoxide detection system, particularly with regard to the detection system competing with the spontaneous dismutation of superoxide. However, using 100 mM DMPO in both the phenazine methosulphate and pyocyanine systems, the efficiency of the detection system was identical and the rates of oxygen consumption in these systems were similar (Fig. 2 and 10). The detection of DMPO-OOH in the pyocyanine system and not in the phenazine methosulphate, phenazine ethosulphate and 1-methoxyphenazine methosulphate systems may rather reflect more superoxide escaping the transient free radical encounter in the pyocyanine system than with the other phenazine derivatives, i.e., r_3/r_2 (pyocyanine) > r_3/r_2 (phenazine methosulphate, phenazine ethosulphate, 1-methoxyphenazine ethosulphate). (From electrochemical measurements [18], phenazine methosulphate and 1-methoxyphenazine methosulphate are presumed to form free radical cations and pyocyanine a neutral free radical, by one-electron reduction of the oxidised forms at pH 7.5.)

The addition of superoxide dismutase to these systems enhances the rate of removal of superoxide (as the product of equilibrium 3) by dismutation. This will displace equilibria 1 and 3, reducing the effect of the back-reaction in equilibrium 1 (r_{-1}). This enhances the net rate of auto-oxidation of PMSH and dihydropyocyanine. This may account for the stimulation of DMPO-OH production in the NADH/phenazine methosulphate or pyocyanine/O_2 systems (the more marked stimu-

lation in the pyocyanine system may reflect the enhanced superoxide production – and hydrogen peroxide by dismutation – in the system).

The mechanism of hydroxyl radical production. Hydroxyl radical production from hydrogen peroxide during the auto-oxidation of PMSH requires one-electron transfer to hydrogen peroxide – the available reductants are PMSH and $PMSH^{+\cdot}$. For PMSH reduction of hydrogen peroxide, the resulting $PMSH^{+\cdot}$ radical cation may disproportionate to PMSH and PMS^+ (oxidised form). $PMSH^{+\cdot}$ was not detected in the NADH/phenazine methosulphate/O_2 system probably because, unlike the electrochemical reduction experiments, the concentration of PMSH is expected to be very low throughout (due to the high instability of PMSH to auto-oxidation).

A mechanism for the oxidation of reduced pyridine nucleotides in aerobic media catalysed by phenazine derivatives. A mechanism for the oxidation of NADH and NADPH by phenazine derivatives in aerobic media, consistent with the experimental observations, is given in Scheme I. Superoxide production in the pyocyanine system is considered as a discrete step whereas in the phenazine methosulphate, phenazine ethosulphate and 1-methoxyphenazine methosulphate systems, the rapid further reduction of superoxide to hydrogen peroxide by $PMSH^{+\cdot}$ is envisaged.

Implications for the use of phenazine derivatives in dehydrogenase assay systems

Early studies using phenazine methosulphate as an artificial electron carrier between respiratory chain enzyme systems and oxygen [1,19], using oxygen manometric measurements to determine the dehydrogenase activities, disclosed that dehydrogenase activities were often decayed by high concentrations of phenazine methosulphate. Such systems would inevitably involve the production of superoxide, hydrogen peroxide and hydroxyl radicals. In the light of current thought on oxygen free radical-mediated toxicity (see, for example, Ref. 20), it is now not surprising that there are serious limitations [13,20] to the use of phenazine methosulphate in dehydrogenase assay protocols.

Acknowledgements

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SCHEME I

A MECHANISM FOR THE OXIDATION OF NADH IN AEROBIC AQUEOUS MEDIA BY PHENAZINE METHOSULPHATE AT pH 7.5

dis., dissociation of species from solvent cage; M, solvent molecule; PMS, phenazine methosulphate; Py, pyocyanine.

(1) $NADH + PMS^+ \rightarrow NAD^+ + PMSH$

(2) $PMSH + O_2 \rightleftharpoons PMSH^+ + O_2^- \xrightarrow{H^+} PMS^+ + H_2O_2$

(3) $PMSH + H_2O_2 + H^+ \rightarrow (PMSH^{+\cdot} + {\cdot}OH) + H_2O$
$\xrightarrow{dis.} PMSH^{+\cdot} + {\cdot}OH$
$\xrightarrow{M} PMS^+ + H_2O$

(4) $2PMSH^{+\cdot} \rightarrow PMSH + PMS^+ + H^+$

(5) $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

For pyocyanine, reaction 2 is expressed in discrete steps:

(2a) $PyH_2 + O_2 \rightleftharpoons PyH{\cdot} + O_2^- + H^+$

(2b) $PyH{\cdot} + O_2 \rightleftharpoons Py + O_2^- + H^+$

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