*Free Rad. Res.*, Vol. 28, pp. 497–505
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# Peroxidase-Mediated Oxidation, a Possible Pathway for Activation of the Fungal Nephrotoxin Orellanine and Related Compounds. ESR and Spin-Trapping Studies

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Accepted by Prof. H. Sies

*(Received 28 July 1997; In revised form 24 November 1997)*



Orellanine is the tetrahydroxylated and di-N-oxidized bipyridine toxin extracted from several *Cortinarius* mushrooms among them *C. orellanus*. The pathogenic mechanism involved in the *C. orellanus*-poisoning by orellanine leading to kidney impairment is not yet fully understood until now. Electron spin resonance (ESR) spectroscopy has been used to study the activation of orellanine by horseradish peroxidase/$H_2O_2$ system at physiological pH. Evidence for a one-electron oxidation of the toxin by this enzymatic system to an *ortho*-semiquinone radical intermediate is presented.

The orellanine *ortho*-semiquinone generated by the peroxidase/$H_2O_2$ system abstracts hydrogen from glutathione, generating the glutathionyl radical which is spin-trapped by 5,5′-dimethyl-1-pyrroline N-oxide (DMPO) and subsequently detected by ESR spectroscopy. Similarly, the *ortho*-semiquinone abstracts hydrogen from ascorbic acid to generate the ascorbyl radical which is detected by direct ESR. The peroxidatic oxidation of orellanine to semiquinone followed by its reduction by glutathione or ascorbic acid does not induce dioxygen uptake. The relationship between chemical structure and HRP oxidation of orellanine-related molecules, namely orelline and $DHBPO_2$ (the parent molecule lacking of hydroxyl groups in 3 and 3′ position) has been investigated in absence or in presence of reducing agents. None of the orellanine-related compounds can be oxidized by the HRP/$H_2O_2$ system, showing that both catecholic moieties and aminoxide groups are necessary for observing the formation of the *ortho*-semiquinone form of orellanine.

As shown for the (photo)chemical oxidation of orellanine, the mechanism of toxicity could be correlated with a depletion of glutathione and ascorbate levels which are implicated in the defence against oxidative damage.

*Keywords:* Orellanine, mushroom nephrotoxin, ESR, horseradish peroxidase, semiquinone radical, spin-trapping

*Abbreviations:* $DHBPO_2$, 2,2′-bipyridine-4,4′-diol-1,1′-dioxide; DMPO, 5,5′-dimethyl-1-pyroline N-oxide, ESR, electron spin resonance, GSH, glutathione, HRP, horseradish peroxidase, Or, orellanine

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## INTRODUCTION

Orellanine, [2,2′-bipyridine]-3,3′,4,4′-tetrol-1,1′-dioxide, is the toxic principle of several *Cortinarius* mushrooms including *C. orellanus*.[1] Orellanine intoxication in man causes acute renal failure, which in its severe form can lead to death. Orellanine has to be activated prior to producing its effect.[2] There is an involvement of free radical production in the mechanism of toxicity of orellanine.[3] The biochemical mechanisms of nephrotoxicity of the toxin are presently under investigation.[4,5] Orellanine is able to be oxidized to the *ortho*-semiquinone radical both enzymatically by the tyrosinase/$O_2$ system and photochemically using visible light. It is also oxidized by some biological agents like cytochrome c and NAD.[4] From these results, we predicted that peroxidase/$H_2O_2$ was probably an efficient means to oxidize this toxin to its *ortho*-semiquinone radical as known for phenols and catechols.[6–8]

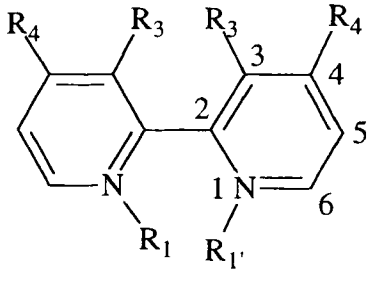
Since horseradish peroxidase (HRP) is a plant enzyme, there is some criticism of its use in studies of the oxidation of toxins because it may not have any pharmacological relevance, but it serves as a model for the peroxidase reaction in biological systems. In this paper, a conventional electron spin resonance (ESR) study has been undertaken to demonstrate production of *ortho*-semiquinone from peroxidatic oxidation of orellanine. The effect of some biological reductants like glutathione (GSH) and ascorbic acid which are involved in the defence against oxidative stress has been investigated.

To assess the effect of substituents on the bipyridine ring on the enzymatic oxidation of orellanine, the study was extended to the other compounds namely orelline and $DHBPO_2$ which present similarity with orellanine in their structures. Their formulae are shown in Figure 1.

## MATERIALS AND METHODS

### Materials

Horseradish peroxidase (HRP, EC 1.11.1.7 type I, 250 units/mg) was supplied by Boehringer. Glutathione (98–100%) was purchased from Sigma. Ascorbic acid was obtained from Carlo Erba (AnalytiCals, grade RPE). Other chemicals were from Merck (Suprapur reagent grade) or from Aldrich. The spin-trap DMPO, also purchased from Sigma, was vacuum-distilled at ambient temperature in order to remove any ESR




| *Compounds* | $R_1$ | $R_{1'}$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| 2,2′-bipyridine-3,3′,4,4′-tetrol-1,1′-dioxixe: **Orellanine** | O | O | OH | OH |
| 2,2′-bipyridine-3,3′,4,4′-tetrol: **Orelline** | — | — | OH | OH |
| 2,2′-bipyridine-4,4′-diol-1,1′-dioxixe: **$DHBOP_2$** | O | O | H | OH |

FIGURE 1 Chemical structure of orellanine and related compounds.

contaminating signal and was stored at −40°C until use. It was used at 100 or 300 mM concentration. Care was taken to ensure that DMPO-containing solutions were subject to minimal light-induced degradation. Orelline was obtained from the laboratory of "Chimie Organique Fine et Hétérocyclique" through the courtesy of Dr. Trécourt.[9] $DHBPO_2$ was synthetized in our laboratory according to Cantin *et al.*[10] Orellanine was extracted from dry powdered carpophores of *C. orellanus* mushrooms and purified as previously described.[11] Considerable attention must be paid to sample preparation.[12] Stock solutions of orellanine (50 mM) were prepared in the dark by dissolving the toxin in 100 mM disodium hydrogen phosphate. Then the pH was adjusted to 7. Stock solutions of this photosensitive and easily oxidizable product were stored in the dark at 4°C and used within two days. All solutions were prepared in phosphate buffer just before use, kept in ice and protected from direct light to avoid any photolytic decomposition.

### ESR Spectroscopy

Electron spin resonance measurements were made at ambient temperature, using a Bruker ER 100 D spectrometer operating at a microwave frequency 9.3 GHz and a magnetic field of 3480 G, with magnetic field modulation at 100 kHz.

### Enzymatic Activation

Enzymatic oxidation of orellanine (Or) was carried out with HRP and hydrogen peroxide. A typical incubation mixture for ESR consisted of Or (1–20 mM), $H_2O_2$ (0–3 mM) and HRP (2–64 $\mu g\,ml^{-1}$) in 100 µl phosphate buffer (100 mM, pH 7). If necessary, spin-trap (DMPO) (100 or 300 mM) and reductants (GSH or ascorbic acid) were added to the medium. The reaction mixture was then immediately introduced into a flat cell and ESR spectra were recorded within 50 or 100 s according to the radical stability. When the reaction was carried out in anaerobic medium, the solution containing Or and $H_2O_2$ was deaerated by bubbling with argon for 5 min prior to the addition of HRP from a solution which was itself deaerated. The same experiments were repeated in aerobic medium after bubbling all solutions with dioxygen. Other experiments were also conducted in phosphate buffers of varying pH. The ESR signal intensity was expressed in arbitrary unit. To assess the stability of *ortho*-semiquinone, glutathionyl and ascorbyl radicals, their ESR signals were recorded every 50 or 100 s until the radicals disappeared. Orelline and $DHBPO_2$ were examined under identical conditions. All experiments were repeated at least three times.

### Dioxygen Consumption

Dioxygen consumption was measured polarographically in 100 mM phosphate buffer (pH 7) at room temperature using a Clark-type electrode system purchased from Hansatech Ltd. HRP (10 and 20 $\mu g\,ml^{-1}$) was added to the mixtures containing Or (5–15 mM), $H_2O_2$ (0.5–2.5 mM) in presence or in absence of biological reductants (5–40 mM), ascorbic acid or GSH.

## RESULTS

### Generation of Orellanine *Ortho*-Semiquinone Radical by the System Or/HRP/$H_2O_2$

#### *Oxidation of Orellanine*

The heme-containing peroxidases, found in animal tissues, are known to catalyze the one-electron oxidation of a wide range of endogenous and exogenous compounds among them catechols.[13] Indeed, by adding HRP (8 $\mu g\,ml^{-1}$) to the colourless Or (5 mM), $H_2O_2$ (0.44 mM) mixture in 100 mM phosphate buffer pH 7 at room temperature, the solution turns yellow. The latter

exhibits a well-resolved nine lines ESR signal if the spectrum is immediately recorded (Figure 2C). The hyperfine coupling constants, identical to those obtained when Or is oxidized by light, are $a_N = 0.31$ mT and $a_H = 0.21$ mT. These values are indicative of interactions of the unpaired electron with the hydrogen atoms in positions 5 and 6 and with the nitrogen atom in position 1. The results are in agreement with the one-electron oxidation of Or to an *ortho*-semiquinone anion radical.[4] The intense signal of *ortho*-semiquinone radical was observed during about 1 min after mixing.

When $H_2O_2$ was omitted from the reaction, the same signal, which was stable for 20 min, was observed at a reduced intensity (Figure 2B). However, no signal was observed in the presence of $H_2O_2$ at 0.44 mM, without HRP and without any incubation time (Figure 2A). The ESR signal was dependent on enzyme activity, since the use of heat-denatured enzyme results in no detectable signal. Addition of EDTA (60 μM) or desferal (60 μM) to the incubation mixture had no effect on the formation of *ortho*-semiquinone suggesting that this activation of Or is not metal-dependent. All of the components of the system Or/$H_2O_2$/HRP were necessary to detect an intense free radical signal. In addition no secondary radical was observed after the *ortho*-semiquinone radical disappeared though the solution remained yellow.

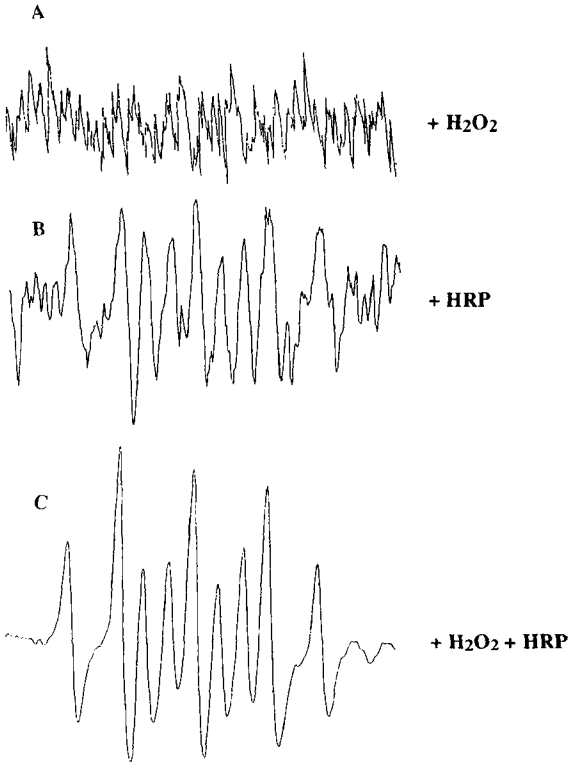



FIGURE 2 ESR spectrum of orellanine semiquinone radical obtained by oxidation of Or by HRP and/or $H_2O_2$. The reaction mixtures contained Or (5 mM) and, where indicated, $H_2O_2$ (0.44 mM) or/and HRP (8 μg ml$^{-1}$) in 100 mM phosphate buffer pH 7. Spectrometer settings: modulation frequency: 100 kHz; modulation amplitude: 0.08 mT; microwave power: 5 mW; receiver gain: $5 \cdot 10^6$; scan time: 200 s for (A), 100 s for (B) and 50 s for (C); scan range: 3 mT; temperature: 20°C.

### *Influence of Or Concentration*

In the presence of increasing concentrations of Or (1–20 mM ) and fixed concentrations of both HRP (8 μg ml$^{-1}$) and $H_2O_2$ (0.44 mM), the intensity of the *ortho*-semiquinone ESR signal was proportional to the toxin concentration up to 4 mM (Figure 3). Above 5 mM, the semiquinone signal intensity decreased significantly.

### *Influence of HRP and $H_2O_2$ Concentrations and pH*

In a medium containing fixed concentrations of Or (4 mM) and $H_2O_2$ (1 mM) and varying HRP

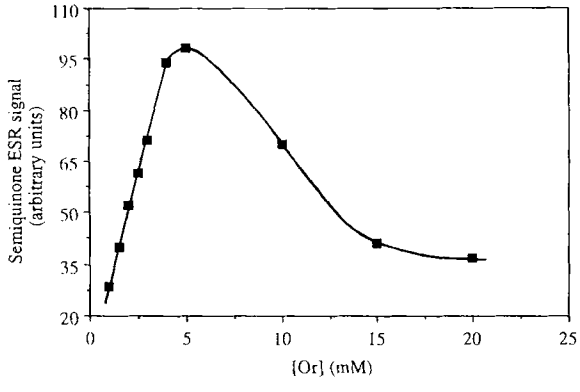



FIGURE 3 Effect of orellanine concentration on the intensity of the ESR signal of the *ortho*-semiquinone anion radical generated by HRP/$H_2O_2$ system. The reaction mixtures contained Or, $H_2O_2$ (0.44 mM) and HRP (8 μg ml$^{-1}$). Spectrometer settings: see Figure 2 (scan time: 50 s).

concentrations (2–64 μg ml$^{-1}$), the semiquinone ESR peak height was proportional to the square root of the enzyme concentration, indicating that radical decay obeyed second-order kinetics, implying non-enzymatic disproportionation or dimerization of the radical as known for hydroquinone and for catechols.[13,14] In addition, at constant concentrations of both HRP (8 μg ml$^{-1}$) and Or (5 mM), the stability of the semiquinone radical decreases with decreasing $H_2O_2$ concentrations (3–0.44 mM). With the lower $H_2O_2$ concentrations, the ESR signal intensity of *ortho*-semiquinone is higher. However, the higher the $H_2O_2$ concentration is, the longer the *ortho*-semiquinone radical half-life is, but the lower is the ESR signal (Figure 4). Thus, it seems that the stability of the *ortho*-semiquinone radical of Or is directly linked to the amount of the radical produced in the medium.

As shown in Figure 5, the amplitude of ESR signal was found to increase with decreasing pH. The ESR signal at pH 6 was twice as high as at pH 7.

### *Influence of Dioxygen*

When oxidation of Or (5 mM) occurred in aerobic or anaerobic medium at pH 6 or 7, the steady-state concentration of *ortho*-semiquinone remained unchanged (results not shown). When the spin-trap DMPO (100–300 mM) was included in the Or (5 mM)/$H_2O_2$ (0.6 mM)/HRP (10 μg ml$^{-1}$) system, neither DMPO–$^{\bullet}$OOH nor DMPO–$^{\bullet}$OH signals were observed. To confirm the results obtained with the previous method, dioxygen consumption measurements were conducted. These experiments show that oxidation of Or (15 mM) by the HRP (10 and 20 μg ml$^{-1}$)/$H_2O_2$ (0.5–2.5 mM) system does not induce dioxygen uptake.

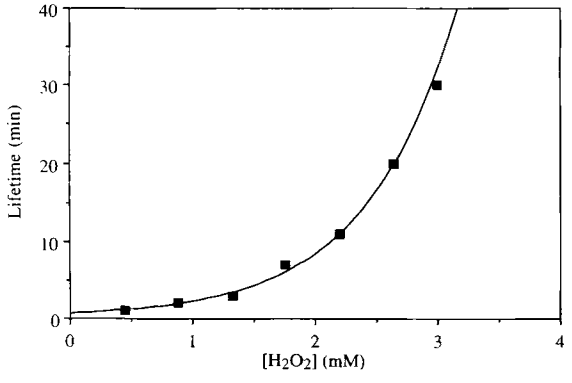



FIGURE 4 Effect of $H_2O_2$ concentration on the lifetime of the orellanine *ortho*-semiquinone anion radical generated by HRP/$H_2O_2$ system. Conditions as in Figure 2.

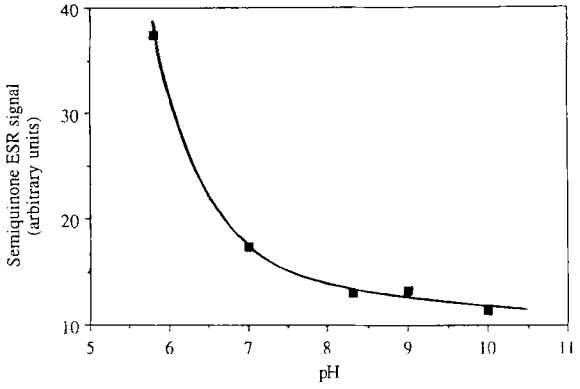



FIGURE 5 Effect of pH on the intensity of the orellanine *ortho*-semiquinone radical generated by HRP/$H_2O_2$ system. The reaction mixtures contained Or (4 mM), $H_2O_2$ (0.7 mM) and HRP (8 μg ml$^{-1}$) in 100 mM phosphate buffer pH 7. Spectrometer settings: modulation frequency: 100 kHz; modulation amplitude: 0.125 mT; microwave power: 5 mW; receiver gain: $5 \cdot 10^5$; scan time: 50 s; scan range: 3 mT; temperature: 20°C.

### Orelline or $DHBPO_2/H_2O_2$ System in Presence of HRP

Concerning the role of aminoxide and *ortho*-dihydroxylated groups borne by the bipyridine cycle, we performed similar experiments with related compounds. Orelline, the fully deaminoxidized Or, is a major metabolite of the toxin. Moreover we studied $DHBPO_2$ which is an aminoxidized 2,2′-bipyridine with two hydroxyl functions in 4 and 4′ position. None of these compounds gave any stable ESR spectrum in presence of the HRP/$H_2O_2$ system even in anaerobic solution or in presence of $Mg^{2+}$ (classically used to stabilize semiquinone radicals). In addition, in a system containing orelline, $H_2O_2$,

GSH or ascorbic acid and HRP, the ESR spectra of ascorbyl or glutathionyl radicals were not different from blanks.

## Reactivity of the *Ortho*-Semiquinone Radical

### *Ascorbate*

Immediately after addition of HRP (8 μg ml$^{-1}$) to the mixture containing Or (5 mM), $H_2O_2$ (0.6 mM) and ascorbic acid (4 mM), the semiquinone radical of Or was completely quenched and a strong signal of ascorbyl radical was observed from the first scan, as shown in Figure 6A. The latter was characterized by its hyperfine coupling constant $a_H = 0.18$ mT. Ten min after mixing the compounds, the ESR signal of the ascorbyl radical decreased by 85%. The signal, measured 30 s after HRP addition, increased as a function of Or concentration varying from 1 to 5 mM (data not shown). When ascorbic acid was not present in the medium, the *ortho*-semiquinone radical of Or was recorded (Figure 6E). Without either Or, $H_2O_2$ or HRP, only a weak signal of ascorbyl radical was observed (Figure 6B, C and D). Ascorbic acid alone in phosphate buffer did not show any ESR radical indicating that, in the medium which is practically free of metals, ascorbate does not autoxidize at pH 7. It is useful to note that after the ascorbyl radical disappeared, the semiquinone ESR signal was not observed and the solution remained colourless.

Only a slight decrease of ascorbate signal occurred when the peroxidatic oxidation of Or took place in presence of both GSH and ascorbic acid at the same concentration. This observation suggests that the reactivity of semiquinone is higher with ascorbic acid than with glutathione. On the other hand, when the reaction was carried out in presence of DMSO and/or DMPO, we did not observe any further signal. In addition, the system containing Or, $H_2O_2$, ascorbic acid and HRP did not induce dioxygen uptake. These findings suggest a direct interaction between

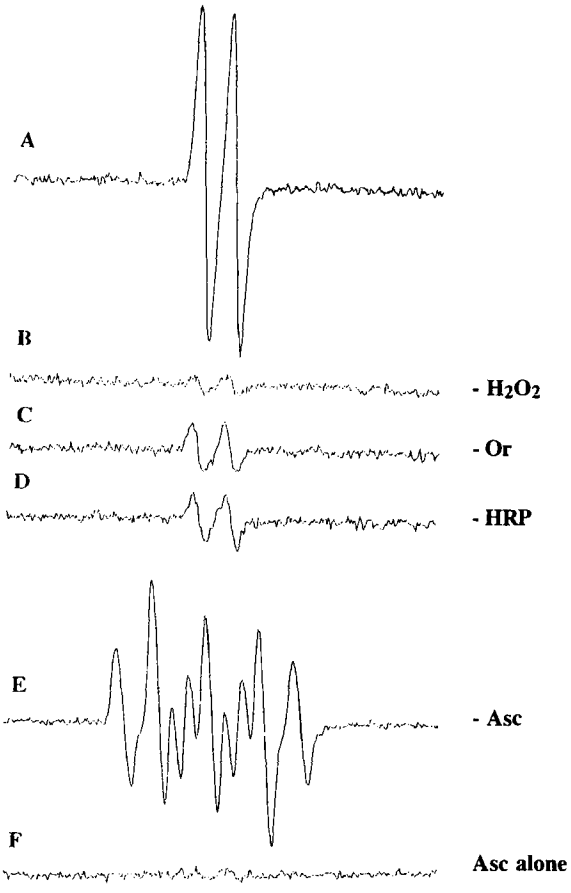



FIGURE 6 Oxidation of orellanine by the system HRP/$H_2O_2$ in presence of ascorbic acid. The reaction mixtures contained Or (5 mM), $H_2O_2$ (0.6 mM), ascorbic acid (4 mM) and HRP (8 μg ml$^{-1}$) in 100 mM phosphate buffer pH 7. A: complete system. The reaction was started by adding HRP to the solution containing Or, $H_2O_2$ and ascorbic acid; B: without $H_2O_2$; C: without Or; D: without HRP; E: without ascorbic acid; F: ascorbic acid alone. Spectrometer settings: modulation frequency: 100 kHz; modulation amplitude: 0.125 mT; microwave power: 20 mW; receiver gain: $5 \cdot 10^5$; scan time: 100 s; scan range: 3 mT; temperature: 20°C.

ascorbic acid and the semiquinone form of Or produced by HRP.

### *Thiols*

Or (5 mM) was mixed with $H_2O_2$ (0.6 mM), GSH (4 mM), DMPO (100 mM) and HRP (8 μg ml$^{-1}$). Figure 7 shows that the system instantaneously gave an intense ESR signal. This signal was assigned to the glutathionyl radical according to its hyperfine coupling constants ($a_N = 1.52$ mT

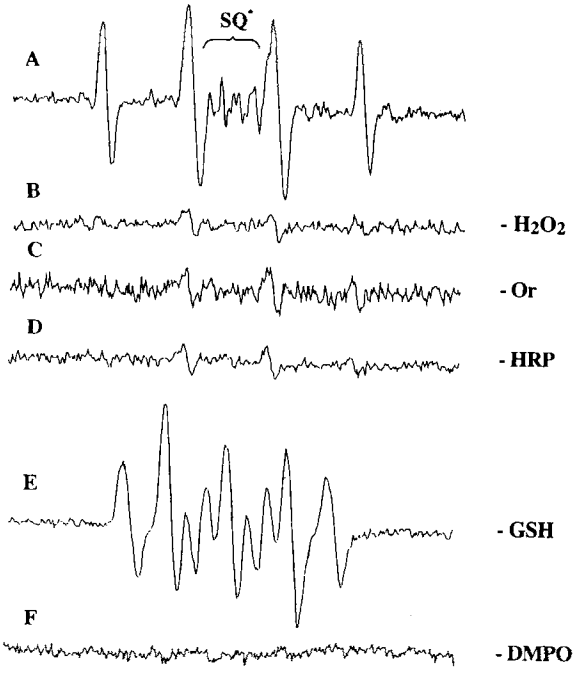



FIGURE 7 Oxidation of orellanine by the system HRP/$H_2O_2$ in presence of glutathione (GSH). The reaction mixtures contained Or (5 mM), $H_2O_2$ (0.6 mM), GSH (4 mM), HRP (8 $\mu g\,ml^{-1}$) and DMPO (100 mM) in phosphate buffer pH 7. A: complete system. B: without $H_2O_2$; C: without Or; D: without HRP; E: without glutathione. F: without DMPO. Spectrometer settings: see Figure 6 (scan range: 10 mT except for E).

and $a_H = 1.61$ mT). In the conditions used, the $t_{1/2}$ of glutathionyl radical is about 5 min. In addition to the glutathionyl radical, two other signals were observed which were very weak. The first one, located between the central lines of the glutathionyl adduct spectrum, corresponds to the *ortho*-semiquinone of Or. The second one probably relates to a degradation product of DMPO.

In the absence of either $H_2O_2$, or HRP, a very weak ESR spectrum of glutathionyl radical was observed (Figure 7B and D). As a control, in the absence of GSH, only the ESR signal of Or *ortho*-semiquinone radical was recorded (Figure 7E). A mixture of GSH, $H_2O_2$ and HRP gave only an insignificant but reproducible ESR signal as shown in Figure 7C, evidencing that GSH is a poor substrate for the HRP/$H_2O_2$ system. Taking into account that the DMPO adduct of glutathionyl radical has ESR splitting constants ($a_N$ = 1.52 mT and $a_H = 1.61$ mT) close to the ones of •OH ($a_N = 1.52$ mT and $a_H = 1.52$ mT), the question has arisen whether thiyl and hydroxyl radicals are simultaneously produced in the reaction. To answer this question, we used the spin-trapping method in presence of ethanol (5–10%, v/v), a known scavenger of hydroxyl radical. We did not observe the ethyl DMPO adduct signal, suggesting that hydroxyl radical was not produced in the medium. To provide further evidence to support the absence of reactive oxygen species production by the system containing Or, $H_2O_2$, GSH and HRP, the oxygen uptake was checked; the oxidation of Or in presence of GSH did not induce dioxygen consumption.

When cysteine was used instead of GSH, in a system containing Or, $H_2O_2$, cysteine, DMPO and HRP, a weak signal of cysteinyl radical was recorded. However, blanks showed a signal of cysteinyl radical in a medium containing $H_2O_2$, cysteine, DMPO and HRP, suggesting that contrarily to glutathione, cysteine is a good substrate for HRP/$H_2O_2$.

## DISCUSSION

The formation of a characteristic *ortho*-semiquinone radical anion derived from Or was demonstrated by direct ESR technique in a system containing HRP and $H_2O_2$. The spectra of the orellanine *ortho*-semiquinone radical generated in the HRP/$H_2O_2$ system appeared without any lag period. The orellanine *ortho*-semiquinone ESR signal decreased when orellanine concentration was increased above 5 mM. This finding could be explained by the inhibition of HRP by Or which is present in excess in the medium, by the fast dimerization or polymerization of *ortho*-semiquinone molecules, by the further oxidation of semiquinone into quinone, or by the scavenging of the *ortho*-semiquinone radical by Or. The pH dependence of the radical formation is likely to be

related to the pH dependence of the enzyme activity.

As demonstrated for the Or/tyrosinase/dioxygen system, stabilization procedures or slow-flow techniques were not required to detect the orellanine *ortho*-semiquinone radical by ESR spectrometry in the system containing Or, $H_2O_2$ and HRP while they are indispensable for observing catechol(amine) semiquinone radicals.[13]

The fact that the semiquinone was obtained in anaerobic as well as in aerobic medium and decays similarly suggests that dioxygen is not involved in the formation of *ortho*-semiquinone and quinone. This finding was supported by the aforementioned dioxygen uptake experiments which show that a solution containing Or, $H_2O_2$ and HRP did not induce any detectable dioxygen consumption. When the spin-trap DMPO was added to the Or/$H_2O_2$/HRP system, neither DMPO–$^{\bullet}$OOH nor DMPO–$^{\bullet}$OH signals were observed. The absence of dioxygen consumption and of oxygen radicals is not surprising because $H_2O_2$ and not $O_2$ is the cosubstrate of HRP. Absence of oxygen radicals formation was noticed during the enzymatic generation of other semiquinone radicals like for catechol(amine)s.[14]

Since peroxidatic oxidation proceeds via two one-electron oxidation steps, this enzyme can oxidize two molecules of Or to the corresponding semiquinone during one enzymatic cycle or one molecule of Or to the corresponding quinone by two successive monoelectronic oxidation steps. The *ortho*-semiquinone form of Or was also generated by the enzymatic system tyrosinase/$O_2$ which proceeds via a two-electron oxidation. In the latter case, the generation of the *ortho*-semiquinone radical likely occurs as a result of an equilibrium between Or and its corresponding quinone.[4] Thus, depending upon the enzymatic system used, *ortho*-semiquinone radical production may occur by either a direct or an indirect mechanism. During oxidation of Or in renal tissue, *ortho*-semiquinone likely accumulates whatever the mode of enzymatic oxidation may be (one or two electrons). The *ortho*-semiquinone as well as the quinone could participate in a variety of reactions including covalent bonding with biological compounds,[15] leading to cell damage.

In addition, when oxidation of Or by HRP/$H_2O_2$ occurred in presence of reducing agents of biological interest such as ascorbic acid or glutathione, the *ortho*-semiquinone ESR signal was almost completely abolished, and the ESR signals of ascorbyl or glutathionyl radicals were observed instead, in agreement with previous work demonstrating a significant decrease in the level of glutathione in the kidneys of rats treated with *orellanus* homogenate.[3]

The findings with parent molecules of Or obviously show that both catecholic moieties and aminoxide groups are necessary for observing the formation of a relatively stable semiquinone form of the toxin. In other words, a slight variation of substituents on the orellanine bipyridine structure can result into major modulation of the overall toxicity of this toxin. This observation is in good agreement with previous work.[16]

### *Acknowledgements*

Thanks are due to A. Jeunet (Laboratoire d'Etudes Dynamiques et Structurales de la Sélectivité URA CNRS 332) and to Professor M. Tissut (Laboratoire de Physiologie Cellulaire Végétale) at the Université J. Fourier of Grenoble for allowing us to use some of their equipment. We are indebted to Professor C. Quéguiner for the gift of synthetic orelline.

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