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RADICAL FORMATION DURING AUTOXIDATION OF 4-DIMETHYLAMINOPHENOL AND SOME PROPERTIES OF THE REACTION PRODUCTS

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Abstract—4-Dimethylaminophenol (DMAP), after intravenous injection, rapidly forms ferrihaemoglobin and has been successfully used in the treatment of cyanide poisoning. Since DMAP produces many equivalents of ferrihaemoglobin, it was of interest to obtain further insight into this catalytic process. DMAP autoxidizes readily at pH regions above neutrality, a process which is markedly accelerated by oxyhaemoglobin. The resulting red-coloured product was identified as the 4-(*N*,*N*-dimethylamino) phenoxyl radical by EPR spectroscopy. The same radical was also produced by pulse radiolysis and oxidation with ferricyanide. The 4-(*N*,*N*-dimethylamino)phenoxyl radical is quite unstable and decays in a pseudo-first order reaction ($k = 0.4\ sec^{-1}$ at pH 8.5, 22°) with the formation of *p*-benzoquinone and dimethylamine. This observed decay rate is identical with the rate of hydrolysis of *N*,*N*-dimethylquinonimine. When a solution containing the phenoxyl radical was extracted with ether, half the stoichiometric amount of DMAP was recovered. Hence it is apparent that the phenoxyl radical decays by disproportionation yielding DMAP and *N*,*N*-dimethylquinonimine. The latter product then quickly hydrolyses. The equilibrium of this disproportionation reaction is far towards the radical side, and the pseudo-first order hydrolysis controls the radical decay rate. *p*-Benzoquinone rapidly reacts with DMAP ($k_2 = 2 \times 10^4\ M^{-1}\ sec^{-1}$) with the formation of the 4-(*N*,*N*-dimethylamino)phenoxyl and the semiquinone radicals. This reaction explains the autocatalytic phenoxyl radical formation during autoxidation of DMAP. DMAP is not oxidized by H_2O_2 or O_2^- but the 4-(*N*,*N*-dimethylamino)phenoxyl radical is very rapidly reduced by O_2^- ($k_2 = 2 \times 10^8\ M^{-1}\ sec^{-1}$). In addition, the phenoxyl radical is quickly reduced by NAD(P)H or GSH with the formation of $NAD(P)^+$ or GSSG. Since DMAP is also able to reduce two equivalents of ferrihaemoglobin (provided that the ferrohaemoglobin produced is trapped by carbon monoxide), electrophilic additon reactions of the phenoxyl radical seem unimportant in contrast to *N*,*N*-dimethylquinonimine. Hence, during the catalytic ferrihaemoglobin formation, DMAP is oxidized by oxygen which is activated by haemoglobin, and the phenoxyl radical oxidizes ferrohaemoglobin. This catalytic process is terminated by covalent binding of *N*,*N*-dimethylquinonimine to SH groups of haemoglobin (and GSH in red cells).

Since the pioneering studies of Michaelis [1], one-electron transfer reactions have attracted increasing attention in recent years, and it has become apparent that many xenobiotics are transformed into free radical intermediates. These radicals may be carbon-, nitrogen- or oxygen-centred and are derived even from popular drugs like nitrofurans, nitroimidazoles, acetamidophenol, estrogens and cytostatic anthraquinones (for review, see Trush *et al.* [2]).

Formation of semiquinones by oxidation of quinols or reduction of quinones is catalysed by a wide variety of enzyme systems (for review, see Mason [3]). As semiquinones are in general autoxidizable compounds, many of the toxic effects observed with these compounds have been attributed to redox cycling and reactive oxygen-reduction products (for review, see Kappus and Sies [4]). In addition, these radical intermediates may be toxic by themselves, and also carcinogenic semiquinone radicals from benzo[*a*]pyrene and anthanthrene have been reported, focusing the scope of chemical carcinogenesis on a new area [5].

The bulk of these investigations have been mainly performed with dihydroxyarenes and the corresponding quinones, less with the analogues phenylenediamines, and least with aminophenols. This latter class, including *N*-acylamines like acetamidophenol, is found in analgesic preparations and has been implicated in the occasionally observed hepatotoxic and nephrotoxic effects. Recently, evidence has been presented that acetamidophenol may be oxidized to the phenoxyl radical by prostaglandin synthetase, microsomes and H_2O_2/horseradish peroxidase [6–8]. The question remains open whether the phenoxyl radical or the *N*-acetylquinonimine is the ultimate electrophilic agent [9, 10].

In studying the reactions of aminophenols with oxyhaemoglobin, we were interested to see whether such one-electron reactions were also involved in both ferrihaemoglobin formation and covalent binding [11–14]. Early studies in Kiese's laboratory on the ferrihaemoglobin-forming activity of various aminophenols revealed that 4-(*N*,*N*-dimethylamino)-phenol (DMAP) was among the most active [15]. This ability to increase the ferrihaemoglobin content of blood has been used as a basis for the treatment of cyanide poisoning [16, 17]. DMAP, which is now marketed as a drug in the F.R.G., produces many

equivalents of ferrihaemoglobin, indicating a catalytic transfer of electrons from ferrohaemoglobin to oxygen. Moreover, DMAP is able to transfer electrons to photosystem I in isolated chloroplast lamellae [18] and to cytochrome *c*.

In red cells, this catalytic cycle is rapidly terminated by thioether formation of oxidized DMAP species with GSH [13] or SH groups of haemoglobin [14]. Such an electrophilic attack might also be responsible for the observed cytotoxicity of large doses of DMAP in the kidney [19–22] and liver. Moreover, toxic effects of DMAP have also been attributed to redox cycling [23, 24], because the electron flow from substrate H_2 to cytochrome oxidase is bypassed by DMAP and cytosolic cytochrome *c* resulting in the loss of respiratory control and energy coupling [25]. Hence it seemed of interest to study the mechanism of DMAP oxidation and further reactions of these intermediates.

For the one-electron oxidation of DMAP, fast-response pulse radiolysis was chosen in order to establish unequivocally the basic characteristics of the phenoxyl radical. For this purpose, all radiolysis products of water were converted ultimately to $(SCN)_2^-$ or formomethyl radicals which quantitatively produced the 4-(*N*,*N*-dimethylamino)phenoxyl radical [26]. To investigate the reactions of DMAP or its phenoxyl radical with the superoxide anion, the latter was also produced radiolytically in order to avoid erroneous results often observed with the xanthine/xanthine-oxidase system [27]. Among the various sources of superoxide radicals, radiolysis offers the advantage that only formate and oxygen have to be additionally present in the system. As long as the concentration of formate is more than two orders of magnitude greater than the other reactive intermediates, more than 98% of the primary radicals of water radiolysis are converted to superoxide [28].

For the studies on further ractions of the phenoxyl radicals, DMAP was either autoxidized or oxidized with equimolar potassium ferricyanide.

MATERIALS AND METHODS

4-(*N*,*N*-Dimethylamino)phenol hydrochloride and the radioactive compounds ([^{14}C-U]phenyl, sp. act. 5 mCi/mmole, and $^{14}CH_3$, sp. act. 1.2 mCi/mmole) were synthesized by Farbwerke Hoechst (Frankfurt, F.R.G.). Purified human haemoglobin, free from catalase, superoxide dismutase and glutathione peroxidase were prepared as described earlier [12]. All other reagents (purest grade commercially available) were purchased from Merck (Darmstadt, F.R.G.) and Boehringer (Mannheim, F.R.G.).

Most of the pulse radiolysis experiments were carried out with a Febetron 705 accelerator [29]. The optical detection system has been especially designed for high resolution kinetic spectroscopic investigations. Rate constants, reaction orders and characteristics were analysed and determined with a Wang 2200 computer. Superoxide and 'OH radicals were produced by short radiation pulses (5×10^{-8} sec) with 1.8 MeV electrons. When the 'OH radicals were of interest, the solutions were saturated with N_2O for hydrated electron conversion. Using nitrous oxide, 90% of all primary radicals of water radiolysis are finally present as 'OH [28]. Superoxide radicals were produced by the addition of high concentrations of formate (0.1–1 M) to oxygen-saturated solutions.

Some of the pulse radiolysis experiments were performed using 0.4–1 μsec pulses from a 3 MeV van de Graaff electron accelerator that delivered doses of *ca.* 2 Gy.

Steady-state radiolysis experiments were performed using a Siemens Dermopan X-ray machine at 50 kV and 25 mA. The EPR spectra were recorded with a Varian E-9 EPR-spectrometer (Palo Alto, CA) equipped with 100 kHz field modulation. Coupling constants and *g*-factors were determined using an NMR technique with simultaneous measurement of microwave frequency. The coupling constants and *g*-factors were accurate to 0.03 G and 5×10^{-5}, respectively.

Stop–flow measurements were performed with a DW-2 dual wavelength spectrophotometer equipped with an Aminco–Morrow stop–flow accessory and a data storage and retrieval system (Dasar, Aminco Silver Spring, MD). Electronic spectra were recorded with a Cary 219 spectrophotometer (Palo Alto, CA).

DMAP and *p*-benzoquinone were determined by isotope dilution technique, and dimethylamine after derivatization as already described [9]. GSH [30], GSSG [31], H_2O_2 [32], NAD^+ and $NADP^+$ [33] were determined enzymatically.

RESULTS

Formation of 4-(N,N-dimethylamino)phenoxyl radicals

Alkaline solutions of 4-dimethylaminophenol (DMAP) developed a transient bright red colour when exposed to oxygen. Repetitive scannings of a solution of 10^{-4} M DMAP in 0.2 M sodium borate, pH 8.5, equilibrated with air, indicated the formation of a short-lived transient with absorbance maxima at 495 and 315 nm (Fig. 1). The stable end-product, with a maximum in absorbance at 245 nm, was identified as *p*-benzoquinone (ether extraction, TLC, UV).

When [^{14}C]DMAP, labelled either in the ring or in the methyl groups (10^{-4} M each), was mixed with potassium ferricyanide (2×10^{-4} M) in 0.2 M sodium borate, pH 8.5, under nitrogen, the solution exhibited the same transient colour, which faded rapidly. After 5 min reaction, no DMAP was found in the ether extracts (TLC, UV). Instead, more than 90% of the radioactivity was found in *p*-benzoquinone (TLC, UV) and dimethylamine, respectively. From these results we presume that DMAP was oxidized to *N*,*N*-dimethylquinonimine, which hydrolysed rapidly giving rise to *p*-benzoquinone and dimethylamine [11].

The kinetics of the rapid transient formation from DMAP in the presence of potassium ferricyanide was studied in a DW-2 dual wavelength spectrophotometer equipped with a stop–flow accessory. Only at equimolar concentrations were maximal yields in the transient obtained. With surplus ferricyanide, the transient faded quickly. Assuming a molar extinction coefficient for the transient of 7×10^3 $M^{-1}cm^{-1}$ at

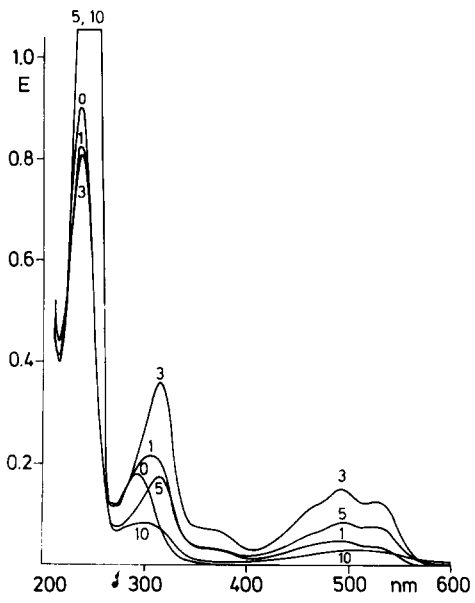

Fig. 1. Changes in the electronic spectra of DMAP solutions during autoxidation. DMAP (10^{-4} M) was dissolved in 0.2 M sodium borate, pH 8.5, equilibrated with air (22°). Repetitive scannings were started after different time intervals (min). Recordings were run in three sections from 600 to 500 nm, from 500 to 330 nm, and from 330 to 210 nm (10 nm/sec each).

495 nm (see below), a second order rate constant of 10^7 $M^{-1}sec^{-1}$ was obtained from initial rate measurements (pH 8.5, 10°). This order of kinetics indicated that the transient was probably a DMAP radical.

When DMAP (0.72 mM) was mixed with potassium ferricyanide (0.23 mM) at pH 9.1 and 37° immediately before the EPR flowcell, formation of a radical was observed, as shown in Fig. 2, upper panel. The *g*-factor measured for the spectrum was 2.00380 ± 0.00005. The hyperfine coupling constants were 7.58 G (for the nitrogen atom), 7.20 G (six equivalent protons, assigned to the methyl groups of Me_2N), and 2.13 G (four equivalent aromatic protons). An identical spectrum was obtained when DMAP (0.29 mM) was mixed with purified human oxyhaemoglobin (0.25 mM Fe) in 66 mM Na_2HPO_4, pH 9.2, at 25° (Fig. 2, middle panel). The experimental spectra can be perfectly simulated by computer, using the indicated coupling constants (Fig. 2, lower panel). The observed *g*-factor of 2.00380 ± 0.00005 is very similar to the value of 2.00377 ± 0.00005 which has been published for the 4-aminophenoxyl radical [34]. The coupling constants are also very similar. Hence, transient phenoxyl radical formation is proved to occur during DMAP oxidation.

To prove whether the observed red-coloured transient of DMAP was the phenoxyl radical, pulse radiolytically generated phenoxyl radicals were

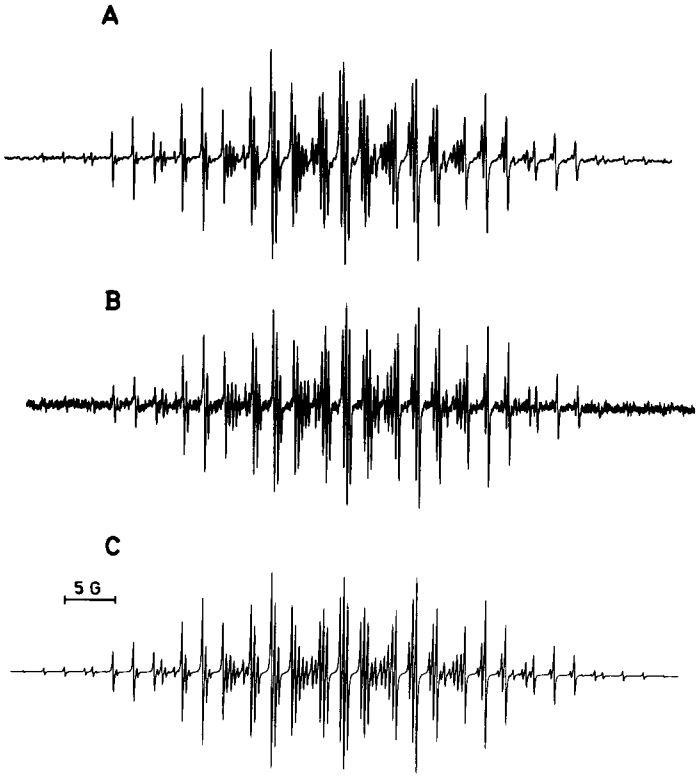

Fig. 2. Formation of 4-(*N*,*N*-dimethylamino)phenoxyl radicals during oxidation of 4-dimethylaminophenol. Upper panel A: DMAP (0.72 mM) was mixed with potassium ferricyanide (0.23 mM) at pH 9.1 and 37° immediately before the EPR flow-cell. Middle panel B: DMAP (0.29 mM) was mixed with purified human haemoglobin (0.25 mM Fe) in 1/15 M Na_2HPO_4, pH 9.2 at 25°. The yield of radical formation was 70% compared to the reaction with ferricyanide. Lower panel C: Computer-simulated EPR spectrum of 4-(*N*,*N*-dimethylamino)phenoxyl radicals using the following splitting constants: $a(H)_{CH_3}$ = 7.20 (6); $a(H)_{o \text{ and } m}$ = 2.13 G (4); $a(N)$ = 7.58 G (1); *g*-factor = 2.00380 ± 0.00005.

examined spectroscopically. A N_2O-saturated solution of DMAP (0.2 mM), pH 9, was irradiated by an electron pulse in the presence of sodium thiocyanate (10 mM) in order to produce quantitatively phenoxyl radicals [35]. The resulting electronic spectrum is presented in Fig. 3. The same spectrum was also obtained upon reaction with other oxidants, e.g. ferricyanide, Tl^{2+}, $^{\cdot}OH$, $SO_4^{\cdot-}$ and $^{\cdot}CH_2CHO$. These compounds generated identical EPR signals (S. Steenken, personal communication). Hence the coloured transient formed by oxidation of DMAP was proved to be the 4-(*N*,*N*-dimethylamino) phenoxyl radical.

Decay of 4-(N,N-*dimethylamino)phenoxyl radicals*

As anticipated, this radical proved to be unstable. An aqueous solution of DMAP (10^{-5} M) in 10^{-3} M sodium borate, pH 8.5, was irradiated with an electron pulse of dose 80.5 Gy in the presence of N_2O in order to convert the hydrated electrons to $^{\cdot}OH$ radicals ($G = 5.4$) [28]. The calculated concentration of $^{\cdot}OH$ formed was 4.5×10^{-5} M. Figure 4 shows the electronic spectra obtained at various intervals after the irradiation. Analysis of the decay kinetics of the phenoxyl radical absorbance at 495 nm revealed no clear reaction order. The assumption of a second order process would indicate a disproportionation reaction of two phenoxyl radicals, giving rise to parent DMAP and *N*,*N*-dimethylquinonimine. This assumption was confirmed by product analysis. When 0.1 mM DMAP was mixed with 0.1 mM ferricyanide, which produced 0.1 mM of the phenoxyl radical, immediate extraction with ether recovered 0.05 mM DMAP. Since the decay kinetics of the phenoxyl radical generated by $^{\cdot}OH$ gave no definitive second order kinetics, we re-examined the kinetics of the phenoxyl radical decay in the ferricyanide system. Table 1 represents the data of a set of experiments performed with equimolar concentrations of DMAP and ferricyanide in the absence of oxygen. Surprisingly, the obtained data indicate a first-order decay of the phenoxyl radical. These puzzling results will be discussed later.

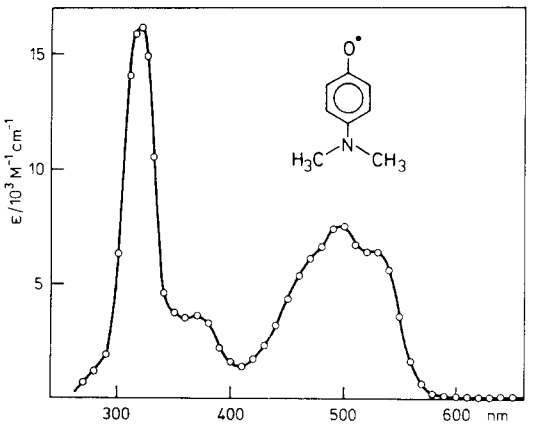

Fig. 3. Electronic spectrum of the 4-(*N*,*N*-dimethylamino)phenoxyl radical. The radical was generated by pulse radiolysis of a DMAP solution (0.2 mM) in the presence of N_2O and sodium thiocyanate (10 mM) at pH 9. The extinction was read 35 μsec after the irradiation pulse.

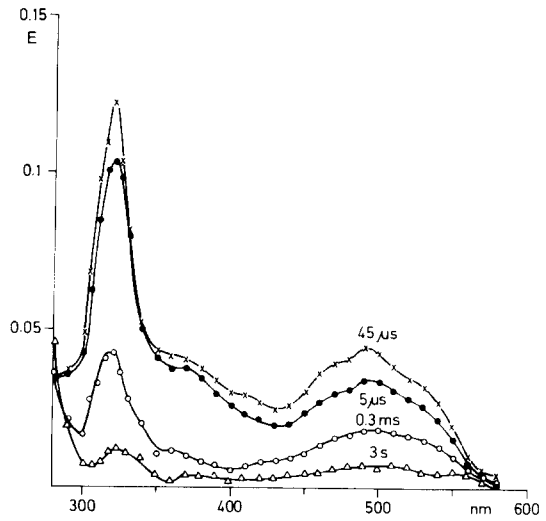

Fig. 4. Electronic spectra of 4-(*N*,*N*-dimethylamino)-phenoxyl radicals generated during pulse radiolysis. A solution of DMAP (10^{-5} M) in sodium borate (10^{-3} M, pH 8.5) was irradiated in the presence of N_2O by an 80 nsec electron pulse. The spectra were recorded at various times after the electron pulse.

Kinetics of DMAP autoxidation

When we examined the autoxidation kinetics of DMAP spectroscopically under air, we became aware of a lag phase in the radical formation, the kinetics of which seemed to proceed autocatalytically. This reaction was poorly reproducible and depended on cleaning the cuvette carefully before reuse. These observations pointed to reaction products catalysing radical formation. In fact, trace amounts of *p*-benzoquinone greatly stimulated the phenoxyl radical formation and shortened the lag phase. Figure 5 is a reproduction of an original test-chart of the autoxidation kinetics of a DMAP solution (0.1 mM, in 0.2 M sodium borate, pH 8.5, 22°, air) and the influence of additional 10^{-7} M *p*-benzoquinone. When DMAP reacted with *p*-benzoquinone in the absence of oxygen, formation of the phenoxyl radical was observed spectroscopically at 495 nm. The reaction was second order ($k_2 = 2 \times 10^4\ M^{-1}\ sec^{-1}$, pH 8.5, 10°). Thus the autocatalytic process of DMAP autoxidation might be sufficiently explained. It had to be proved, however, whether the reduced oxygen species were also involved in DMAP oxidation, since formation of $O_2^{\cdot-}$ and H_2O_2 seems to occur inevitably during autoxidation of DMAP.

As already published [18], the determination of superoxide radicals formed during one electron oxidation of DMAP was unsuccessful since DMAP interfered with all the available test systems. Rather, formation of hydrogen peroxide was observed during autoxidation of DMAP. During the initial stages of DMAP autoxidation, the yield of hydrogen peroxide equalled the amount of DMAP consumed (Fig. 6). Later on, hydrogen peroxide recovery was less than DMAP consumption. This was assumed to be indicative of a competition reaction between oxygen and hydrogen peroxide for DMAP oxidation. However,

Table 1. Decay kinetics of the 4-(*N*,*N*-dimethylamino)phenoxyl radical

DMAP (μM)	Fe^{3+} (μM)	Phenoxyl radical formed (μM)	Phenoxyl radical decayed (μM/sec)	k (sec^{-1})
100	100	99	45	0.45
50	50	48	22	0.46
20	20	19	7.7	0.40
10	10	9.7	4.2	0.43
5	5	5.1	1.9	0.37
2.5	2.5	2.5	1.0	0.40

DMAP reacted with stoichiometric amounts of potassium ferricyanide in 0.2 M sodium borate, pH 8.5, at 22° under an atmosphere of nitrogen. The initial rate of the decrease in absorbance at 495 nm was used to calculate the kinetic constants. All measurements were run in triplicate.

when hydrogen peroxide (1 mM) was incubated with DMAP (1 mM) in the absence of oxygen (0.2 M sodium borate, pH 8.5, 22°), no decrease in DMAP was observed within 10 min. Thus hydrogen peroxide reacted preferentially with breakdown products of DMAP and not with DMAP itself.

To prove whether superoxide radicals were able to oxidize DMAP, a solution of DMAP (5×10^{-5} M) in 0.5 mM sodium borate, pH 6.7, and 0.1 M sodium formate equilibrated with air was irradiated with 50 kV X-rays (56 Gy/min). At this dose rate, superoxide radicals were produced at a rate of 6×10^{-7} M sec^{-1}. No decrease in DMAP concentration was observed during 10 min irradiation. During this period, a total of 3.6×10^{-4} mole/l. of superoxide radicals was produced (during irradiation, the sample was purged with air). This experiment shows that superoxide radicals can be ruled out as primary oxidants of DMAP. On the contrary, superoxide radicals preferentially reduce 4-(*N*,*N*-dimethylamino)phenoxyl radicals as shown below.

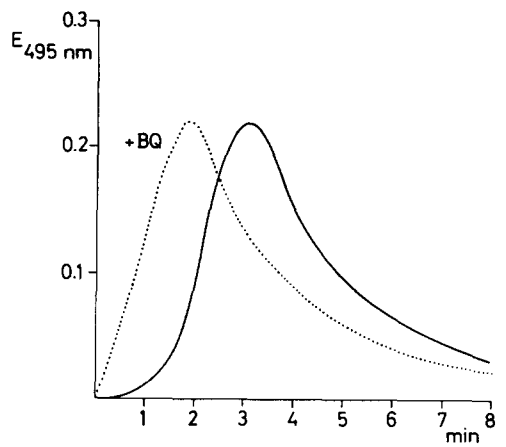

Fig. 5. Kinetics of autoxidation of 4-dimethylaminophenol and the influence of *p*-benzoquinone. A solution of DMAP (10^{-4} M) in sodium borate (0.2 M, pH 8.5) was followed spectroscopically at a wavelength of 495 nm at 22° under air. *p*-Benzoquinone (10^{-7} M) shortened the lag phase in 4-(*N*,*N*-dimethylamino)phenoxyl radical formation considerably.

Reactions of the 4-(N,N-*dimethylamino)phenoxyl radical with reducing agents*

Superoxide radicals were generated by pulse radiolysis of an aqueous solution containing 0.1 M sodium formate in order to convert all primary radicals to O_2^- [28]. Additional 0.5 mM sodium borate maintained the pH at 8.5. This buffer did not change the spontaneous superoxide dismutation rate ($k_2 = 1.3 \pm 0.1 \times 10^5$ M^{-1} sec^{-1}). The experimental conditions were set to generate a superoxide radical concentration of 3×10^{-5} M within 80 nsec. DMAP (5×10^{-5} M) in the above solution equilibrated with oxygen was allowed to autoxidize, the course of which was followed spectroscopically at 495 nm. In these experiments, no lag phase of the autoxidation kinetics was observed (cf. Fig. 5). Since the optical cuvette was automatically purged and refilled, some contaminating benzoquinone was obviously present. About 60 sec after injection of the DMAP stock solution kept under nitrogen, the phenoxyl radical concentration passed its maximum and then decayed gradually. At various time intervals, this solution was irradiated, which resulted in a nearly complete quenching of the phenoxyl radical absorbance (Fig. 7). Thereafter, the phenoxyl radical concentration increased again. The initial reaction rate was then markedly faster, probably due to the accumulated *p*-benzoquinone concentration (see above). Hence early irradiations caused no significant delay in the

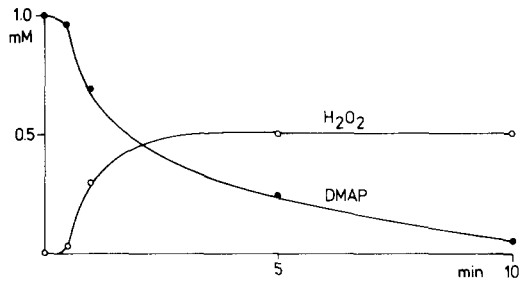

Fig. 6. Autoxidation of 4-dimethylaminophenol and formation of hydrogen peroxide. 4-Dimethylaminophenol (1 mM) was incubated in 0.2 M sodium borate, pH 8.5, at 22° under air. DMAP in ether extracts was determined spectroscopically; hydrogen peroxide was determined enzymatically (see Materials and Methods).

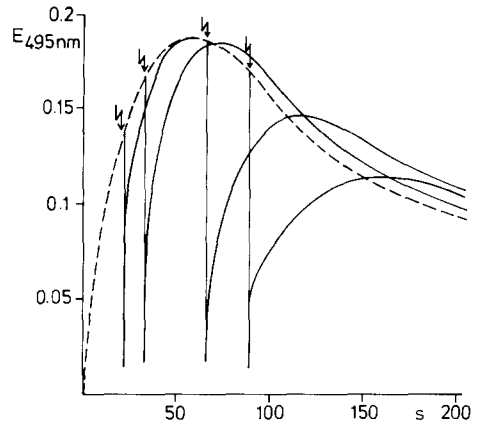

Fig. 7. Autoxidation of DMAP and the influence of pulse radiolytically generated superoxide radicals. A solution of DMAP (5×10^{-5} M) in 0.5 mM sodium borate, pH 8.5, and 0.1 M sodium formate under an atmosphere of oxygen was allowed to autoxidize. 4-(*N,N*-Dimethylamino)phenoxyl radical formation was detected at 495 nm. At the indicated intervals (flash) the solution was pulse-irradiated, resulting in the formation of 3×10^{-5} M superoxide radicals within 80 nsec. (Overlay of five different reaction curves; the dashed curve was obtained without irradiation.)

overall reaction and the envelope curves were similar to the curve obtained during undisturbed autoxidation (dashed curve, Fig. 7). Thus superoxide radicals apparently reduce the phenoxyl radicals back to the parent DMAP.

From the rate of the phenoxyl radical decrease, its concentration and the generated superoxide radical concentration, a second order rate constant of $1.9 \pm 0.3 \times 10^8\ M^{-1}\ sec^{-1}$ was calculated.

In addition to O_2^-, the phenoxyl radicals were also quenched by other reducing agents [ascorbic acid, hydroxylamine, NAD(P)H, and GSH]. DMAP (0.1 mM) was added to a solution containing NADPH (0.1 mM) in 0.2 M sodium borate, pH 8.5, equilibrated with oxygen, and the reaction was followed at 350 nm. After a short lag phase, the reduced nucleotide was re-oxidized within 15 min (Fig. 8).

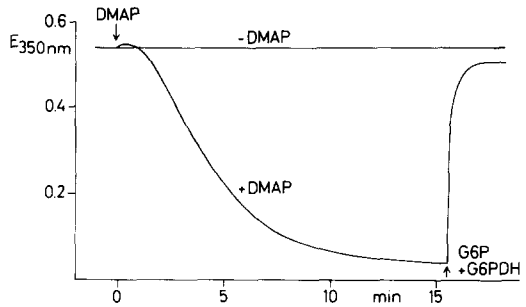

Fig. 8. Oxidation of NADPH in the presence of 4-dimethylaminophenol and oxygen. A solution of NADPH (10^{-4} M) in 0.2 M sodium borate, pH 8.5, was mixed with 4-dimethylaminophenol (10^{-4} M) and followed at 350 nm at 22° under air. In the absence of DMAP, NADPH was not oxidized significantly. After 15 min reaction in the presence of DMAP, almost all NADPH was oxidized and was re-reduced by the addition of glucose-6-phosphate and glucose-6-phosphate dehydrogenase.

By enzymatic reduction (glucose-6-phosphate + glucose-6-phosphate dehydrogenase), 0.095 mM NADPH was recovered. The same results were obtained with NADH (reduction of NAD^+ with ethanol + ADH). Whether the small loss in the nucleotides was due to electrophilic attack of the radical or the *N,N*-dimethylquinonimine yielding an adduct with the nucleotide awaits further investigation. When DMAP reacted with a 10-fold excess of NAD(P)H in oxygen-saturated solutions under the above conditions, all the reduced nucleotides disappeared, indicating a cyclic process in which DMAP transferred electrons from NAD(P)H to oxygen [36].

The kinetics of disappearance of the phenoxyl radical by GSH is shown in Fig. 9. In this experiment, DMAP was oxidized by a stoichiometric amount of ferricyanide. Addition of a stoichiometric amount of GSH resulted in the immediate disappearance of the radical. When GSH was premixed with DMAP, the addition of ferricyanide produced only small amounts of the phenoxyl radicals after a lag phase (Fig. 10). It should be noted that reduction of ferricyanide by GSH was insignificant under these conditions ($k_2 = 2 \times 10^2\ M^{-1} sec^{-1}$). Similar to these experiments, GSH apparently inhibited autoxidation of DMAP. When DMAP was incubated in 0.2 M phosphate, pH 7.4, under air, DMAP autoxidized at 37° with an apparent half-life of 45 min. When GSH (2.5 mM) was present, 0.6 mM DMAP did not decrease significantly within 3 hr. During that time, part of the GSH was oxidized, with the formation of GSSG (Fig. 11).

DISCUSSION

During the oxidation of 4-dimethylaminophenol, transient radical formation was observed. This radical was identified as the 4-(*N,N*-dimethylamino)-phenoxyl radical by EPR spectroscopy, and exhib-

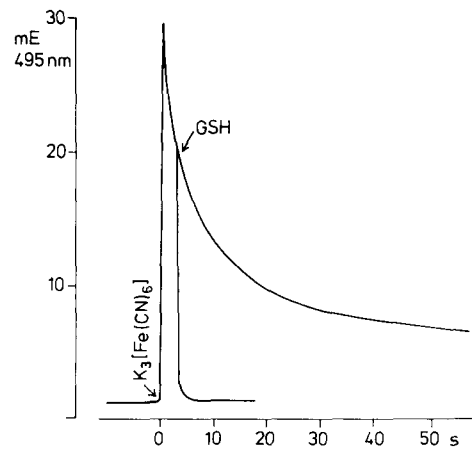

Fig. 9. Rapid disappearance of 4-(*N,N*-dimethylamino) phenoxyl radicals by the addition of GSH. 4-Dimethylaminophenol (5×10^{-6} M) was oxidized by potassium ferricyanide (5×10^{-6} M) in 0.2 M sodium borate, pH 8.5, at 22° under nitrogen. Three seconds after the addition of ferricyanide, GSH (5×10^{-6} M) was added, which resulted in the immediate disappearance of 4-(*N,N*-dimethylamino)phenoxyl radicals.

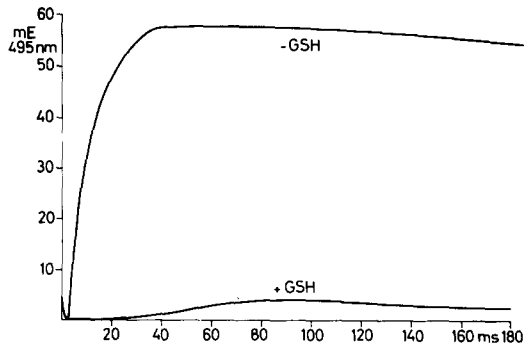

Fig. 10. Inhibition of 4-(*N*,*N*-dimethylamino)phenoxyl radical appearance by ferricyanide in the presence of GSH. 4-Dimethylaminophenol (10^{-5} M) in 0.2 M sodium borate, pH 8.5, was mixed with potassium ferricyanide (10^{-5} M), which resulted in the rapid appearance of the phenoxyl radical. Addition of GSH (10^{-5} M) to DMAP prior to mixing with ferricyanide almost completely inhibited radical formation. It should be noted that the ferricyanide reduction rate by GSH was only 10^{-4} that of DMAP oxidation.

ited strikingly similar characteristics to the spectrum of the *p*-aminophenoxyl radical [33]. The electronic spectrum of the 4-(*N*,*N*-dimethylamino)phenoxyl radical was markedly shifted to a higher wavelength as compared to DMAP and exceeded even the extent of shift seen with the hydroquinone–semiquinone couple [37]. The intensive absorbance around 500 nm allowed the convenient optical detection of the radical without interference by the parent DMAP and the stable end-product *p*-benzoquinone.

Product analysis after oxidation of DMAP revealed mainly *p*-benzoquinone and dimethylamine, as published earlier [11]. Such a product pattern points to hydrolysis of *N*,*N*-dimethylquinonimine as the most probable intermediate. Unfortunately, this compound has escaped exact detection so far because the steady-state concentration has been apparently too low. As reported previously [38], *N*,*N*-dimethylquinonimine has its UV absorbance maximum at 280 nm ($\log \varepsilon = 4.27$). The pulse radiolysis experiments shown in Fig. 4 indicate a relative increase in extinction at that wavelength during the radical decay. As is well known from other examples, such radicals tend to disproportionate. In fact, when a solution containing phenoxyl radicals was extracted immediately with ether, ethyl acetate or dichloromethane, half the stoichiometric amount was recovered as the parent DMAP. Obviously, this disproportionation giving rise to DMAP and *N*,*N*-dimethylquinonimine is in a rapid equilibrium greatly favouring radical formation.

Disturbance of this equilibrium, however, by rapid hydrolysis of *N*,*N*-dimethylquinonimine leads to the observed pseudo-first order kinetics of the radical decay, $k = 0.4\ \text{sec}^{-1}$ (cf. Table 1). From these data, a half-life of 1.5 sec was calculated for the phenoxyl radical (pH 8.5, 22°). This value agrees well with the reported rate of hydrolysis of *N*,*N*-dimethylquinonimine [38]. These authors reported $k = 0.6\ \text{sec}^{-1}$ under slightly different conditions (pH 7.4, phosphate, 25°). Hence the steady-state concentration of *N*,*N*-dimethylquinonimine can be predicted to be low.

From these data the following reaction scheme is derived:

$$\text{DMAP} - 1e \rightarrow \text{DMAP}^{\cdot} \quad (1)$$

$$2\,\text{DMAP}^{\cdot} \rightleftharpoons \text{DMAP} + N,N\text{-dimethylquinonimine} \quad (2)$$

$$N,N\text{-Dimethylquinonimine} + H_2O \rightarrow p\text{-benzoquinone} + \text{dimethylamine} \quad (3)$$

During autoxidation of DMAP, the electron is transferred to oxygen. Superoxide radicals were not detected hitherto in the presence of DMAP and phenoxyl radicals, both of which reacted with the various detection systems for superoxide radicals [18]. Moreover, $O_2^{\cdot-}$ very rapidly reduces 4-(*N*,*N*-dimethylamino)phenoxyl radicals back to DMAP

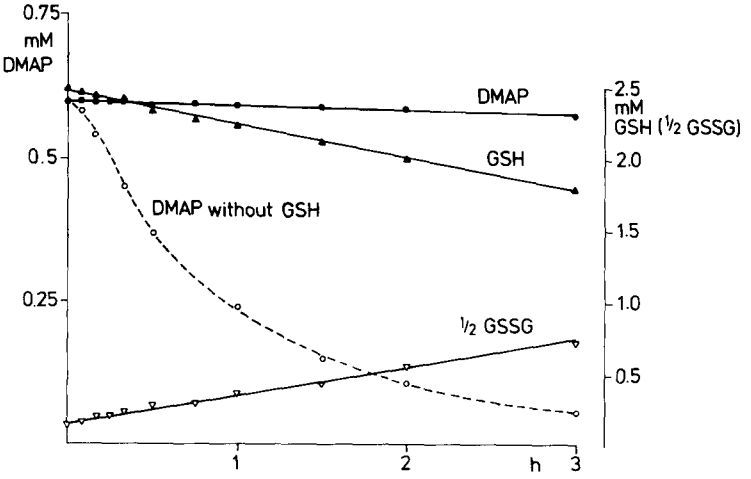

Fig. 11. Inhibition of autoxidation of 4-dimethylaminophenol by GSH. 4-Dimethylaminophenol (0.6 mM) in 0.2 M sodium phosphate, pH 7.4, was incubated at 37° under air in the presence or absence of 2.5 mM GSH.

($k_2 \sim 10^8\,M^{-1}\,sec^{-1}$). This reaction greatly exceeds the spontaneous dismutation of O_2^- ($k \sim 10^5\,M^{-1}\,sec^{-1}$). Hence DMAP is rather stable in the presence of oxygen because the reverse reaction is kinetically favoured. This reaction also seems to be favoured thermodynamically. The redox potential of the phenoxyl radical/DMAP is 0.174 V at pH 13.5 [26] and *ca.* 0.4 V at pH 8.5 (pK 4-dimethylaminophenol/phenoxide = 10.1; pK dimethylamino group = 6.15). At pH 8.5, the redox potential of O_2/O_2^- is −0.33 V and of O_2^-/H_2O_2 + 0.68 V [39]. Hence, O_2^- reduces the 4-(*N*,*N*-dimethylamino)phenoxyl radical only, whereas other semiquinones with low redox potentials are preferably oxidized by O_2^- (for review, see ref. [39]). Superoxide radicals have also been implicated in the production of phenoxyl radicals from various catechols, which should explain some toxic effects of superoxide radicals [4]. DMAP, however, is not attacked by superoxide radicals even during long time exposure as shown by the above experiments.

During autoxidation, a small fraction of the phenoxyl radicals decay ultimately giving rise to *p*-benzoquinone, and some O_2^- dismutates with the formation of hydrogen peroxide. Both reactions are irreversible. Since *p*-benzoquinone reacts rapidly with DMAP ($k_2 = 2 \times 10^4\,M^{-1}\,sec^{-1}$) yielding 4-(*N*,*N*-dimethylamino)phenoxyl radicals and the semiquinone radical (as indicated by EPR, data not shown), the oxidation rate of DMAP is accelerated autocatalytically. Hence only traces of *p*-benzoquinone are required to accelerate phenoxyl radical formation (see Fig. 5). Therefore, careful cleaning of the cuvette is essential in order to follow the true autoxidation kinetics of DMAP. Under our experimental conditions, hydrogen peroxide, the other reaction product during autoxidation of DMAP, apparently did not react with DMAP or with the phenoxyl radical, because the addition of hydrogen peroxide did not significantly change the phenoxyl radical decay kinetics.

During autoxidation of DMAP, the maximal steady-state concentration of 4-(*N*,*N*-dimethylamino)phenoxyl radicals was found to be only 0.3 of the theoretical yield in the presence of air, 0.5 in the presence of one atmosphere of oxygen, and 0.7 in the presence of oxyhaemoglobin under air, again indicating that oxygen is activated in oxyhaemoglobin. Similarly, dimethylamine formation from DMAP was markedly increased in the presence of oxyhaemoglobin [11].

Besides disproportionation, 4-(*N*,*N*-dimethylamino)phenoxyl radicals are reduced by a variety of substances other than O_2^-, of which NAD(P)H and GSH are among the most important biologically. Preliminary experiments indicated that the phenoxyl radical is simply reduced by NAD(P)H and GSH. When we determined the products, however, we found some loss both in DMAP and nucleotides or total glutathione, respectively (cf. Fig. 8). This might be due to electrophilic attack of the phenoxyl radical or the quinonimine on NAD(P)H and GSH. As already published, DMAP forms thioethers with GSH and the reactive SH groups in haemoglobin [13, 14] in the presence of oxyhaemoglobin as in the red cell. Because of the apparent rapid equilibrium between the phenoxyl radicals and the disproportionation products, it is at present difficult to evaluate which species of oxidized DMAP is the more reactive towards nucleophiles. As DMAP is able to reduce two equivalents of ferrihaemoglobin (provided that the resulting ferrohaemoglobin is trapped with carbon monoxide [11]), the reactivity of the phenoxyl radical towards haemoglobin SH groups seems to be low. This was already expected because DMAP is able to produce up to one hundred equivalents of ferrihaemoglobin. The involvement of phenoxyl radicals as ferrihaemoglobin-forming intermediates, as anticipated earlier [11], is plausible in the knowledge of the value of the redox potential of the radical now available [26] and the potential of $HbFe^{+3}/HbFe^{2+}$ = 0.15 V at pH 7.4 [40], and has now been proven by EPR measurements.

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