PHENYL RADICAL PRODUCTION DURING THE OXIDATION OF PHENYLHYDRAZINE AND IN PHENYLPHYDRAZINE-INDUCED HAEMOLYSIS

H. A. O. HILL and P. J. THORNALLEY
Inorganic Chemistry Laboratory, South Parks Road, Oxford OX1 3QR, England

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1. Introduction

Phenylhydrazine is a well-known haemolytic agent in vivo and in vitro [1–3]. In both instances the formation of hydrogen peroxide has been demonstrated to be associated with the haemolytic action of phenylhydrazine [4,5]. It was proposed [6] that hydrogen peroxide is formed at concentrations which exceed the detoxification capacity of erythrocytes, and this, in part, leads to cytoxicity of phenylhydrazine. There is no doubt that several of the effects of phenylhydrazine are oxygen-dependent; the fate of the aromatic moiety is less clear.

Kinetic studies on the oxidation of phenylhydrazine in buffered aqueous solutions [7] have suggested a free-radical chain reaction. Initiation of the reaction can be by metal ions, e.g., by Cu(II), in which case EDTA acts as an inhibitor, or by oxyhaemoglobin, in which case chelating ligands have no effect. The chain carriers in the reaction are presumed to be the superoxide ion and phenylhydrazyl and phenyldiazenyl radicals. Evidence for a phenyldiazene intermediate has been given [8].

The report which follows provides conclusive proof of the formation of phenyl radicals during phenylhydrazine-induced haemolysis.

2. Materials and methods

2.1. *Preparation of erythrocytes*

Blood was drawn from normal human subjects by venous puncture using a 4% citrate solution as an anticoagulant. The plasma was separated after centrifugation at 3000 × *g* for 10 min and the erythrocytes were washed 3 times with 0.9% NaCl in 0.05 M phosphate buffer at pH 7.0. The final packed erythrocyte suspension was diluted with an equal volume of isotonic buffer to give a 50% stock solution and stored on ice.

2.2. *Spin trap*

The spin trap, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO, I) was prepared by the method in [9] and purified as in [10]. A high final concentration of spin trap, 100 mM, was used throughout to ensure efficient spin-trapping.

2.3. *Other reagents*

All other reagents were of standard laboratory grade except phenylhydrazine (Fisons Lab. Reagent) and phenylhydrazine-4-sulphonic acid hemihydrate (Fluka, Basle) which were both recrystallized twice, m.p., 20°C and 286°C, respectively) before use and subsequently stored at 0–4°C in the dark. The chelating ligand, diethylenetriaminepentaacetic acid (DETAPAC) was obtained from Aldrich, Gillingham Dorset. Haemoglobin was supplied by Sigma Chemicals, London. It was reduced with the minimum amount of sodium dithionite and solutions were flushed with oxygen to produce oxyhaemoglobin.

2.4. *Oxidation experiments*

The autoxidation of phenylhydrazine was studied: (a) Cu(II)-initiated aerobic oxidation; (b) oxyhaemoglobin-initiated aerobic oxidation; and (c) during haemolysis. Typical reaction mixtures contained: (a) 100 mM DMPO, 1 mM $PhNHNH_2$, 10 μM $CuCl_2$ in sodium borate/sodium hydroxide buffer, at pH 10.2; (b) 100 mM DMPO, 1 mM oxyhaemoglobin, 1 mM $PhNHNH_2$, 1 mM DETAPAC in 0.05 M phosphate buffer, at pH 7.0; (c) 100 mM DMPO, 1 mM $PhNHNH_2$,

1% erythrocyte suspension, 1 mM DETAPAC in phosphate buffer (pH 7.0).

2.5. *Electron paramagnetic resonance experiments*

EPR spectra of the nitroxide spin adducts were recorded using a Varian E104, X-band EPR spectrometer with a Varian E900-3 data acquisition system. The production of spin adducts in phenylhydrazine-treated erythrocytes was successfully monitored by locking-on to one of the peaks of the EPR spectrum of the spin adduct. It was possible to begin monitoring 1 min after the start of each experiment. The yields of radicals were determined by double-integration of the EPR spectra and compared to that of a standard solution of a nitroxide.

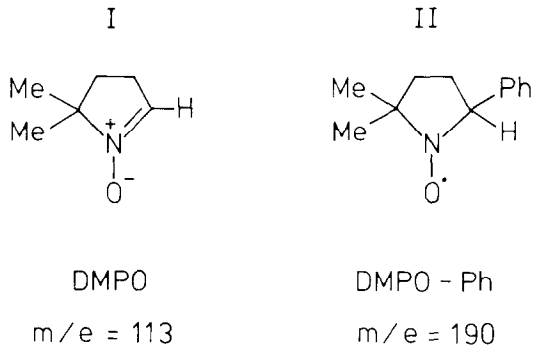
2.6. *Mass spectral analysis*

Spin adducts were extracted from the aqueous solutions with chloroform and eluted through a silica gel 60 column with methanol:chloroform (1:19). The eluent was monitored by EPR spectroscopy for spin adducts. The samples were sent for mass spectral analysis, after removal of the solvent. The mass spectra were recorded on a V.G. Micromass 12B (accelerating voltage 2.51 eV, electron energy 25 eV, 100 μA emission and with a source temperature of 240°C).

3. Results

3.1. *Cu(II) initiation of phenylhydrazine autoxidation*

The autoxidation of phenylhydrazine, in the presence of DMPO, produces a small amount of a nitroxide spin adduct. Autoxidation in the presence of Cu(II) ions greatly increases the yield of the spin adduct, such that 25% of the phenylhydrazine can be accounted for. The spin adduct gives a 6 line EPR spectrum (fig.1a), a triplet of doublets [11,12], with $g = 2.0045$, $a_N = 15.9$ G, $a_H = 24.8$ G, *pk–pk* width = 1.15 G, and a half-life of ~24 h. The relatively large a_N and a_H values are consistent with a carbon radical having been trapped. Since the EPR parameters are solvent-dependent [11] the phenylhydrazine-derived spin adduct was extracted into benzene; the EPR spectrum had the following hyperfine values: a_N = 13.8 G and a_H = 19.2 G, consistent with the literature values [11] for the phenyl radical spin adduct of DMPO, i.e., (5,5-dimethyl-2-phenyl-pyrrolidino-1-oxy), (II), DMPO-Ph.



Isolation and purification of the phenylhydrazine-derived spin adduct by silica gel chromatography produced a pure, concentrated sample of spin adduct suitable for mass spectrometric analysis. The sample gave a mass spectrum with molecular ion $m/e = 190$ and a fragment ion pattern consistent with the presence of a phenyl moiety in the parent molecule. All the properties of the phenylhydrazine-derived spin adduct cited above are consistent with a trapped phenyl radical (II).

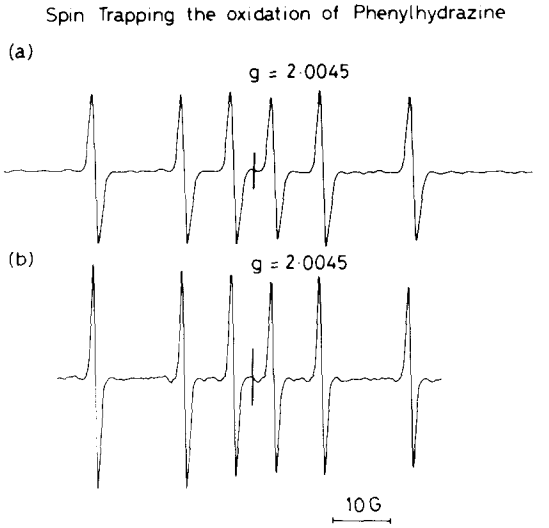


Fig.1. EPR spectra of radicals produced during: (a) Cu(II)-initiated aerobic autoxidation – 1 mM phenylhydrazine, 100 mM DMPO, 10 μM $CuCl_2$ in 50 mM borate/hydroxide buffer (pH 10.2); (b) haemolysis 1 mM phenylhydrazine, 1% erythrocyte suspension, 1 mM DETAPAC in isotonic buffer (pH 7.0). EPR spectrometer settings: field set, 3385 G; field scan, 200 G; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; reciever gain, 5×10^3; time constant, 0.128 s; scan time, 240 s; microwave frequency 9.278 GHz; microwave power, 10 mW; temp., 20°C.

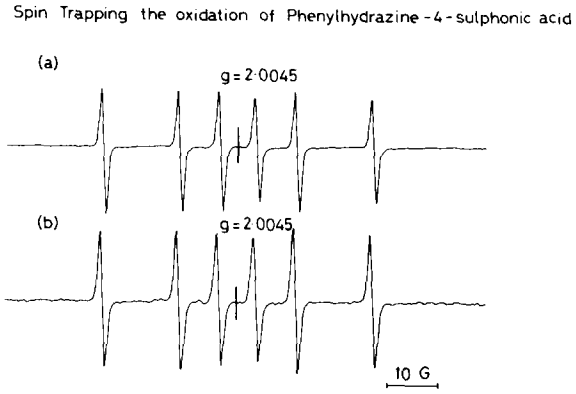


Fig.2. EPR spectra of radicals produced during: (a) Cu(II)-initiated aerobic autoxidation 1 mM phenylhydrazine-4-sulphonate, 100 mM DMPO, 10 μM $CuCl_2$ in 50 mM borate/hydroxide buffer (pH 10.2); (b) haemolysis 1 mM phenylhydrazine-4-sulphonate, 1% erythrocyte suspension, 100 mM DMPO, 1 mM DETAPAC in isotonic buffer (pH 7.0). EPR spectrometer settings: field set, 3385 G; field scan, 200 G; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; reciever gain, 5×10^3 for (a) and 5×10^4 for (b); time constant, 0.128 s; scan time, 240 s; microwave frequency, 9.478 GHz; microwave power, 10 mW; temp., 20°C.

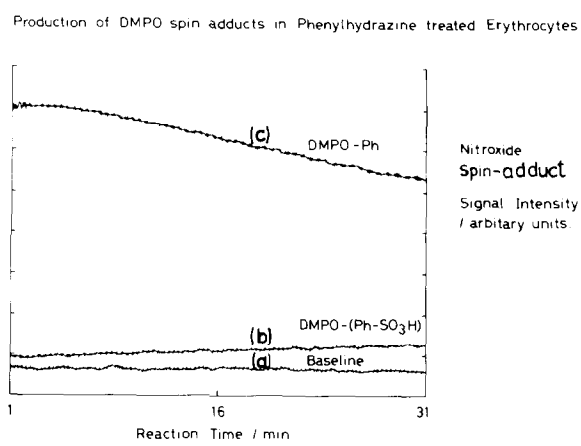


Fig.3. Intensities of EPR spectra as a function of time during aryl-hydrazine-induced haemolysis: (a) spectrometer baseline; (b) 1 mM phenylhydrazine-4-sulphonate, 1% erythrocyte suspension, 100 mM DMPO, 1 mM DETAPAC in isotonic buffer (pH 7.0); (c) 1 mM phenylhydrazine, 1% erythrocyte suspension, 100 mM DMPO, 1 mM DETAPAC in isotonic buffer (pH 7.0). EPR spectrometer settings: field set, 3354.18 G (low-field hyperfine peak); field scan, 0; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; reciever gain, 1×10^4; time constant, 4.0 s; scan time, 30 min; microwave frequency, 9.478 GHz; microwave power, 10 mW; temp., 20°C.

Phenylhydrazine-4-sulphonic acid gives similar results (fig.2a). The DMPO–$PhSO_3H$ spin adduct has EPR parameters: $g = 2.0045$, $a_N = 15.9$ G, $a_H = 24.8$ G, *pk–pk* width = 0.98 G, half-life ~24 h. The sulphonate group on the aromatic moiety has little effect on the autoxidation of the hydrazine function.

3.2. *Oxyhaemoglobin initiation of phenylhydrazine autoxidation*

It has been reported [7] that oxyhaemoglobin initiates the autoxidation of phenylhydrazine. Experiments identical to those described in section 3.1., except that oxyhaemoglobin replaced Cu(II) ions as the initiator of autoxidation, gave rise to the same results, i.e., the DMPO adducts of the phenyl and sulphonated phenyl radicals were trapped in approximately the same yields.

3.3. *The reaction of human erythrocytes with phenyl hydrazines*

The reaction of $PhNHNH_2$ with washed human erythrocytes in the presence of DMPO gives rise to a product with the following EPR parameters: $g = 2.0045$, $a_N = 15.8$ G, $a_H = 24.4$ G, *pk–pk* width = 1.96 G; the half-life depends on the particular erythrocyte preparation; values between 15 min and 12 h were found. By the time it was possible to begin monitoring the EPR signal, the intensity had already reached (fig.3b) a maximum; it then slowly decreases.

In contrast, phenylhydrazine-4-sulphonic acid-treated erythrocytes produced only small amounts of DMPO–$PhSO_3H$ (see fig.2b,3b), despite the almost identical amounts of spin adduct production from phenylhydrazine and phenylhydrazine-4-sulphonic acid under Cu(II)- and oxyhaemoglobin-initiated autoxidation.

4. Discussion

The haemolytic action of hydrazine derivatives has been the subject of much research [1–3,6,13] and interest has usually centred on the production of potentially toxic oxygen species, e.g., O_2^- and H_2O_2. In addition, Misra and Fridovich [7] stressed the importance of metal ion initiation and suggested the involvement of a phenyldiazene intermediate. Goldberg and Stern [8] studied the haemolytic properties of

phenyldiazene, benzenediazonium ion and benzene, finding only phenyldiazene to possess hemolytic activity. They concluded that the active hemolytic agent produced from phenylhydrazine in red cells is, a phenyldiazine-derived reactive intermediate produced by the rapid reaction of phenyldiazine with dioxygen [14]. These observations are summarised in the following mechanism for the autoxidation:

$$PhNHNH_2 + M^{n+} \rightarrow H^+ + PhNHNH^{\cdot} + M^{(n-1)+} \quad (a)$$

$$PhNHNH^{\cdot} + O_2 \rightarrow H^+ + O_2^- + PhN{=}NH \quad (b)$$

$$O_2^- + H^+ + PhNHNH_2 \rightarrow H_2O_2 + PhNHNH^{\cdot} \quad (c)$$

$$2\ PhNHNH^{\cdot} \rightarrow PhNHNH_2 + PhN{=}NH \quad (d)$$

$$PhN{=}NH + O_2 \rightarrow PhN{=}N^{\cdot} + HO_2^{\cdot} \quad (e)$$

$$PhN{=}N^{\cdot} \rightarrow Ph^{\cdot} + N_2 \quad (f)$$

$$Ph^{\cdot} + \text{Substrate-H} \rightarrow PhH + \text{Substrate} \quad (g)$$

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

Which of the postulated radical intermediates ($PhNHNH^{\cdot}$, O_2^-, $PhN{=}N^{\cdot}$, $Ph^{\cdot}$) is trapped will depend on the steady state concentration and the rate of reaction with DMPO. Thus whilst $PhN{=}N^{\cdot}$ is extremely short-lived and its steady state concentration is expected to be low, the rate of reaction of O_2^- with DMPO is known [15] to be slow. It is reasonable therefore that it should be the phenyl radical that is actually trapped.

No conclusive evidence for phenyl radical involvement in chain (e)–(g) in haemolysis has been cited although some evidence for a phenyl radical intermediate in chemical oxidation has been noted [16]. Our results provide conclusive proof for the generation of substantial quantities of phenyl radicals during both chemical oxidations and in the reactions which lead to haemolysis. Furthermore we interpret the low yield of radicals trapped in the reaction of the sulphonated phenylhydrazine with erythrocytes as indicating that the reactions take place intracellularly, the negative charge on the sulphonated derivative impeding transport across the cell wall.

Acknowledgements

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