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Phenoxyl Free Radical Formation during the Oxidation of the Fluorescent Dye 2′,7′-Dichlorofluorescein by Horseradish Peroxidase

POSSIBLE CONSEQUENCES FOR OXIDATIVE STRESS MEASUREMENTS*

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The oxidation of the fluorescent dye 2′,7′-dichlorofluorescein (DCF) by horseradish peroxidase was investigated by optical absorption, electron spin resonance (ESR), and oxygen consumption measurements. Spectrophotometric measurements showed that DCF could be oxidized either by horseradish peroxidase-compound I or -compound II with the obligate generation of the DCF phenoxyl radical (DCF$^{\bullet}$). This one-electron oxidation was confirmed by ESR spin-trapping experiments. DCF$^{\bullet}$ oxidizes GSH, generating the glutathione thiyl radical (GS$^{\bullet}$), which was detected by the ESR spin-trapping technique. In this case, oxygen was consumed by a sequence of reactions initiated by the GS$^{\bullet}$ radical. Similarly, DCF$^{\bullet}$ oxidized NADH, generating the NAD$^{\bullet}$ radical that reduced oxygen to superoxide ($O_2^{\bullet-}$), which was also detected by the ESR spin-trapping technique. Superoxide dismutated to generate H_2O_2, which reacted with horseradish peroxidase, setting up an enzymatic chain reaction leading to H_2O_2 production and oxygen consumption. In contrast, when ascorbic acid reduced the DCF phenoxyl radical back to its parent molecule, it formed the unreactive ascorbate anion radical. Clearly, DCF catalytically stimulates the formation of reactive oxygen species in a manner that is dependent on and affected by various biochemical reducing agents. This study, together with our earlier studies, demonstrates that DCFH cannot be used conclusively to measure superoxide or hydrogen peroxide formation in cells undergoing oxidative stress.

2′,7′-Dichlorofluorescin (DCFH)[1] is widely used to measure oxidative stress in cells. The diacetate form of DCFH enters the cell and is hydrolyzed by intracellular esterases to liberate DCFH. Upon reaction with oxidizing species, the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF) is formed. The fluorescence intensity can be measured and is the basis of the popular cellular assay for oxidative stress (1).

Although this assay is commonly used, there are many controversies regarding its real validity. It is not clear which oxidative species is responsible for the oxidation of DCFH. Furthermore, there are some disagreements in the literature concerning the effect of superoxide dismutase on oxidative stress in cells monitored by the DCF fluorometric assay. Some studies report an inhibitory effect by superoxide dismutase (2–15), while almost the same number of studies report no effect (16–26). A recent paper by LeBel *et al.* (27) mentioned that the interpretation of specific reactive oxygen species involved in the oxidation of DCFH to DCF in biological systems should be approached with caution. It has also been demonstrated that the photoreduction of DCF results in the formation of the DCF semiquinone free radical (DCF$^{\bullet-}$), which, under aerobic conditions, is oxidized by oxygen to its parent dye, DCF, concomitantly forming superoxide radical (28). Moreover, it was reported that DCFH, upon reacting with horseradish peroxidase-compound I or -compound II, was oxidized to DCF$^{\bullet-}$, which was subsequently air-oxidized to DCF with the concurrent generation of superoxide radical (29).

In the present work, the reaction of DCF with horseradish peroxidase in the presence of reduced glutathione or NADH was studied to continue our investigation of the DCF fluorometric assay. We employed the ESR spin-trapping technique and measured the oxygen consumption to evaluate free radical formation during the reactions along with the formation of compound I and compound II monitored by UV-visible spectrophotometry.

Our results indicate that when DCF reacts with compound I and compound II, DCF is oxidized to the phenoxyl free radical DCF$^{\bullet}$, reducing the respective horseradish peroxidase enzyme intermediates (Scheme 1). In the presence of a reducing agent, such as GSH or NADH, DCF$^{\bullet}$ is then reduced back to DCF with the formation of GS$^{\bullet}$ or NAD$^{\bullet}$, respectively, and the subsequent generation of superoxide.

EXPERIMENTAL PROCEDURES

Chemicals—Horseradish peroxidase type VI-A (EC 1.11.1.7), porcine liver esterase (EC 3.1.1.1), diethylenetriamine pentaacetic acid, DCF, and the spin trap 2-methyl-2-nitrosopropane (MNP) were purchased from Sigma. Tris and Chelex 100 resin were purchased from Bio-Rad. Catalase (from beef liver, 65,000 units/mg) (EC 1.11.1.6) and superoxide dismutase (from bovine erythrocytes, 5,000 units/mg) (EC 1.15.1.1) were purchased from Roche Molecular Biochemicals. Hydrogen peroxide (30%) was purchased from Fisher.

All of the reactions were carried out in a 50 mM Tris buffer adjusted to pH 7.4 with hydrochloric acid. The Tris-HCl buffer was treated with Chelex 100 resin to remove traces of transition metal ions and contained 50 μM diethylenetriamine pentaacetic acid to minimize the possibility of trace metal catalysis. DCF was dissolved with methanol to form a 12.5 mM solution, which was then diluted to 500 μM with a pH 7.4 Tris-HCl buffer (*i.e.* methanol-buffer solution). In the experiments

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[1] The abbreviations used are: DCFH, 2′,7′-dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DCF, 2′,7′-dichlorofluorescein; DCF$^{\bullet}$, DCF phenoxyl free radical; DCF$^{\bullet-}$, DCF semiquinone radical; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; ESR, electron spin resonance; GS$^{\bullet}$, glutathione thiyl radical; MNP, 2-methyl-2-nitrosopropane; SOD, superoxide dismutase; HRP, horseradish peroxidase.

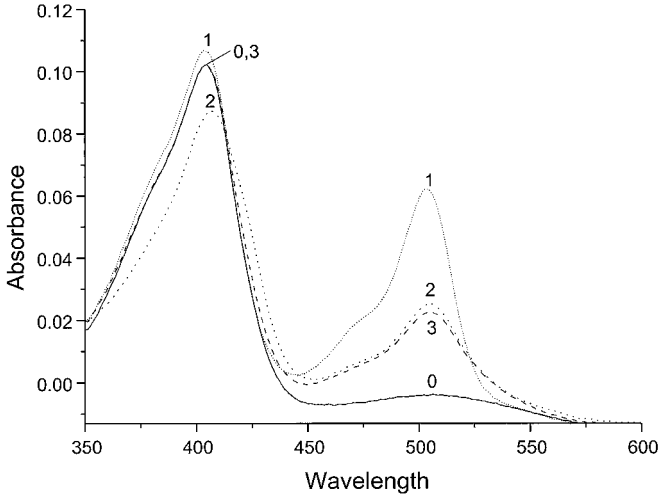


FIG. 1. **Absorption spectra changes during the reaction of DCF, H_2O_2, and horseradish peroxidase.** Horseradish peroxidase (1 μM) in Tris-HCl buffer is represented by *scan 0*. *Scan 1* was taken immediately after the addition of 1 μM DCF; *scans 2* and *3* were taken immediately and 2 min after the subsequent addition of 1 μM H_2O_2, respectively. All spectra were recorded at a rate of 5 nm/s, yielding a complete spectrum in 50 s.

shown in Fig. 2, a 25 mM DCF methanol solution was directly added to the samples. MNP was prepared in methanol and kept in the dark during the experiments. The spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Sigma, purified by vacuum sublimation at ambient temperature, and stored at −70 °C until use. 2′,7′-Dichlorofluorescin diacetate (also known as 2′,7′-dichlorodihydrofluorescein diacetate, DCFH-DA) was purchased from Molecular Probes, Inc. (Eugene, OR). DCFH-DA was enzymatically deesterified; *i.e.* 500 μM DCFH-DA in a methanol-buffer solution reacted with esterase (100 units) in the dark at room temperature for 1 h at neutral pH. Concentrations of horseradish peroxidase were determined by using the extinction coefficient ϵ = 102 mM^{-1} cm^{-1} at 403 nm (30). Stock concentrations of H_2O_2 in deionized water were determined by using the extinction coefficient ϵ = 43.6 M^{-1} cm^{-1} at 240 nm (31).

DCF and DCFH were stored and kept from room light. Sample preparation and experiments were performed in the dark. All reactions were initiated with the addition of horseradish peroxidase. In the samples where DCFH or DCF was omitted, an equivalent volume of methanol-buffer solution was added.

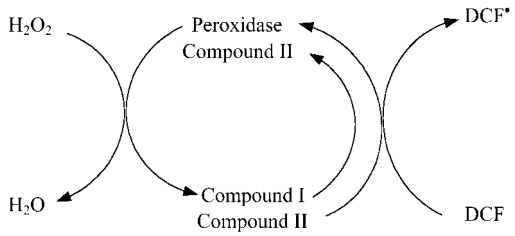
Electron Spin Resonance Experiments—ESR spectra were recorded on a Bruker ECS-106 ESR spectrometer (Billerica, MA) operating at 9.77 GHz with a modulation frequency of 50 kHz equipped with a TM_{110} cavity. All experiments were performed at room temperature with a 17-mm quartz flat cell. In the experiments described in Figs. 2, 3, and 8, samples were aspirated into the flat cell with ESR data acquisition started approximately 15 s after sample preparations. The data analysis and spectral simulation were performed using programs developed in our laboratory and are available through the Internet. The details of the program are described elsewhere (32). The low energy structure of MNP-DCF radical adduct was obtained using the MOPAC with MM2 minimization.

Horseradish Peroxidase UV-visible Spectra—All optical measurements were carried out with an SLM Aminco DW-2000 UV-visible dual beam spectrophotometer (Urbana, IL). The samples were prepared and recorded in deionized water (used in place of Tris buffer to prevent any reductant in the solution).

Oxygen Consumption Experiments—Oxygen consumption measurements were made using a Clark-type oxygen electrode fitted to a 1.8-ml Gilson sample cell and monitored by a YSI Inc. model 53 oxygen monitor. Oxygen consumption data were recorded by a computer interfaced with a DT2801 Data Translation board connected to the oxygen monitor. All of the experiments were performed at room temperature, and the incubation conditions are described in the figure legends.

RESULTS

The UV-visible spectra of the resting state of compound I and compound II reacting with DCF are shown in Fig. 1. The UV-visible spectrum of the resting state of horseradish peroxidase (1 μM) is characterized by its maximum absorption at 403 nm (Fig. 1, *scan 0*). The addition of DCF to horseradish peroxidase solution resulted in a small increase in the absorption at 403 nm due to the fact that DCF has absorption at this frequency (maximum at 502 nm) as shown in Fig. 1, *scan 1*. The significant decrease in intensity at 502 nm when H_2O_2 was added to the system demonstrated that DCF had been transformed during the reaction of DCF with horseradish peroxidase in the presence of H_2O_2 (Fig. 1, *scan 2*). In this case, compound I formed by the addition of H_2O_2 to horseradish peroxidase rapidly reacted with DCF to form the DCF$^{\bullet}$ radical and compound II, which is characterized by the appearance of an isosbestic point for horseradish peroxidase and its compound II at 412 nm (33). *Scan 3* in Fig. 1 shows the conversion of compound II back to the horseradish peroxidase resting state. These results are consistent with the peroxidase-mediated metabolism of DCF to the corresponding DCF$^{\bullet}$ radical as shown in Scheme 1.



SCHEME 1

The ESR spin-trapping technique was employed to characterize the free radical formed during the turnover of horseradish peroxidase with H_2O_2 and DCF in the presence or absence of a reducing agent. When DCF was reacted with horseradish peroxidase and H_2O_2 in the presence of spin trap MNP, an isotropic three-line spectrum with a hyperfine coupling constant of 14.9 G was detected (Fig. 2*A*). The absence of any hydrogen or chlorine hyperfine couplings in the three-line spectrum excluded these atoms at positions α or β to the nitroxide nitrogen and indicated that the radical was located on a tertiary carbon (34, 35). The structure of the radical adduct based on our ESR data and energy minimization is shown in Fig. 2. The radical formation was completely dependent on the presence of DCF (Fig. 2*B*). The absence of either horseradish peroxidase or H_2O_2 resulted in a very weak signal (Fig. 2, *C* and *D*) consisting, for the most part, of the di-*tert*-butylnitroxide from the decomposition of MNP characterized by a hyperfine coupling constant of 17 G (36). This weak signal is also present in the solution of MNP in buffer (Fig. 2*E*). Based on the characteristics of its ESR signal and the similarity of the hyperfine coupling constant compared with that previously obtained from the trapping of MNP/tyrosyl radical (37), we assigned the radical as the phenoxyl free radical of DCF.

When DCF was incubated with a solution of GSH, H_2O_2, and horseradish peroxidase in the presence of the spin trap DMPO, a four-line ESR spectrum of the DMPO/GS$^{\bullet}$ adduct was detected (Fig. 3*A*), characterized by hyperfine coupling constants of a^N = 15.15 G and a^H_β = 16.17 G, consistent with those previously reported (38, 39). The same radical adduct was obtained from a system where DCF was omitted, but the ESR spectrum was of lower intensity (Fig. 3*B*). The radical adduct depended on the presence of GSH or horseradish peroxidase (Fig. 3, *C* and *D*). The addition of superoxide dismutase did not have any effect (data not shown), eliminating the possibility of superoxide-dependent glutathione thiyl radical formation. However, the addition of catalase completely eliminated the ESR signal (Fig. 3*E*), indicating the radical adduct formation is H_2O_2-dependent. When H_2O_2 was omitted, a weak spectrum of the DMPO/GS$^{\bullet}$ adduct was detected (Fig. 3*F*) and was com-

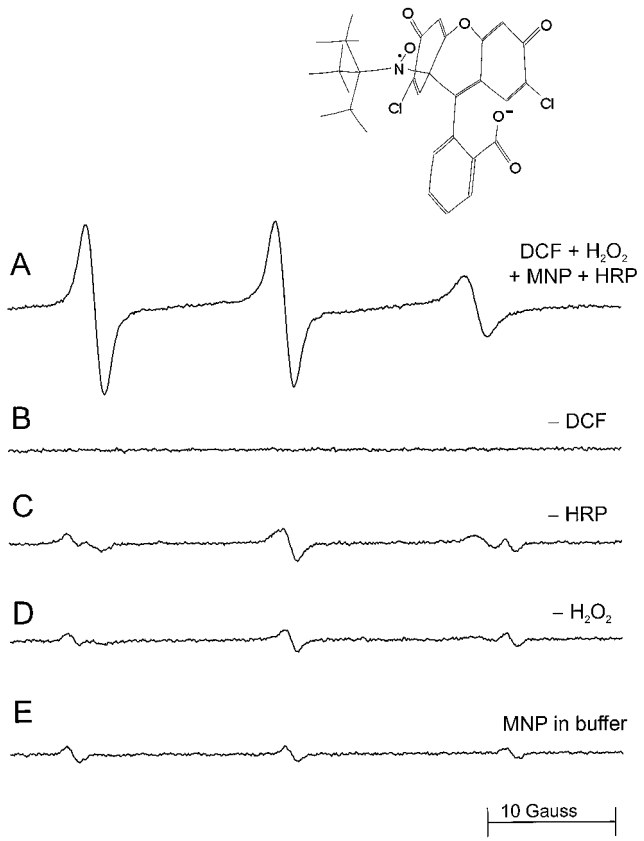


FIG. 2. **ESR spectra of the MNP radical adduct produced during the reaction of DCF, H_2O_2, and horseradish peroxidase.** *A*, the ESR spectrum obtained from a reaction mixture containing 4 mM DCF, 1 mM H_2O_2, 50 mM MNP, and 1 μM horseradish peroxidase (HRP). *B*, the same as *A*, except DCF was omitted. *C*, the same as *A*, but with the omission of HRP. *D*, the same as *A*, but with the omission of H_2O_2. *E*, the spectrum obtained from a solution of MNP alone in Tris buffer. The hyperfine coupling constants are provided under "Results." Spectrometer conditions were as follows: modulation amplitude, 1 G; microwave power, 40 milliwatts; time constant, 0.328 s; conversion time, 0.164 s; scan time, 168 s; receiver gain, 5×10^4.

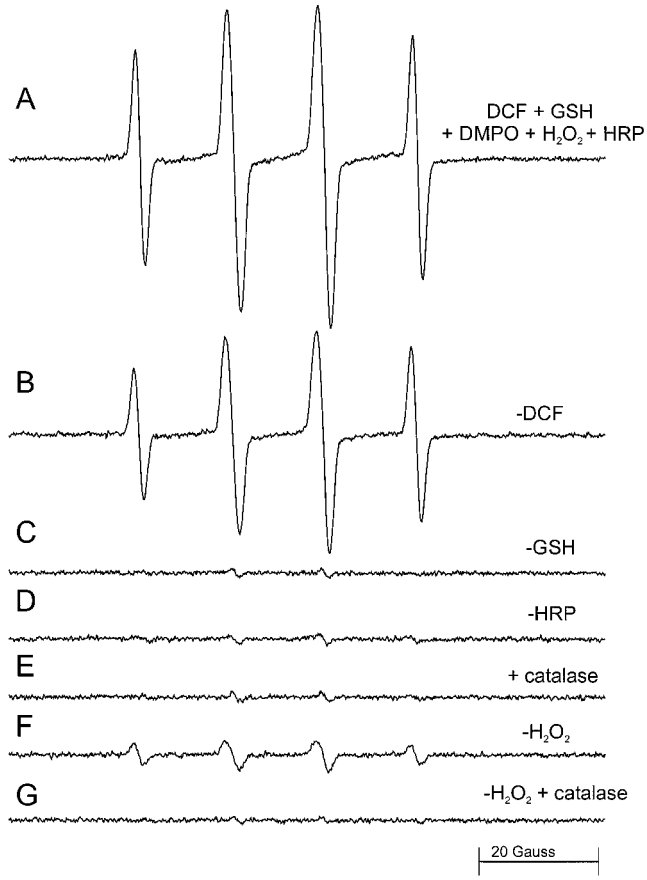


FIG. 3. **ESR spectra of the DMPO/GS$^{\cdot}$ radical adduct produced during the reaction of DCF, GSH, H_2O_2, and horseradish peroxidase.** *A*, the ESR spectrum obtained from a reaction mixture containing 10 μM DCF, 1 mM GSH, 32 μM H_2O_2, 200 mM DMPO, and 0.2 μM horseradish peroxidase. *B*, the same as *A*, except DCF was omitted. *C*, the same as *A*, but with the omission of GSH. *D*, the same as *A*, but with the omission of HRP. *E*, the same as *A*, but with the addition of 150 μg/ml catalase. *F*, the spectrum obtained using the conditions of *A*, except H_2O_2 was substituted with an equal volume of deionized water. *G*, the same as *F*, but with the addition of 150 μg/ml catalase. The hyperfine coupling constants are provided under "Results." Spectrometer conditions were as follows: modulation amplitude, 1 G; microwave power, 20 milliwatts; time constant, 0.164 s; conversion time, 0.164 s; scan time, 168 s; receiver gain, 5×10^4.

pletely suppressed by the addition of catalase, indicating the formation of H_2O_2 via oxidation of GSH by trace metal catalysis.

Moreover, molecular oxygen was consumed in a reaction mixture containing DCF, H_2O_2, and GSH (Fig. 4*a*). The duration of the fast rate of oxygen consumption strongly depended on the concentration of hydrogen peroxide (Fig. 4*a*, *A* and *C*). When DCF was reacted with GSH, horseradish peroxidase, and H_2O_2, the rate of oxygen consumption was very high for ~1 min, and then it decreased (Fig. 4*a*, *A*). This is due to the consumption of H_2O_2 in this first minute, as is demonstrated by the fact that the addition of more H_2O_2 restored the initial rate of oxygen consumption (Fig. 4*a*, *B*) until all of the oxygen in the solution was consumed.

When DCF, GSH, or horseradish peroxidase was omitted, no oxygen consumption was observed (Fig. 4*b*, *E–G*). However, when ascorbic acid (1 mM) was added to the reaction mixture prior to or after the horseradish peroxidase initiation, the oxygen consumption was completely inhibited (Fig. 4*b*, *H* and *B*, respectively). The same inhibition was obtained even when 100 μM of ascorbate was used (data not shown). In addition, the oxygen consumption was inhibited when DMPO (200 mM) was added prior to or after the horseradish peroxidase initiation (Fig. 4*b*, *D* and *C*, respectively). When superoxide dismutase (100 μg/ml) was added to the reaction mixture before horseradish peroxidase initiation (Fig. 4*c*, *A*), the initial rate of oxygen consumption was found to be identical to that of the complete system (Fig. 4*c*, *B*). However, in the presence of superoxide dismutase (Fig. 4*c*, *A*), the oxygen consumption did not slow after 1 min, in contrast to that of the complete system, but completely stopped approximately 2 min after the horseradish peroxidase initiation (Fig. 4*c*, *A*). Apparently, superoxide dismutase, catalyzing the disproportionation of superoxide radicals, facilitated H_2O_2 formation, keeping the consumption rate high for a longer time. The addition of superoxide dismutase 90 s after horseradish peroxidase initiation did completely inhibit the oxygen consumption (Fig. 4*c*, *C*). In this case, superoxide dismutase had been added when the H_2O_2 was already consumed, and this slower oxygen consumption came almost completely from a free radical chain reaction that is completely superoxide-dependent. The addition of catalase (150 μg/ml) prior to or after horseradish peroxidase initiation completely inhibited the oxygen consumption (Fig. 4*c*, *D* and *E*).

When added hydrogen peroxide was omitted from the complete system, the rate of oxygen consumption was much slower (Fig. 5*A*). The addition of more diethylenetriamine pentaacetic acid (from 250 to 800 μM in final concentration) slowed the rate of oxygen consumption further without stopping it completely

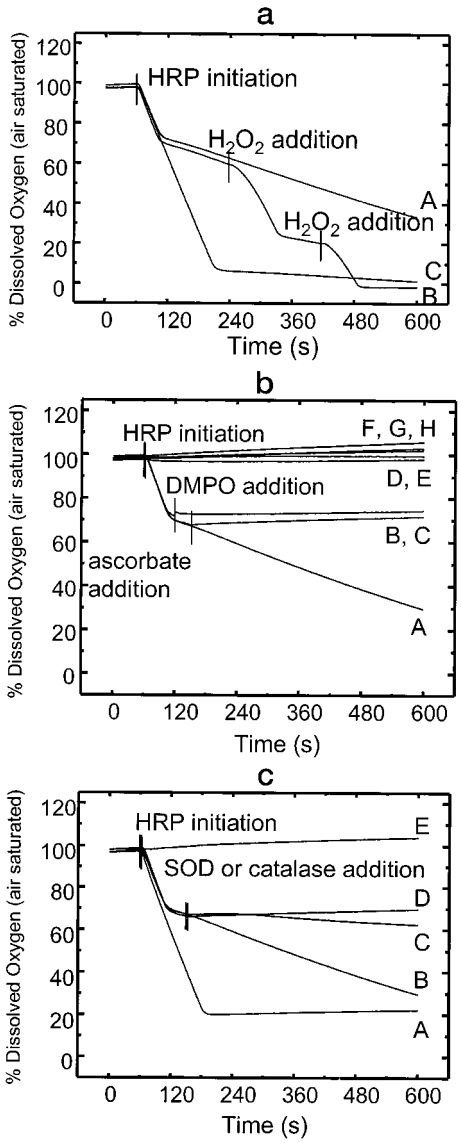


FIG. 4. **Rate of oxygen consumption during the oxidation of DCF by horseradish peroxidase in the presence of GSH and H_2O_2.** Effect of the addition of DMPO, ascorbate, superoxide dismutase, and catalase. The *vertical bars* mark the time of the addition of HRP, H_2O_2, DMPO, ascorbate, superoxide dismutase (*SOD*), and catalase to the system. *a*, rate of oxygen consumption during the oxidation of DCF by HRP in the presence of GSH and H_2O_2. *A*, complete system containing 100 μM DCF, 5 mM GSH, 32 μM H_2O_2, and 1 μM HRP. *B*, as in *A*, but with additional H_2O_2 (32 μM). *C*, as in *A*, but with the addition of 150 μM H_2O_2. *b*, effect of ascorbate or DMPO on the rate of oxygen consumption during the oxidation of DCF by HRP in the presence of GSH and H_2O_2. *A*, complete system containing 100 μM DCF, 5 mM GSH, 32 μM H_2O_2, and 1 μM HRP. *B*, as in *A*, but with the addition of 1 mM ascorbate 90 s after the addition of HRP. *C*, as in *A*, but with the addition of 200 mM DMPO 1 min after the addition of HRP. *D*, as in *A*, but with the addition of 200 mM DMPO before the addition of HRP. *E*, as in *A*, but with the omission of DCF. *F*, as in *A*, but with the omission of GSH. *G*, as in *A*, but with the omission of HRP. *H*, as in *A*, but with the addition of 1 mM ascorbate before the addition of HRP. *c*, effect of SOD or catalase on the rate of oxygen consumption during the oxidation of DCF by HRP in the presence of GSH and H_2O_2. *A*, complete system with the addition of 100 μg/ml SOD before the addition of HRP. *B*, complete system containing 100 μM DCF, 5 mM GSH, 32 μM H_2O_2, and 1 μM HRP. *C*, as in *A*, but with the addition of 100 μg/ml SOD 90 s after the addition of HRP. *D*, as in *B*, but with the addition of 50 μg/ml catalase 90 s after the addition of HRP. *E*, as in *B*, but with the addition of 50 μg/ml catalase before the addition of HRP.

(data not shown), demonstrating the involvement of metal catalysis. Moreover, the addition of superoxide dismutase (100 μg/ml) to the reaction mixture right before horseradish peroxidase completely inhibited the oxygen consumption (Fig. 5*D*). A similar inhibition was obtained when superoxide dismutase was added 2 min after the addition of horseradish peroxidase (Fig. 5*B*). These results show that, in the absence of H_2O_2, oxygen consumption arose completely from a superoxide-dependent reaction. When catalase was added to the reaction mixture before or after horseradish peroxidase initiation, the oxygen consumption was completely inhibited (Fig. 5, *C* and *E*), indicating that the presence of a small amount of hydrogen peroxide in the system was also necessary for oxygen consumption.

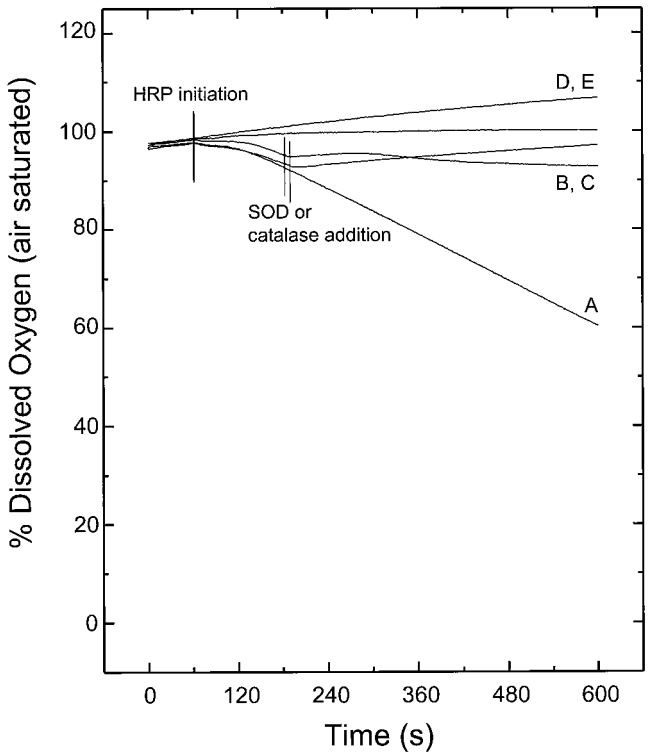


FIG. 5. **Effect of superoxide dismutase and catalase on the rate of oxygen consumption during the oxidation of DCF by horseradish peroxidase in the presence of GSH.** The *vertical bars* mark the time of the addition of HRP, SOD, or catalase to the system. *A*, complete system containing 100 μM DCF, 5 mM GSH, and 1 μM HRP. *B*, as in A, but with the addition of 100 μg/ml SOD 2 min after the addition of HRP. *C*, as in *A*, but with the addition of 50 μg/ml catalase 2 min after the addition of HRP. *D*, as in *A*, but with the addition of 100 μg/ml SOD before the addition of HRP. *E*, as in *A*, but with the addition of 50 μg/ml catalase before the addition of HRP.

When DCF was incubated with NADH and horseradish peroxidase in the presence of the spin trap DMPO, a composite ESR spectrum was detected (Fig. 6*A*). A similar ESR spectrum, with much lower intensity, was observed in the same system without DCF (Fig. 6*B*). When NADH (Fig. 6*D*) or horseradish peroxidase (Fig. 6*E*) was omitted from the system, no signal or only a very weak signal was detected. The addition of superoxide dismutase completely inhibited the radical formation (Fig. 6*F*), confirming the involvement of superoxide radicals in the formation of the DMPO radical adducts. The addition of catalase had the same inhibitory effect as superoxide dismutase (Fig. 6*G*), demonstrating that the presence of H_2O_2, at least in traces, was necessary for the radical reaction to proceed. The hydrogen peroxide, essential for the initial activation of horseradish peroxidase, presumably resulted from autoxidation of NADH.

In order to characterize the radical formed during the spin-trapping study of DCF oxidation by horseradish peroxidase in the presence of NADH, the ESR spectrum of the system with-

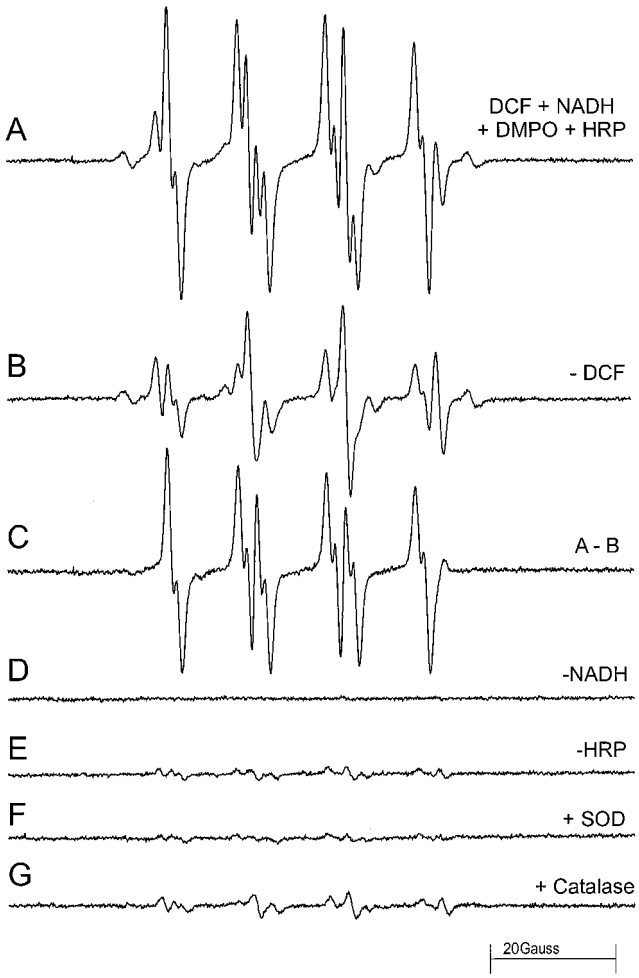


FIG. 6. **ESR spectra of DMPO radical adducts produced by the reaction mixture containing DCF, NADH, and horseradish peroxidase.** *A*, the ESR spectrum obtained from a reaction mixture containing 45 μM DCF, 1 mM NADH, 200 mM DMPO, and 0.2 μM HRP. *B*, the same as *A*, except DCF was omitted. *C*, the spectrum obtained by the subtraction of spectrum B from spectrum A. *D*, the same as *A*, but with the omission of NADH. *E*, the same as *A*, but with the omission of HRP. *F*, the same as *A*, but with the addition of 50 μg/ml SOD. *G*, the same as *A*, but with the addition of 150 μg/ml catalase. The hyperfine coupling constants of each species are provided under "Results." Spectrometer conditions were as follows: modulation amplitude, 1 G; microwave power, 20 milliwatts; time constant, 0.328 s; conversion time, 0.655 s; scan time, 671 s; receiver gain, 4×10^5.

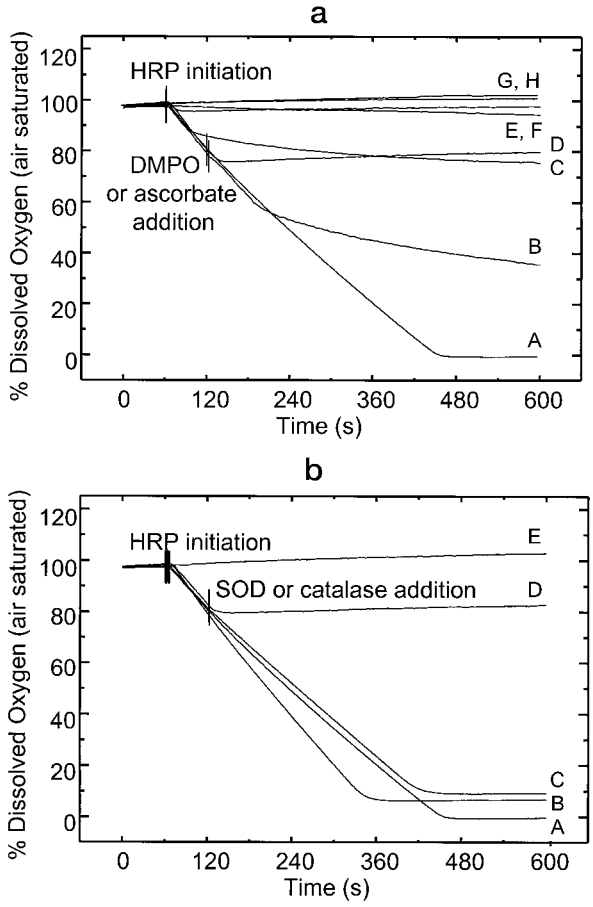


FIG. 7. **Rate of oxygen consumption during the oxidation of DCF by HRP in the presence of NADH.** The *vertical bars* mark the time of the addition of HRP, DMPO, ascorbate, SOD, and catalase to the system. *a*, effect of ascorbate and DMPO on the rate of oxygen consumption during the oxidation of DCF by HRP in the presence of NADH. *A*, complete system containing 100 μM DCF, 2 mM NADH, and 1 μM HRP. *B*, as in *A*, but with the addition of 200 mM DMPO 1 min after the addition of HRP. *C*, as in *A*, but with the addition of 200 mM DMPO before the addition of HRP. *D*, as in *A*, but with the addition of 1 mM ascorbate 1 min after the addition of HRP. *E*, as in *A*, but with the omission of DCF. *F*, as in *A*, but with the addition of 1 mM ascorbate before the addition of HRP. *G*, as in *A*, but with the omission of NADH. *H*, as in *A*, but with the omission of HRP. *b*, effect of SOD and catalase on the rate of oxygen consumption during the oxidation of DCF by HRP in the presence of NADH. *A*, complete system containing 100 μM DCF, 2 mM NADH, and 1 μM HRP. *B*, as in *A*, but with the addition of 100 μg/ml SOD before the addition of HRP. *C*, as in *A*, but with the addition of 100 μg/ml SOD 1 min after the addition of HRP. *D*, as in *A*, but with the addition of 50 μg/ml catalase 1 min after the addition of HRP. *E*, as in *A*, but with the addition of 50 μg/ml catalase before the addition of HRP.

out DCF (Fig. 6*B*) was subtracted from that of the complete system (Fig. 6*A*) to yield a single radical species (Fig. 6*C*). Based on the analysis of hyperfine coupling constants and characteristics of the radical, it was assigned as the DMPO-superoxide radical adduct, DMPO/$^{\cdot}$OOH, with hyperfine coupling constants of $a^{N} = 14.16$ G, $a^{H}_{\beta} = 11.25$ G, and $a^{H}_{\gamma} = 1.2$ G, consistent with those previously reported (40).

Furthermore, oxygen consumption was investigated in the same reaction mixture containing DCF, NADH, and horseradish peroxidase with the addition of catalase, SOD, or ascorbate. The results are shown in Fig. 7*a*. Molecular oxygen in the reaction mixture was completely consumed in less than 8 min (Fig. 7*a*, *A*). When DCF, NADH, or horseradish peroxidase was omitted from the system, no oxygen consumption was observed (Figs. 7*a*, *E*, *G*, and *H*, respectively). When ascorbic acid (1 mM) was added prior to or after horseradish peroxidase initiation, the oxygen consumption was completely inhibited (Figs. 7*a*, *F* and *D*, respectively). In addition, the same inhibition was observed when 100 μM ascorbate was added before horseradish peroxidase or 250 μM ascorbate was added after horseradish peroxidase (data not shown). When the spin trap DMPO was added after or prior to horseradish peroxidase initiation, the oxygen consumption was inhibited (Figs. 7*a*, *B* and *C*, respectively). These results are consistent with radical formation during the oxidation of DCF by horseradish peroxidase in the presence of NADH as demonstrated by ESR. The addition of catalase to the complete system also strongly inhibited oxygen consumption (Fig. 7*b*, *D* and *E*). When superoxide dismutase was added to the reaction mixture prior to horseradish peroxidase initiation, the rate of oxygen consumption was found to be slightly faster than that of the complete system and was completely inhibited after 6 min (Fig. 7*b*, *B*). The addition of superoxide dismutase 1 min after the addition of horseradish peroxidase possibly had a weak inhibitory effect (Fig. 7*b*, *C*).

Ascorbate is known to undergo one-electron oxidation to produce the relatively stable ascorbate free radical that can be

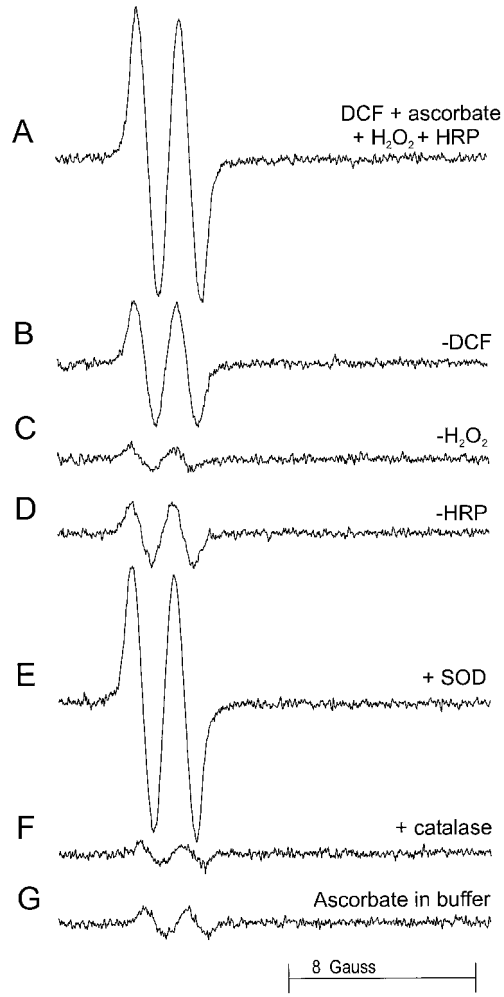


FIG. 8. **ESR spectrum of ascorbate anion radical obtained from a reaction mixture containing DCF, ascorbate, horseradish peroxidase, and H_2O_2.** *A*, the ESR spectrum obtained from a reaction mixture containing 45 μM DCF, 100 μM ascorbate, 5 μM H_2O_2, and 0.04 μM HRP. *B*, the same as *A*, except DCF was omitted. *C*, the same as *A*, but with the omission of H_2O_2. *D*, the same as *A*, but with the omission of HRP. *E*, the same as *A*, but with the addition of 50 μg/ml SOD. *F*, the same as *A* but with the addition of 150 μg/ml catalase. *G*, the ESR spectrum obtained from a solution containing 100 μM ascorbate in buffer. The hyperfine coupling constant of the radical species is provided under "Results." Spectrometer conditions were as follows: modulation amplitude, 1 G; microwave power, 20 milliwatts; time constant, 0.164 s; conversion time, 0.164 s; scan time, 168 s; receiver gain, 4×10^5. The center field was offset to a higher field so the ascorbate ESR spectrum would be scanned earlier in the spectral sweep.

detected by direct ESR. A reaction mixture of DCF, H_2O_2, and horseradish peroxidase in the presence of ascorbate gave a typical doublet ESR signal of the ascorbate anion free radical as shown in Fig. 8*A* with the characteristic hyperfine coupling constant of $a^H = 1.79$ G (41). No changes in the ascorbate anion radical spectrum were observed upon the addition of GSH (same concentration as ascorbate) to the reaction mixture (data not shown), indicating that DCF$^{\bullet}$ phenoxyl radical reacted with ascorbate instead of GSH (42). In this case, ascorbate was a better radical scavenger than GSH. The omission of DCF, H_2O_2, or horseradish peroxidase resulted in a weaker signal (Fig. 8, *B–D*). The addition of catalase (50 μg/ml) to the reaction mixture inhibited the formation of ascorbate anion radical (Fig. 8*F*) to the level found with ascorbate alone (Fig. 8*G*). The presence of superoxide dismutase (50 μg/ml) in the reaction mixture had no effect on the ESR signal (Fig. 8*E*), suggesting that superoxide radicals were not involved in either ascorbate radical formation or decay.

In order to better understand the chemistry involved between DCFH and DCF, we measured oxygen consumption from a reaction mixture containing the reduced leuco compound DCFH and horseradish peroxidase. When DCFH reacted with horseradish peroxidase in the presence of NADH under room light, oxygen was consumed within 12 min (Fig. 9*A*). Fig. 9*B* shows that for approximately 12 min, the rate of oxygen consumption in the dark was similar to that of the complete system without DCFH (Fig. 9*C*). Then the consumption rate increased dramatically and consumed all of the oxygen within the next 5 min. This 12-min lag phase probably represents the time required to oxidize DCFH to produce enough DCF (29) to support the oxygen-consuming reactions between DCF, horseradish peroxidase, and NADH (Fig. 7, *a* and *b*). When NADH was omitted from the system in the dark, no oxygen was consumed (Fig. 9*E*). When the experiment was repeated under room light, no lag phase was detected, and the rate of oxygen consumption of the complete system was much faster (Fig. 9*A*). This result demonstrates that the oxidation of DCFH to DCF is also catalyzed by room light. When NADH was omitted from the reaction performed under room light, no detectable oxygen consumption was observed (Fig. 9*D*).

DISCUSSION

Our previous studies demonstrated the formation of DCF semiquinone ($DCF^{\bullet-}$) free radical by photoreduction of DCF (28) or by horseradish peroxidase-catalyzed oxidation of the reduced leuco compound DCFH (29). The photoreduction of DCF to $DCF^{\bullet-}$ in the presence of NADH or GSH under aerobic conditions resulted in the formation of superoxide radical that was detected by the ESR spin-trapping technique (28). The enzymatic oxidation of DCFH in the dark to $DCF^{\bullet-}$ by the reaction of horseradish peroxidase in the presence or absence of added H_2O_2 resulted in hydroxyl and superoxide radical formation, and H_2O_2 was demonstrated to be formed during the deacetylation of DCFH-DA (29).

In the present study, we analyzed the oxidation of the fluorescent dye DCF by horseradish peroxidase in the dark. Our results provide strong evidence for the formation of the novel phenoxyl radical intermediate during the peroxidase-mediated oxidation of DCF as demonstrated by the ESR spin-trapping technique (Fig. 2). UV-visible spectrophotometry confirmed the existence of compound II during the oxidation, and significant changes in the DCF absorption band at 502 nm were observed (Fig. 1), providing evidence for one-electron peroxide-mediated oxidation of DCF (Scheme 1); *i.e.* when DCF reacts with horseradish peroxidase in the presence of H_2O_2, DCF undergoes one-electron oxidation, with the obligate formation of the DCF phenoxyl radical (Fig. 2). This free radical is structurally and chemically distinct from the DCF semiquinone $DCF^{\bullet-}$. In the presence of reducing agents such as NADH and GSH, the DCF phenoxyl radical is reduced back to its parent compound DCF with the concomitant formation of $NAD^{\bullet}$ or $GS^{\bullet}$, respectively, which, in the presence of oxygen, form superoxide radical.

DCF, as shown in Scheme 2, catalytically stimulates the formation of $GS^{\bullet}$ and oxygen consumption in the glutathione-H_2O_2-horseradish peroxidase system (Figs. 3 and 4*b*). The rate of oxygen consumption was detectable in the absence of added H_2O_2 (Fig. 5*A*). In this case, oxygen consumption can occur when $GS^{\bullet}$ reacts with another GSH (GS^-) molecule to form the $(GSSG)^{\bullet-}$, which reacts rapidly with oxygen to produce superoxide radical (43). When the system contained only traces of H_2O_2, superoxide dismutase almost completely inhibited the rate of oxygen consumption, suggesting that, at least in part, superoxide radical is produced by a free radical chain reaction (Figs. 4*c*, *C*, and 5, *B* and *D*). No superoxide was detected by

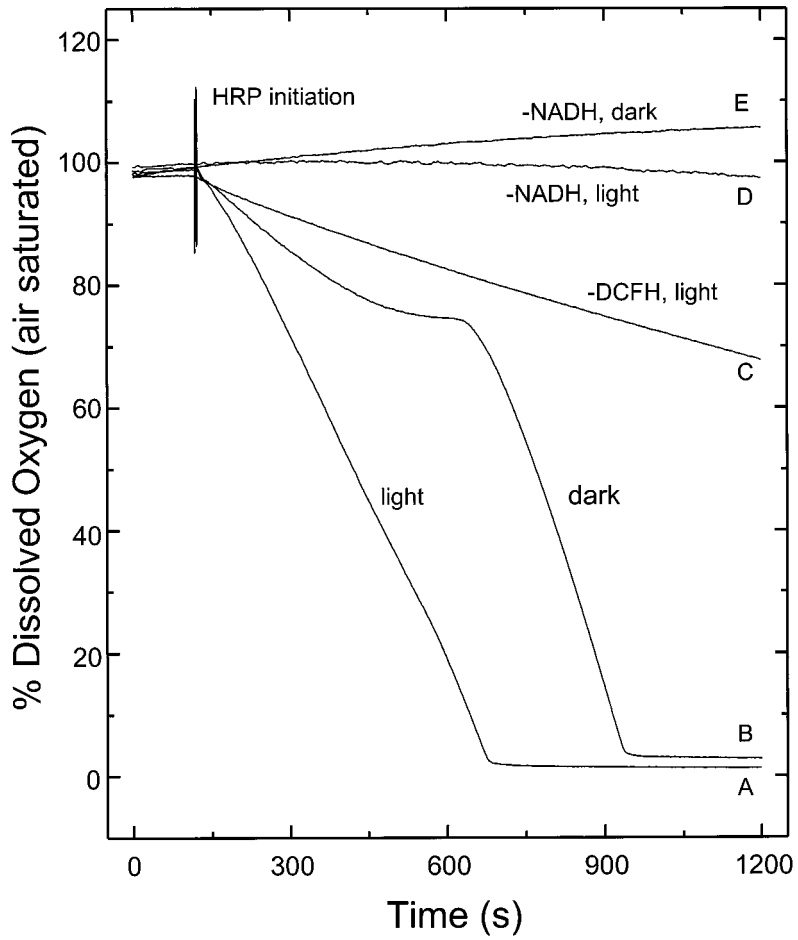
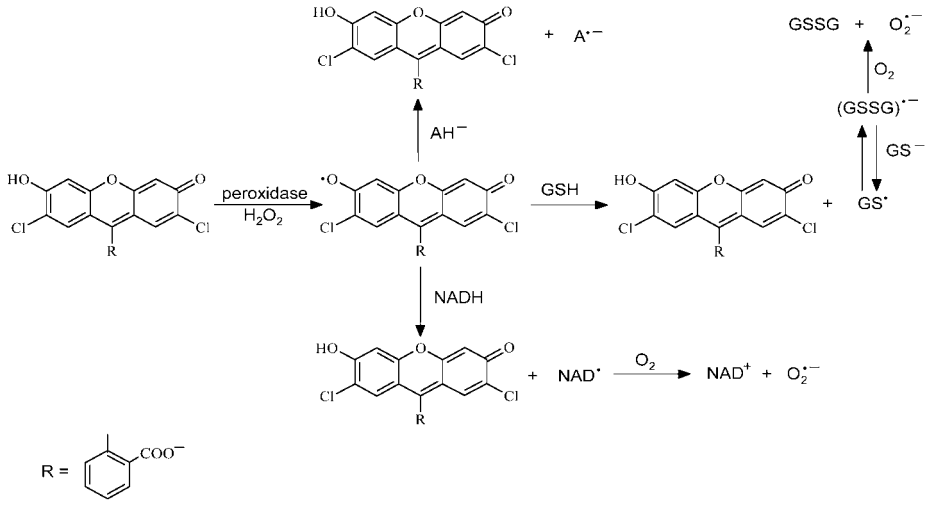


FIG. 9. **Effect of room light on the rate of oxygen consumption during the reaction of DCFH with horseradish peroxidase in the presence of NADH.** The *vertical bars* mark the time of addition of HRP to the system. *A*, complete system containing 100 μM DCFH, 3 mM NADH, and 1 μM HRP under room light. *B*, as in *A*, but the experiment was carried out in the dark. *C*, as in *A*, but with the omission of DCFH. *D*, as in *A*, but with the omission of NADH. *E*, as in *B*, but with the omission of NADH.



SCHEME 2

ESR in this system, probably because the precursor of this radical, GS$^{\bullet}$, is very efficiently scavenged by DMPO ($k = 2.6 \times 10^8$ M^{-1} s^{-1}) (44), as demonstrated also by the total inhibition of oxygen consumption by the addition of DMPO (Fig. 4b, *C* and *D*). At pH 7.8, superoxide radical can react with GSH with a second-order rate constant of $k = 6.7 \times 10^5$ M^{-1} s^{-1}, producing H_2O_2 and regenerating GS$^{\bullet}$, thus continuing the chain reaction (38).

DCF greatly enhanced the rate of oxygen consumption by horseradish peroxidase and NADH (Fig. 7*a*). The resulting superoxide radical was detected by ESR spin trapping (Fig. 6). The inhibition of free radical formation and oxygen consumption by the addition of catalase demonstrated that a trace of hydrogen peroxide was present in the system (Figs. 6*G* and 7*b*, *D* and *E*). The reactions leading to the formation of superoxide radical by the reaction of DCF and horseradish peroxidase/H_2O_2 in the presence of the reducing agents NADH and GSH are summarized in Scheme 2. Similar to GSH, superoxide radical can oxidize additional NADH with a second-order rate constant of $k = 9.3 \times 10^4$ M^{-1} s^1 at pH 7.4, producing H_2O_2 and regenerating NAD$^{\bullet}$, thus continuing the chain reaction (45).

The complete suppression of oxygen consumption by the addition of ascorbate to both the DCF-GSH-horseradish peroxidase (Fig. 4*b*) and the DCF-NADH-horseradish peroxidase

systems (Fig. 7*a*) and the DCF-dependent increase of ascorbate anion radical formation (Fig. 8) also support the DCF phenoxyl radical formation. Ascorbate radical is relatively stable once formed and does not further oxidize any other biochemical reductant in the system nor reduce oxygen.

The reaction of the reduced leuco compound DCFH with horseradish peroxidase in the presence of NADH consumed oxygen in a light-dependent manner because DCFH was more efficiently oxidized to DCF in the presence of light (Fig. 9). This experiment demonstrates that there are many reactions involving DCFH, DCF, and/or light in biological systems that can potentially lead to artificial radical formation.

Several issues concerning the measurement of DCF formation as an index of intracellular hydrogen peroxide (or other reactive species) formation must be considered in future work. First, the same species that forms DCF from DCFH (in this case horseradish peroxidase compounds I and II) will probably oxidize DCF to the novel DCF phenoxyl radical DCF$^{\bullet}$. Second, DCF$^{\bullet}$ oxidizes many biochemical reducing agents to free radicals, which may or may not (depending on their chemistry) react with molecular oxygen to form superoxide and, ultimately, hydrogen peroxide. Consequently, the potential for DCF$^{\bullet}$ oxidation forming reactive oxygen species will strongly depend on the intracellular concentration of the various biochemical reducing agents.

This study, combined with our earlier studies (28, 29), clearly shows that results from the DCF fluorometric assay are difficult to interpret in cells undergoing oxidative stress.

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