Free Rad. Res. Comms., Vol. 17, No. 2, pp. 133–141
Reprints available directly from the publisher
Photocopying permitted by license only

QUANTITATIVE DETERMINATION BY ESR OF THE ARYLAMINYL FREE RADICAL DURING THE REACTION OF N,N,N′,N′-TETRAMETHYL-p-PHENYLENEDIAMINE WITH OXYHEMOGLOBIN

CHRISTIAN STÖRLE*, KURT STETTMEIER† and PETER EYER*

** Walther-Straub-Institut für Pharmakologie und Toxikologie der Ludwig-Maximilians-Universität München, Nußbaumstraße 26, D-8000 München 2*
† Institut für Strahlenbiologie, GSF-Forschungszentrum für Umwelt und Gesundheit, Ingolstädter Landstraße 1, D-8042 Neuherberg

(Received March 18, 1992; in final form May 16, 1992)

Aromatic amines with electron-donating substituents are directly activated by pure oxyhemoglobin with formation of ferrihemoglobin. Of these xenobiotics the N-alkylated p-phenylenediamines are particularly active. With N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) cooxidation with oxyhemoglobin was observed by ESR spectroscopy with formation of the arylaminyl free radical ($TMPD^{+}$*). Since the radical is rapidly reduced by ferrohemoglobin, a catalytic cycle of ferrihemoglobin formation is sustained with initially very low steady-state concentrations of the radical, e.g. below 0.1%. Ferrihemoglobin is also able to oxidize TMPD to the radical, hence the steady-state concentration of $TMPD^{+}$* rises with increasing ferrihemoglobin. Radicals of the Wurster's type tend to disproportionate at high rates generating reactive quinonediiminium cations which oxidize and arylate cellular thiols like GSH and protein SH groups. Because the disproportionation rate depends on the square of the radical concentration, quenching of the radicals by ferrohemoglobin to protect cellular thiols will be effective as long as the capacity of the methemoglobin reductase system is not overwhelmed. The results indicate that erythrocytes may play a critical role in activation and detoxication of p-phenylenediamines.

KEY WORDS: p-Phenylenediamines, oxyhemoglobin, arylaminyl free radicals, ESR.

INTRODUCTION

Evidence is accumulating that activation of aromatic amines of toxicological significance does not exclusively occur by the cytochrome P-450 and flavin-containing monooxygenase systems, predominantly in the liver, but additionally in extrahepatic tissues equipped with peroxidatic activities. During the peroxidase-coupled biotransformation of aromatic amines formation of arylaminyl free radicals has been observed.[1–6] These intermediates are potential promutagens, procarcinogens and responsible for target tissue toxicity (for recent reviews see ref. 7). Hence it was of interest to investigate whether oxyhemoglobin is also capable of producing arylaminyl free radicals.

Previously we reported on activation of aromatic amines by oxyhemoglobin which is particularly active on aromatic amines with electron-donating substituents.[8] Of these, N-alkylated p-phenylenediamines gained most interest, because they are known

Correspondence to: Prof. Dr. Peter Eyer, Walther-Straub-Institut, Nußbaumstraße 26, D-8000 München 2, Germany.

to form rather stable radical cations, the Wurster's dyes. When we investigated reactions of N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) and its free radical cation ($TMPD^{+}$*) in more depth, we found that the radical is rapidly quenched by ferrohemoglobin and GSH. Ferrohemoglobin quickly reacts with $TMPD^{+}$* under formation of ferrihemoglobin and the parent amine ($k = 10^3\ M^{-1}\ s^{-1}$), in agreement with thermodynamics.[9] In contrast, the direct reaction of $TMPD^{+}$* with GSH is very sluggish ($k = 5\ M^{-1}\ s^{-1}$). In this case, GSH at physiological concentrations reacts mainly with the quinonediiminium dication with formation of a thioether and GSSG ($k = 10^5\ M^{-1}\ s^{-1}$).[10]

Recent experiments with N,N-dimethyl-p-phenylenediamine (DMPD) have revealed similar findings, i.e. reduction of the radical by ferrohemoglobin and arylation of GSH via the quinonediiminium cation. The unambiguous attribution of the radical to hemoglobin oxidation and the disproportionation product to addition reactions was achieved by shifting the disproportionation equilibrium by excess amine. Looking at these reactions in intact red blood cells, we became aware that ferrohemoglobin largely prevented GSH from alkylation. At increasing amounts of ferrihemoglobin, however, GSH was depleted in an exponential way. When ferrihemoglobin had been performed prior to addition of DMPD, GSH depletion occurred immediately. This reaction could be completely blocked by complexing ferrihemoglobin with cyanide.[11]

These experiments prompted us to investigate the role of ferro- and ferrihemoglobin on the steady-state concentration of the arylaminyl free radicals. Thermodynamic and kinetic considerations[9,11] suggested very low steady-state concentrations of the radical from DMPD (nmolar range) which would escape detection under equilibrium conditions. With TMPD, however, we expected radical concentrations in the micromolar range, suitable for direct ESR spectroscopy.

To this end, we performed experiments with TMPD and its radical cation to evaluate ferrohemoglobin as a potential scavenger of arylaminyl free radicals. It appears once more that erythrocytes with the abundant hemoglobin (25 mM) play a dual role in the biotransformation of aromatic amines:[12–14] oxyhemoglobin activates these compounds like an oxidase, while ferrohemoglobin (particularly in the unliganded form) effectively quenches the radical intermediates.

MATERIALS AND METHODS

TMPD (98% purity) from Aldrich Chemie (Steinheim, D-7924) was used without further purification.

The stable radical cation of TMPD was prepared according to Michaelis and Granick.[15] The purity of the crystalline $TMPD^{+}$* perchlorate was checked by TLC (silica gel $60F_{254}$, 0.2 mm, Merck (Darmstadt, D-6100) with methanol/methylene chloride 95:5 as mobile phase), and the compound showed the electronic spectrum as published by Rao and Hayon[16] with $\varepsilon_{611\,nm} = 12.5 \times 10^3\ M^{-1}\ cm^{-1}$. At −20°C under an atmosphere of argon $TMPD^{+}$* perchlorate was stable for months, and in acetonitrile the radical cation was stable at 0°C for a working day. Solutions in sodium phosphate, pH 7.4, were prepared immediately before use ($t_{1/2} = 90$ min at 22°C).

All other chemicals were purchased from Merck at the highest grade available.

Hemolysate was freshly prepared from outdated human red blood cell concentrates of a local blood bank by hemolysis in two volumes of distilled water containing

TABLE I
Calibration of the $TMPD^{+}*$ signal by ESR spectroscopy

$TMPD^{+}*$ (μM)	Amplitude (rel. intens.)
1	729
3	2040
10	7130
30	

$TMPD^{+}*$ at different concentrations in 0.2 M sodium phosphate, pH 7.4 was subjected to ESR spectroscopy at 22°C. The amplitude of the most intensive radical signal correlated with the $TMPD^{+}*$ concentration. The mean error at 3 μM $TMPD^{+}*$ was 4% (single scans, n = 4 samples).

saponine. The hemolysate was extensively dialysed against sodium phosphate buffer (0.2 M, pH 7.4). Hemolysate with hemoglobin in the ferric state was prepared by oxidation of hemolysate with 1.1 equivalents ferricyanide followed by extensive dialysis.

Ferrihemoglobin and total hemoglobin, expressed in iron equivalents, were determined according to Kiese.[17] ESR spectra were recorded at ambient temperature (22 ± 2°C) using a Bruker ESP-300 spectrometer (data system ESP 1600) equipped with a cylindrical TM_{110} cavity. Parameters were chosen in a way that all measurements could be carried out without any change (MA = 1 G, sweep 2.6 G/s, power 5 mW). The first ESR spectrum was read within 30 s after mixing the compounds.

Because of the unusually high stability of $TMPD^{+}*$, it was possible to correlate the $TMPD^{+}*$ concentration with the most intensive absorption amplitude. As shown in Table I, a linear relationship of the radical concentration and the signal amplitude was obtained from 1 to 30 μK. The lower useful detection limit was 0.1 μM $TMPD^{+}*$ (single scan).

RESULTS

Figure 1 shows the ESR spectrum of the authentic Wurster's blue radical cation ($TMPD^{+}*$) and the spectrum obtained in hemolysate (3 mM hemoglobin) 5 min after addition of TMPD (1 mM; 0.2 M sodium phosphate buffer, pH 7.4, 22°C, air = standard conditions).

When authentic $TMPD^{+}*$ (0.1 mM) was mixed with hemolysate, the intensity of the radical signal quickly faded. Figure 2 illustrates the $TMPD^{+}*$ concentration vs. time curve when $TMPD^{+}*$ was added to hemolysate. After 2 min reaction, the radical concentration fell below 2 μM and re-increased thereafter. A similar increase was also observed when TMPD was mixed with hemolysate. These data confirmed our previous working hypothesis that the steady-state concentration of $TMPD^{+}*$ should be low, because the rate of radical reduction by ferrohemoglobin was about 3 orders of magnitude faster than the rate of radical formation by oxyhemoglobin.[9] Hence, a steady-state concentration of $TMPD^{+}*$ of about 0.1 μM was expected under the conditions of Figure 2. The 20-times higher concentration after 2 min reaction (start with $TMPD^{+}*$) was therefore somewhat surprising.

Although thermodynamics were not in favour of the back reaction, we examined

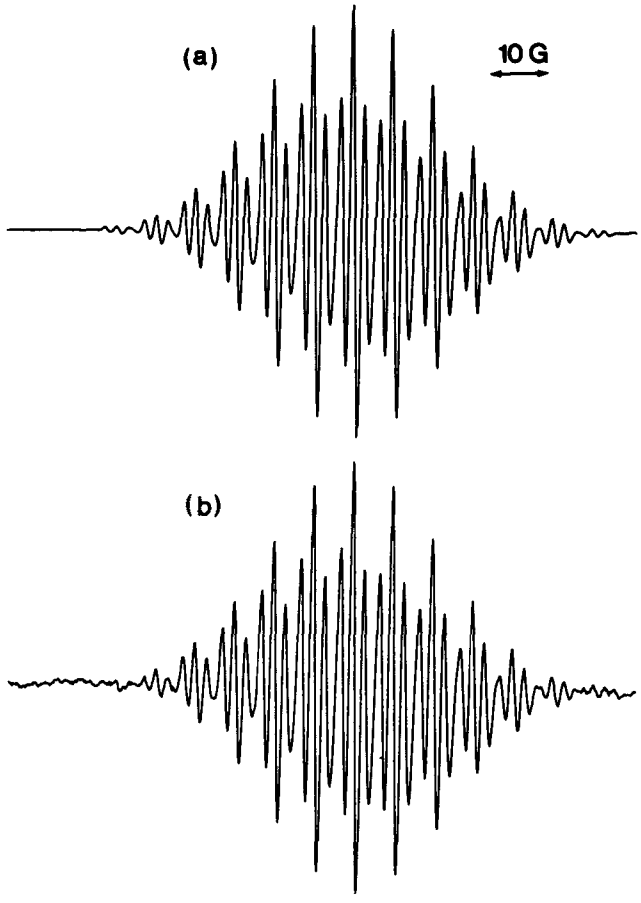

FIGURE 1 ESR spectrum of authentic N,N,N′,N′-tetramethyl-p-phenylenediamine radical cation ($TMPD^{+}$*) and an incubate of N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) with hemolysate. (a) Authentic $TMPD^{+}$* in 0.2 M sodium phosphate, pH 7.4 and (b) TMPD (1 mM) in dialysed hemolysate (3 mM oxyhemoglobin) in 0.2 M sodium phosphate, pH 7.4, at 22°C under air (after 5 min reaction, 3 scans).

whether TMPD was able to reduce ferrihemoglobin ($TMPD^{+}$*/TMPD = 0.27 V, $HbFe^{3+}/HbFe^{2+}$ = 0.13 V).[11] To this end, human ferrihemoglobin (0.1 mM) was reacted with TMPD (0.1 mM) in 0.2 M sodium phosphate, pH 7.4, at 20°C under an atmosphere of CO. As shown in Figure 3a, the ferrihemoglobin spectrum (broken line) gradually turned into the composite spectrum of HbCO and $TMPD^{+}$* (repetitive scannings every min). The control spectra of $TMPD^{+}$* (0.1 mM, full line), HbCO (0.1 mM, broken line) and of a mixture of both (under CO, dashed line) are presented in Figure 3b. Thus it was evident that $TMPD^{+}$* can be formed in the reaction of ferrihemoglobin and TMPD (back reaction).

To investigate the influence of ferrihemoglobin on the radical steady-state concentration, oxyhemoglobin was mixed with ferrihemoglobin to give 10% and 20% ferrihemoglobin, respectively. To these mixtures (3 mM final concentration)

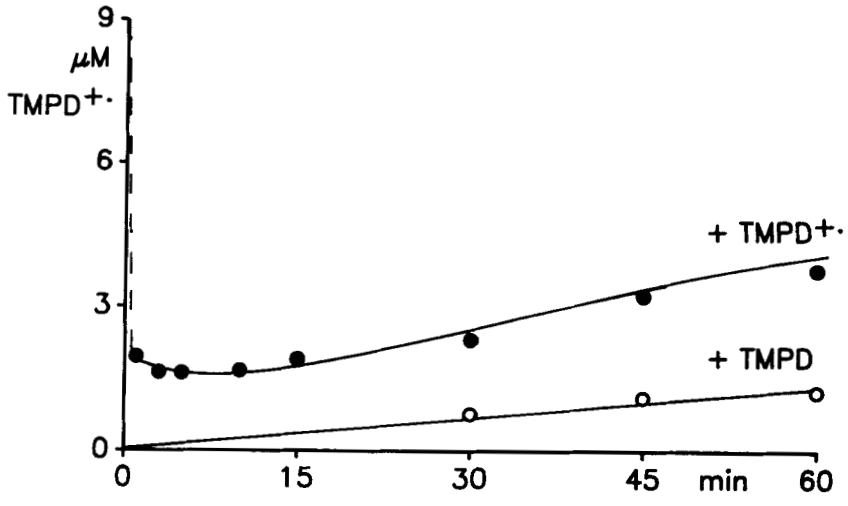

FIGURE 2 Concentration of $TMPD^{+}$* vs. time during the reaction of TMPD (0.1 mM) and $TMPD^{+}$* (0.1 mM), respectively, with hemolysate (conditions as in Figure 1; means of 2 experiments).

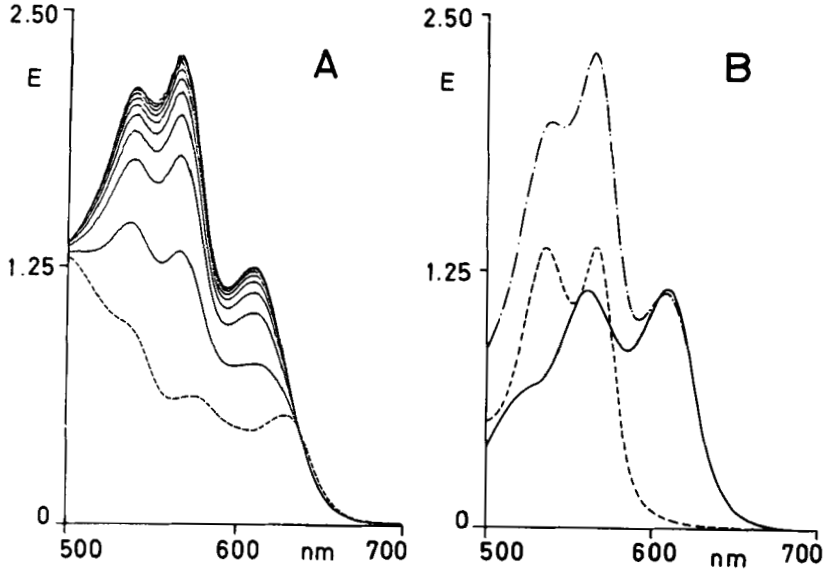

FIGURE 3 Reduction of ferrihemoglobin by TMPD. (a) Ferrihemoglobin (0.1 mM; broken line) was allowed to react with TMPD (0.1 mM) at 22°C under an atmosphere of carbonmonoxide: formation of HbCO and $TMPD^{+}$*, scans every min. (b) Visible spectra of HbCO (0.1 mM, broken line), $TMPD^{+}$* (0.1 mM, full line) and a mixture of both under an atmosphere of carbon monoxide (dashed line).

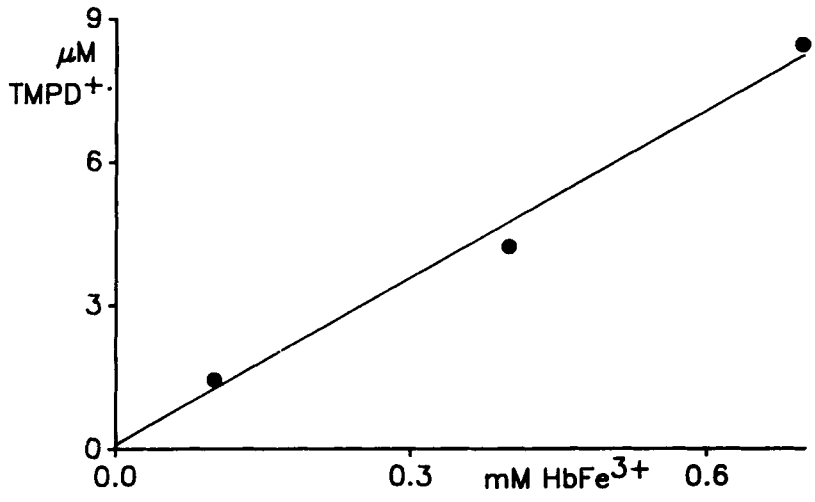

FIGURE 4 Influence of the ferrihemoglobin content of hemolysate on the steady-state concentration of $TMPD^{+}$*. $TMPD^{+}$* (0.1 mM) was incubated with hemolysate (3 mM) containing various proportions of initial ferrihemoglobin (0%, 10%, 20%) for 10 min. Incubation conditions as in Figure 1. Because nearly equivalent amounts of ferrihemoglobin are formed during the reaction with $TMPD^{+}$*, this amount added to the initial one.

$TMPD^{+}$* (0.1 mM) was added and allowed to react for 10 min prior to ESR spectroscopy. As shown in Figure 4, the $TMPD^{+}$* concentration was linearly correlated with the ferrihemoglobin content. Since $TMPD^{+}$* nearly stoichiometrically produces ferrihemoglobin, this amount, i.e. 0.1 mM, added to the initial amount of ferrihemoglobin. The intercept of the regression line at 0% ferrihemoglobin corresponded roughly to 0.1 μM $TMPD^{+}$*, a concentration three orders of magnitude lower than the initial one, as expected.

Last we examined the influence of cyanide on the steady-state concentration of $TMPD^{+}$*, in order to remove ferrihemoglobin by strong ligation. As shown in Figure 5, the steady-state concentration of $TMPD^{+}$* fitted the expected amount in solution of 3 mM hemolysate and 1 mM TMPD, provided ferrihemoglobin was complexed by cyanide (10 mM). A steady-state of $TMPD^{+}$* at 0.07% of the concentration of TMPD was kept for 10 min. The slow increase afterwards, about 0.2% per h, was much slower than in the absence of cyanide (about 1.3% per h, notice the different initial $TMPD^{+}$* concentrations in Figures 2 and 5, respectively). These results confirmed the importance of free ferrihemoglobin on the steady-state concentration of $TMPD^{+}$*.

DISCUSSION

During the reaction of TMPD with hemolysate an ESR signal was observed identical with that of authentic $TMPD^{+}$*, with coupling constants of 6.9 and 2.0 Gauss as described by Hausser.[18] The radical concentration, determined by the amplitude of the most intensive resonance, steadily increased and paralleled ferrihemoglobin formation. To determine the recovery of the $TMPD^{+}$* signal, authentic $TMPD^{+}$*

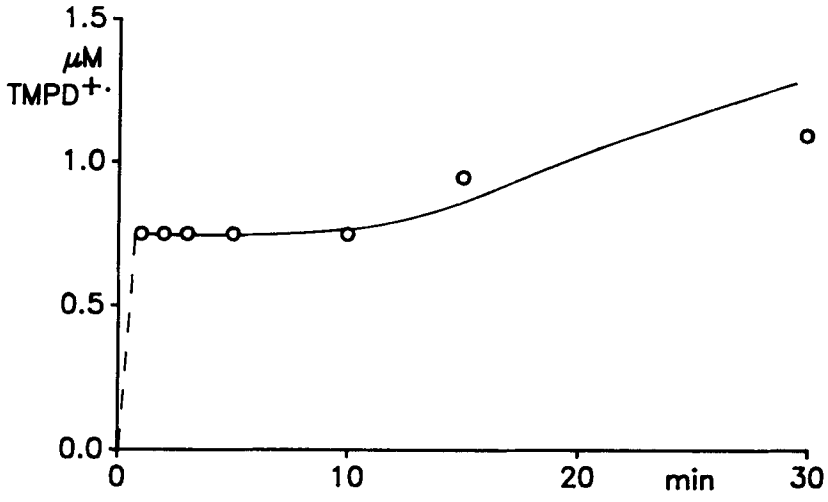

FIGURE 5 $TMPD^{+}$* concentration vs. time during the reaction of TMPD with hemolysate in the presence of cyanide. TMPD (1 mM) was allowed to react with hemolysate (3 mM) in the presence of cyanide (10 mM). Other conditions as in Figure 1 (means of two experiments).

was mixed with hemolysate whereby the resonance intensity quickly faded. Such a behaviour was expected since $TMPD^{+}$* is rapidly reduced by ferrohemoglobin. From the rates of $TMPD^{+}$* formation and consumption, respectively, the steady-state concentration should be predicted.

Under the conditions of our experiments (dialysed human hemolysate, 22°C) ferrihemoglobin formation was slower than previously reported for purified human hemoglobin at 37°C.[9] Assuming a temperature coefficient of 2.5 for Δ10°C, the reaction rate constant was expected to drop from 5.5 $M^{-1} s^{-1}$ (37°C) to 1.4 $M^{-1} s^{-1}$ (22°C). Because catalase diminished ferrihemoglobin formation by 33%,[9] a reaction rate constant of 0.93 $M^{-1} s^{-1}$ for ferrihemoglobin formation was expected and experimentally confirmed. As recently published,[9] the total reaction sequence was supposed to consist of 4 microscopic events:

$$HbO_2 + TMPD \rightleftharpoons HbFe^{3+}[-OO]^{2-} + TMPD^{+}* \quad (1)$$

$$HbFe^{2+} + TMPD^{+}* \rightleftharpoons HbFe^{3+} + TMPD \quad (2)$$

$$HbFe^{3+}[-OO]^{2-} + 2H^{+} \rightleftharpoons HbFe^{3+} + H_2O_2 \quad (3)$$

$$HbFe^{2+} + H_2O_2 \dashrightarrow HbFe^{3+} + \text{products} \quad (4)$$

In the presence of catalase (which prevents ferrihemoglobin formation via reaction 4) reaction 1 runs half as fast as ferrihemoglobin formation which is brought about by reaction 2 and 3. Thus a $TMPD^{+}$* formation rate of 0.47 $M^{-1} s^{-1}$ is calculated. The rate constant of reaction 2 is 10^3 $M^{-1} s^{-1}$ at 25°C[19] and presumably 0.75×10^3 $M^{-1} s^{-1}$ at 22°C. Hence, the steady-state concentration of $TMPD^{+}$* was expected to be $0.47/750 = 0.63 \times 10^{-3}$ of the concentration of initial TMPD. Such a radical proportion was observed when the back reaction 2 was inhibited by complexing ferrihemoglobin with cyanide, and the calculated steady-state concentration remained constant for a 10-min period (Figure 5). The gradual increase

thereafter was probably due to the consumption of ferrohemoglobin. Such a consumption *per se* should not alter the steady-state concentration of $TMPD^{+}$*, because both, reaction 1 and 2 are equally affected. In contrast, autoxidation of TMPD becomes increasingly important when ferrohemoglobin is consumed.

Specifically, after 20 min reaction (standard conditions) about 70% ferrihemoglobin has been formed. $TMPD^{+}$* formation then proceeds at about 0.42 μM/s via reaction 1, $TMPD^{+}$* consumption at 0.68 mM/s and autoxidation of TMPD at 0.17 μM/s.[9] From these data a steady-state radical concentration of $(0.42 + 0.17)/680 = 0.9 \times 10^{-3}$ of the concentration of TMPD is expected, which agrees with the observation depicted in Figure 5 (at 20 min).

The influence of the ferrihemoglobin content on the steady-state concentration of the free arylaminyl radical has toxicological implications. p-Phenylenediamine radical cations tend to disproportionate[20,21] giving rise of the highly reactive quinonediiminium cations which readily react with cellular sulfhydryls like GSH and protein SH groups.[9,11] Because the rate of disproportionation depends on the square of the radical concentration, the latter critically controls GSH oxidation and adduct formation. In the case of DMPD we have recently found[11] that disturbance of the glutathione status in erythrocytes depends on the ferrihemoglobin content and could be prevented when cyanide inhibited the back reaction 2. It is anticipated that thioether formation with TMPD in human red cells[10] can be equally prevented by cyanide.

On the other hand, it can be deduced that below critical concentrations of the p-phenylenediamines a certain threshold might exist, below which cellular defense systems are able to reduce the radical quickly enough (pseudo-first-order reactions with respect to the radicals) to prevent the disproportionation (second-order reaction). Of these cellular defense systems the abundant ferrohemoglobin coupled with the methemoglobin reductase system will be of paramount importance in human red cells.

Acknowledgment

This study was supported by the Deutsche Forschungsgemeinschaft.

References

1. D. Job and H.B. Dunford. (1976) Substituent effect on the oxidation of phenols and aromatic amines by horseradish peroxidase compound I. *European Journal of Biochemistry*, **66**, 607–614.
2. P.D. Josephy, T.E. Eling and R.P. Mason. (1982) The horseradish peroxidase catalyzed oxidation of 3,5,3′,5′-tetramethylbenzidine. Free radical and charge transfer complex intermediates. *Journal of Biological Chemistry*, **257**, 3669–3675.
3. P.D. Josephy, T.E. Eling and R.P. Mason. (1983) An electron spin resonance study of the activation of benzidine by peroxidases. *Molecular Pharmacology*, **23**, 313–320.
4. R.P. Mason. (1984) Free-radical intermediates in the metabolism of toxic chemicals. In *Free Radicals in Biology* (ed. W.A. Pryor), Academic Press Inc., Orlando, Florida, Vol. V, pp. 161–222.
5. T.E. Eling, J.A. Boyd, R.S. Krauss and R.P. Mason. (1985) Metabolism of aromatic amines by prostaglandin H synthetase. In *Biological Oxidation of Nitrogen in Organic Molecules: Chemistry, Toxicology and Pharmacology* (eds. J.W. Gorrod and L.A. Damani), Ellis Horwood, Chichester, U.K., pp. 350–363.
6. T.E. Eling, J.F. Curtis, D.C. Thompson, J. Van der Zee and R.P. Mason. (1991) Formation of aromatic amine free radicals by prostaglandin hydroperoxidase and peroxyl radicals: Analysis by ESR and stable end products. In *N-Oxidation of Drugs. Biochemistry, Pharmacology, Toxicology* (eds. P. Hlavica and L.A. Damani), Chapman & Hall, London, pp. 3–18.

7. P. Hlavica and L.A. Damani. (1991) *N-Oxidation of Drugs. Biochemistry, Pharmacology, Toxicology.* Chapman & Hall, London.
8. P. Eyer. (1991) Activation of aromatic amines by oxyhemoglobin. In *N-Oxidation of Drugs. Biochemistry, Pharmacology, Toxicology* (eds. P. Hlavica and L.A. Damani), Chapman & Hall, London, pp. 371–391.
9. C. Störle and P. Eyer. (1991) Formation and reactions of the Wurster's blue radical cation during the reaction of N,N,N′,N′-tetramethyl-p-phenylenediamine with oxyhemoglobin. *Chemico-Biological Interactions*, **78**, 321–331.
10. C. Störle and P. Eyer. (1991) Reactions of the Wurster's blue radical cation with thiols, and some properties of the reaction products. *Chemico-Biological Interactions*, **78**, 333–346.
11. C. Störle and P. Eyer. (1992) Reaction of the Wurster's red radical cation with hemoglobin and glutathione during cooxidation of N,N-dimethyl-p-phenylenediamine and oxyhemoglobin in human red cells. *Chemico-Biological Interactions*, in press.
12. P. Eyer. (1983) The red cell as a sensitive target for activated toxic arylamines. *Archives Toxicology, Supplement*, **6**, 3–12.
13. K.-G. Eckert and P. Eyer. (1986) Formation and transport of xenobiotic glutathione-S-conjugates in red cells. *Biochemical Pharmacology*, **35**, 325–329.
14. P. Eyer. (1988) Detoxication of N-oxygenated arylamines in erythrocytes. An overview. *Xenobiotica*, **11**, 1327–1333.
15. L. Michaelis and S. Granick. (1943) The polymerization of the free radicals of the Wurster dye type: The dimeric resonance bond. *Journal of the American Chemical Society*, **65**, 1747–1755.
16. P.S. Rao and E. Hayon. (1975) Oxidation of aromatic amines and diamines by OH radicals. Formation and ionization constants of amine cation radicals in water. *Journal of Physical Chemistry*, **79**, 1063–1066.
17. M. Kiese. (1959) Oxydation von Anilin zu Nitrosobenzol im Hunde. *Naunyn-Schmiedeberg's Archiv der experimentellen Pathologie und Pharmakologie*, **235**, 354–359.
18. K.H. Hausser. (1960) Uber den Einfluß des gelösten Sauerstoffs auf die Linienbreite der Elektronen-Spin-Resonanz in Lösung. *Naturwissenschaften*, **47**, 251.
19. C. Störle. (1989) *Bildung und Reaktionen des Radikal-Kations bei der Ferrihämoglobin-Bildung durch N,N,N′,N′-Tetramethyl-p-phenylendiamin.* Diplomarbeit, München.
20. L. Michaelis, M.P. Schubert and S. Granick. (1939) The free radicals of the type of Wurster's salts. *Journal of the American Chemical Society*, **61**, 1981–1992.
21. U. Nickel, E. Haase and W. Jaenicke. (1977) Optische Spektren und Gleichgewichtskonstanten der Semichinone und Chinodiimine von substituierten p-Phenylendiaminen. *Berichte der Bunsengesellschaft für Physikalische Chemie*, **81**, 849–853.

Accepted by Prof. H. Sies