

# Physiological Aspects of Free-Radical Reactions

by Isao Yamazaki,\* Mamoru Tamura,\* Ryo Nakajima,\* and Masao Nakamura\*

Enzymes which catalyze the formation of free radicals *in vitro* will catalyze similar reactions *in vivo*. We believe that the formation of some kinds of free radicals has definite physiological meanings in metabolism. In this sense, the enzymes forming such free radicals are concluded to be in evolutionally advanced states. Elaborated structure and function of enzymes such as horseradish peroxidase and microsomal flavoproteins support the idea. Deleterious and side reactions caused by free radicals are assumed to be minimized *in vivo* by localizing the reactions, but this assumption should be verified by future studies.

Free radicals are regarded to be toxic to cells and have been studied mostly to elucidate their deleterious effects in cells. This tendency of studies of free radicals has been strengthened since the finding of McCord and Fridovich (1), who reported an enzyme that decomposes superoxide radicals. Numerous experimental results have led one to conclude that the enzyme called superoxide dismutase is an indispensable component of the system of defenses which make aerobic life possible (2-5). Semidehydroascorbate reductase is another type of free-radical scavenging enzyme, which has been studied a long time (6-13). In this case, however, it is not known whether this enzyme also serves as a defense enzyme.

An enzyme is believed to catalyze a unique reaction which has a definite physiological role or an evolutionary meaning in biology. For instance, the relationship between structure and function of oxygen-metabolizing enzymes has been explained in terms of evolution of enzymes (14). There are a group of enzymes which catalyze the formation of free radicals. It seems, therefore, of primary importance to discuss the question whether enzymatic formation of free radicals has any physiological meanings. If such free radicals are really deleterious, the free-radical formation may be a defect in the enzyme catalysis, which is still in an evolutionary stage. We shall treat the problem from the two points of view; mechanism of enzymatic formation of free radicals and possible physiological meanings of free-radical reactions.

## Mechanism of Free-Radical Formation

In some enzymatic redox reactions, free radicals are derived from substrates and prosthetic groups of enzymes. Enzymes which catalyze free-radical reactions contain transition metal ion(s), flavin(s) or rarely quiones(s) as the prosthetic group. These enzymes are grouped into apparently two contrasting types, one that scavenges free radicals using them as a substrate and the other that catalyzes the formation of free radicals from a substrate. Superoxide radical and semidehydroascorbate are the only known free radicals used as the substrate of enzymes, while many free radical species are reported to be formed during enzyme catalysis (Table 1). Free radicals derived from substrates usually decay fast through a dimerization or dismutation pathway and accumulate only a little during the reaction. It is not always possible to detect them directly with an optical or ESR spectrometer. In order to analyze the mechanism of electron transfer between enzyme and substrate, it is necessary to clarify whether free radicals are primary products in enzyme catalysis and whether they are formed through a main enzyme reaction. The experimental methods for the quantitative measurement of one-electron flux in enzymatic redox reactions were discussed in detail previously (41,42). After careful consideration of the above problems and on the basis of quantitative analysis, it is possible to classify the electron-transfer mechanism into one-electron and two-electron types. In a few cases, the electron transfer occurs through a mixed mechanism (41,42).

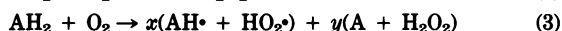
\*Biophysics Division, Research Institute of Applied Electricity, Hokkaido University, Sapporo 060, Japan.

Then, the question may arise whether any unique structural factors are needed for enzymes to catalyze the formation of substrate free radicals. Since it is known that the superoxide radical is formed through autoxidation of various divalent reductants, nonenzymatic electron transfer might be by nature a one-electron type. Therefore, it may also be necessary to consider the evolutionary state of free-radical-forming enzymes from the points of catalytic efficiency and specificity.

Here, we shall discuss kinetics of enzymatic and nonenzymatic electron transfers, and enzymatic devices which may control the formation of free radicals.

### Nonenzymatic Formation of Free Radicals

There are numerous reports concerning the formation of free radicals in nonenzymatic reactions (43). It is beyond doubt that a free radical is formed when a divalent molecule is oxidized or reduced by a monovalent molecule. The problem to be discussed here is how much free radicals are formed in electron-transfer reactions between two divalent molecules. For example, the primary process of autoxidation of a divalent reductant ( $AH_2$ ) will be formulated as shown in Eqs. (1)-(3).



where  $x + y = 1$ . The mechanism of the reactions is termed, from the top, one-electron, two-electron and mixed types (41). The fraction of one-electron flux,  $x/(x + 2y)$  has to be measured for characterization of re-

action (3) (42). The formation of superoxide radical has been shown in autoxidations of reduced flavin (44,45), pyrogallol (46), dyes (47,48), adrenalin (49,50), and ascorbate (51). The amount of superoxide radical formed during the reaction is measured directly with an ESR spectrometer (44) or indirectly from secondary reactions of the superoxide radical with electron acceptors and adrenalin (45-50). The difficulty of quantitative measurement of one-electron flux is due to nonspecific reactions caused by free radicals.

A kinetic study has been carried out on a reaction between ascorbate and 2,6-dichlorophenolindophenol (DCIP). During the oxidation of ascorbate by DCIP, only ascorbate free radical is observed. The dependency of steady-state concentration of ascorbate free radical on the DCIP concentration is shown in Figure 1 (52). Based on the rate constant measured for dismutation of ascorbate free radicals (15,53-55) it can be concluded that ascorbate is oxidized mostly by way of one-electron transfer, the one-electron flux being about 80%. Figure 1 shows also that ascorbate free radicals decay mostly by dismutation at low concentrations of DCIP and the decay through a reaction with DCIP increases as the dye concentration increases.



where  $AH_2$  denotes ascorbic acid. The DCIP free radical,  $DCIPH\cdot$ , appears to decay very fast because the ESR signal observed is only that of ascorbate free rad-

Table 1. Free radicals generated from substrates by enzymes.

Free radical	Enzyme		Reference
	Oxidative	Reductive	
Semidehydroascorbate	Peroxidase Ascorbate oxidase Dopamine $\beta$ -monooxygenase		(15-17) (15) (18)
Semidehydroreductate	Ascorbate oxidase		(15)
Semiquinones	Laccase Peroxidase		(19) (17,20,21)
		Microsomal flavoproteins	(8)
		NADH dehydrogenase	(22)
		Ferredoxin-NADP reductase	(22)
		Xanthine oxidase	(23)
		Xanthine dehydrogenase	(24)
		Lipoamide dehydrogenase	(25)
Superoxide anion		Xanthine oxidase	(26,27)
		Flavoproteins in phagocytosis	
Phenoxy radicals	Peroxidase		(17,28-30)
Free radicals from Amines	Peroxidase Prostaglandin endoperoxide synthetase		(31-33) (34-37)
	Cytochrome P-450		(38)
Chlorpromazine	Peroxidase		(39)
Indoleacetate	Peroxidase		(40)

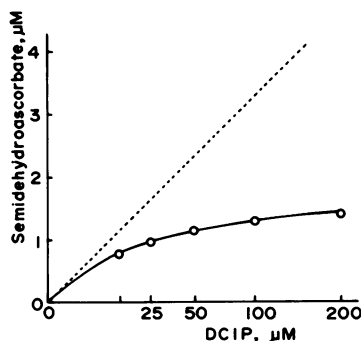


FIGURE 1. Steady-state concentration of semidehydroascorbate formed in the oxidation of ascorbate by 2,6-dichlorophenolindophenol (DCIP) at varied concentrations: (—) calculated on the assumption that ascorbate is oxidized to semidehydroascorbate, which decays only through dismutation.

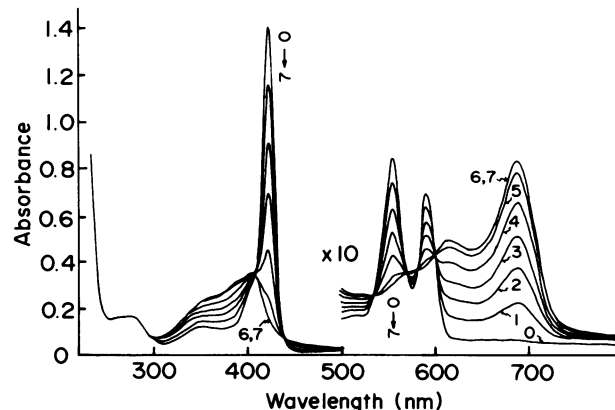


FIGURE 3. Oxidation of Mg-substituted horseradish peroxidase by potassium hexachloroiridate and formation of porphyrin  $\pi$ -cation radical. The Mg enzyme (0) is titrated stepwise with hexachloroiridate, and the final spectrum (6,7) is obtained at the equimolar concentration (78).

ical. Although Misra and Fridovich (45) have reported two-electron reduction of oxygen by reduced menadi-one, it may be concluded that free-radical species are more or less formed in nonenzymatic electron transfers between two divalent redox molecules.

### Enzymatic Formation of Free Radicals

For most enzymatic reactions it is possible to estimate the exact fraction of one-electron flux (41,42). The mechanism of electron transfer can be concluded to depend mostly on the structure of enzymes, including interaction between protein and prosthetic groups. For instance, ascorbate oxidase, laccase and tyrosinase are all copper proteins, but the former two catalyze one-electron oxidations of donor molecules (15,19) and tyrosinase catalyzes a two-electron oxidation of catechol (56). The oxygen is freed from these copper enzymes after it is reduced to water. It is, therefore, concluded that the electron-transfer mechanism is not determined by prosthetic group itself. This conclusion is valid for heme- and flavin-containing enzymes.

The most characteristic feature of enzyme catalysis which yields free radicals may be that free radicals are derived from only one of the two substrates, oxidant and reductant, but not from both of them (42). It is also important to note that an electron-transfer mechanism

is not necessarily fixed to an enzyme. In some cases, the mechanism varies with the kind of substrate and the experimental conditions (41,42).

**Horseradish Peroxidase.** Peroxidases distributed widely in tissues of plants are similar to each other and can be represented by horseradish peroxidase, which has been studied extensively (57,58). The enzyme has five oxidation states from 2 to 6, where the oxidation state of ferrous enzyme is set at 2 (74). The conversion between these states is caused by suitable oxidants and reductants, as shown in Figure 2 and Table 2. The reactions are essentially the same as those of animal peroxidases prepared from thyroid, milk, intestine and leucocyte. The 5 state of animal peroxidases, however, is unstable and converted to 4 within a second, probably by endogenous reductants. Characteristic of horseradish peroxidase regarding the formation of free radicals is as follows.

In the 3  $\rightarrow$  5 reaction, upon the reaction of the 3 state with hydroperoxides (ROOH), the O—O bond of ROOH is split heterolytically by acid-base catalysis of the iron and two amino acid residues in the heme crevice (75). The two oxidizing equivalents of ROOH are retained at the heme moiety, one as a ferryl ion and the other as a porphyrin  $\pi$ -cation radical (76). This radical can be detected quantitatively by optical and ESR spectroscopy when the iron of horseradish peroxidase is replaced by Zn or Mg (77,78). Spectrophotometric titration with potassium hexachloroiridate is shown in Figure 3. As the porphyrin  $\pi$ -cation radical is not formed in myoglobin, it is concluded that the heme crevice of peroxidase has a structure suitable for stabilizing the porphyrin  $\pi$ -cation radical (77,78).

In the 5  $\rightarrow$  4  $\rightarrow$  3 and 5  $\rightarrow$  3 reactions, various electron donors are oxidized by 5, via a route of either 5  $\rightarrow$  4  $\rightarrow$  3 or 5  $\rightarrow$  3. The mechanism is dependent on the kind of electron donors and, in a few cases, on pH also. The 5  $\rightarrow$  4  $\rightarrow$  3 mechanism is common to most peroxidase re-

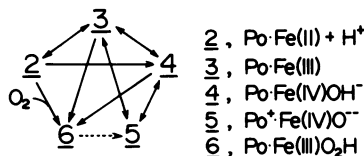


FIGURE 2. Five oxidation states of horseradish peroxidase: (2) ferrous; (3) ferric; (4) Compound II or ferryl; (5) compound I or ferryl + porphyrin  $\pi$ -cation radical; and (6) compound III or oxy-form. Po denotes porphyrin. Correlation is shown in Table 2.

Table 2. Correlation between five oxidation states of peroxidase.

Reaction <sup>a</sup>	Oxidant	Reductant	Reference
2→3	Ferricyanide		(59)
2→4	H <sub>2</sub> O <sub>2</sub>		(60)
2→6	O <sub>2</sub>		(61,62)
3→2		Dithionite	(59)
3→4	Hexachloroiridate, ferricyanide <sup>b</sup>		(63)
3→5	H <sub>2</sub> O <sub>2</sub> , alkylhydroperoxides, peracids		(59,64,65)
3→6	Superoxide		(23,66,67)
4→3		Numerous donors	<sup>c</sup>
4→5	Hexachloroiridate		(63)
4→6	H <sub>2</sub> O <sub>2</sub>		(68)
5→3		Iodide, thiocyanate H <sub>2</sub> O <sub>2</sub> , sulfite <sup>d</sup>	(69-71)
5→4	Numerous donors		<sup>c</sup>
6→5		Hydrated electron	(72)
6→3 <sup>e</sup>		<i>p</i> -Phenylenediamine	(73)

<sup>a</sup> Numbers denote oxidation states (See Fig. 2).

<sup>b</sup> Only at alkaline pH.

<sup>c</sup> See Table 1.

<sup>d</sup> At acidic pH.

<sup>e</sup> The reaction appears to occur via 6→5→4→3, although the intermediates are not detected.

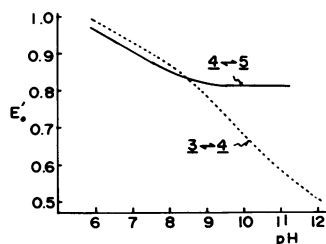


FIGURE 4. pH dependence of redox potentials of horseradish peroxidase (63).

actions and electron donors are all oxidized to free radicals. For the same electron donor, the 5→4 reaction is approximately 100 times faster than the 4→3 reaction (79,80). This difference cannot be explained in terms of redox potentials because the  $E'_0$  values for the 5/4 and the 4/3 couples are about the same at pH values where the enzyme activity is measured (Fig. 4). The relation between the rate constant and the redox potential is demonstrated in the result of Figure 5, which shows that the oxidation of 4 by hexachloroiridate is faster than that of 3 by hexachloroiridate (63). These results have led us to conclude that an electron is transferable from and to porphyrin much faster than from and to the heme iron. One of the reasons may be that the edge of porphyrin ring in peroxidases is exposed to the solvent. And, this might be a structural factor that enables peroxidases to catalyze typical one-electron oxidations of donor molecules. In most cases, peroxidases exist as the 4 form during the catalytic reactions because the 3→5 reaction is very fast under ordinary experimental conditions. Free radicals formed during the reactions have been measured by ESR spectroscopy (15-17,20,21,28,29,31,33,39,81,82).

The oxidations of halides, thiocyanate and hydrogen peroxide are coupled with the 5→3 path of peroxidases,

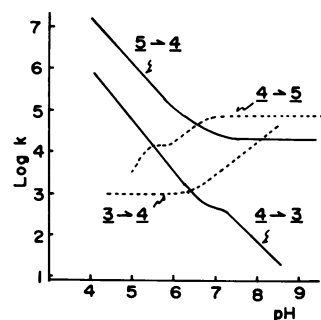


FIGURE 5. Second-order rate constants  $k$  for oxidation by potassium hexachloroiridate and reduction by potassium hexachloroiridite of horseradish peroxidase A<sub>2</sub> (63).

occurring by way of two-electron transfer (Table 2). Horseradish peroxidase catalyzes the oxidation of sulfite through a mixed mechanism, which varies from a two-electron type at acidic pH to a one-electron type at alkaline pH (69). Although it is in general true that peroxidases catalyze one-electron oxidations of organic molecules, such as phenol derivatives and aromatic amines, an interesting exception has been found in the reactions of thyroxine synthesis catalyzed by thyroid peroxidase, which will be discussed later.

The 3→6 reaction was suggested to explain the stoichiometric formation of 6 during the aerobic oxidation of dihydroxyfumarate catalyzed by horseradish peroxidase (66). The formation of 6 from 3 + O<sub>2</sub><sup>-•</sup> has been confirmed with lactoperoxidase (23), intestinal tryptophan 2,3-dioxygenase (83) and myeloperoxidase (67), and recently by pulse radiolysis with horseradish peroxidase (84) and catalase (85). Since the reaction occurs with a rate constant of 10<sup>6</sup>-10<sup>7</sup>/M-sec (86,87), it can be used for the measurement of the amount of superoxide radical formed (23). As the stability of 6 is dependent

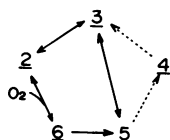
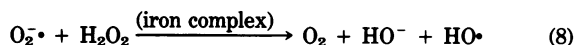


FIGURE 6. Pentagonal representation of the function of cytochrome P-450 in comparison with that of horseradish peroxidase.

on the experimental conditions, this method is not valid for some cases (88).

**Cytochrome P-450.** Cytochrome P-450 is a unique monooxygenase. Since it has a peroxidase activity (89) and a spectral property similar to chloroperoxidase (90), the structural and functional comparison of cytochrome P-450 with peroxidases is an interesting subject (91–93). The catalytic pathway of cytochrome P-450 can also be shown in a pentagonal diagram (Fig. 6). As compared with peroxidases, several important points still remain to be solved in the case of cytochrome P-450 (Table 3). Porphyrin  $\pi$ -cation radical is formed in peroxidases, but is not detected in cytochrome P-450. The ferryl form is a common catalytic intermediate of peroxidases, and a similar form is found in cytochrome P-450 (95) but its participation is uncertain in cytochrome P-450 reactions. The cleavage of the O—O bond accompanying the reactions of 6→5 and 3 + ROOH is heterolytic in peroxidases, but is still a controversial subject in cytochrome P-450 (96–98).

The formation of aminopyrrole free radical is directly confirmed by optical and ESR spectroscopic methods in cytochrome P-450 reactions (99,100). The involvement of free radical intermediate is also confirmed with the uses of spin traps (101–103) and radical scavengers (103–105). Particularly, several workers have concluded that the hydroxyl radical which is formed through an iron-catalyzed Haber-Weiss reaction causes the hydroxylation of aniline (106) and benzene (107), and the oxidation of ethanol (108–111).



The source of superoxide radical may be either the autoxidation of cytochrome P-450 reductase (110) or of cytochrome P-450 (106,107,111,112).

The key problem in the mechanism of cytochrome P-450 reactions is whether cleavage of the O—O bond is heterolytic or homolytic. As the homolytic cleavage yields the hydroxyl radical, it is inconsistent with the mechanism involving the Haber-Weiss pathway in the formation of hydroxyl radical. On the other hand, the heterolytic cleavage is accepted by workers who insist an oxene-transfer mechanism for hydroxylation by cytochrome P-450 (113,114). In the heterolytic cleavage, like peroxidases, cytochrome P-450 is to be oxidized to the 5 form (compound I) upon reactions of 6→5 and 3 + ROOH. Then, in cytochrome P-450 reactions, the oxygen atom coordinated at the 6th position of heme iron is to be transferred to an oxygen acceptor. Although the formation of a higher valence state of cytochrome P-450 is reported (95,114), there is no direct evidence to support the oxene-transfer mechanism.

Another serious question as to the mechanism of cytochrome P-450 reactions would be the time of the OO bond cleavage. In the oxene mechanism, the heterolytic cleavage is to precede the oxygen transfer. In the mechanism of homolytic cleavage, the 5 form is regarded as a complex of 3 with hydroperoxide (96,97) and the O—O bond cleavage will be accompanied with the hydroxylation. A free radical derived from the homolytic cleavage of the O—O bond abstracts a hydrogen atom from an oxygen acceptor (RH) and the R $\cdot$  thus formed reacts with the iron-bound hydroxyl radical to produce ROH. After a series of these reactions in the heme crevice a hydroxylated molecule will be released from the enzyme. This mechanism, which may be called an intramolecular free-radical mechanism, will explain various aspects of liver microsomal cytochrome P-450 reactions (97,98,115). It seems possible that electron donors and spin-trapping reagents are released from the enzyme as free radicals just after reacting with free-radical species formed by the homolytic cleavage. The flexible mechanism of liver microsomal cytochrome P-450 will be related to its low specificity for substrate. Since the release of free radicals does not appear to be physiological in this case, such flexibility of the mechanism is of special interest from the physiological and evolutionary points of view.

**Flavoprotein Enzymes.** Flavoproteins occupy key positions in biological electron transport systems. In general, a flavoprotein is reduced by a specific substrate

Table 3. Comparison between peroxidase and cytochrome P-450.

	Peroxidase	Cytochrome P-450
Heme	Protoheme <sup>a</sup>	Protoheme
5th ligand	Imidazol <sup>b</sup>	Thiol
Cleavage of the O-O bond	Heterolytic	Homolytic and coupled with hydroxylation <sup>c</sup>
5 state	Ferryl plus porphyrin $\pi$ -cation radical <sup>d</sup>	complex of 3 with hydroperoxide?
4 state	Ferryl	Not confirmed <sup>e</sup>

<sup>a</sup> A slightly modified form of protoheme is involved in animal peroxidases except for myeloperoxidase which contains a formyl heme.

<sup>b</sup> The 5th ligand of chloroperoxidase is thiol.

<sup>c</sup> See McCarthy and White (93)

<sup>d</sup> The oxidizing equivalent is transferred from the porphyrin to an amino acid residue in the case of cytochrome c peroxidase (94).

<sup>e</sup> See text.

and reoxidized by relatively nonspecific electron acceptors although *in vivo* it mediates a specified electron transfer from donor to acceptor. The mechanism of electron transfer from donor to flavoprotein is regarded to be a hydride or two-electron transfer (41,116) and the one-electron transfer mechanism is frequently involved in the reactions between flavoproteins and electron acceptors. Of numerous electron acceptors, *p*-benzoquinone serves as good electron acceptor for most flavoproteins, and the fraction of one-electron reduction can easily be measured by either ESR or scavenger method (8,22,41,42).

The dependence of the one-electron flux on the experimental conditions is summarized in Table 4. The results suggest that (i) when the one-electron flux depends on pH, it increases as the pH increases; (ii) when the one-electron flux depends on the acceptor concentration, it increases as the acceptor concentration increases; (iii) the one-electron flux increases when the enzyme suffers mild modification. Although it is premature to deduce generalization from a few examples, there are reasons to rationalize the rules. For (i), upon the stepwise reduction of oxygen or quinone (A) at neutral pH, the two protons are incorporated at the second step.



The release of  $AH_2$  becomes difficult as the pH increases. For (ii), if the rate of electron supply to a flavoprotein is limited, the one-electron transfer from the flavoprotein to A will predominate over the two-electron transfer as the concentration of A increases. This can be seen in the reaction of xanthine oxidase in which molybdenum and iron ions are contained in addition to flavin. For (iii), the two-electron transfer tends to occur in a reaction of an enzyme with a highly specific substrate and the loosening its interaction will result in a shift from two-electron to one-electron transfer mechanism.

We have shown that the primary reduction products

We have shown that the primary reduction products of quinones are semiquinones in the reactions catalyzed by flavoprotein enzymes of electron transport systems and hydroquinone forms in the reaction catalyzed by DT diaphorase (8,22). The difference is not completely explained in terms of molecular structure of the flavoproteins, but clear experimental evidence has been given to answer the question why microsomal flavoproteins catalyze one-electron reduction of quinones (119–121). Cytochrome P-450 reductase contains two flavins, FAD and FMN (119). The FMN acts like flavodoxin and is converted to a stable FMNH $\cdot$  form during the reaction (119,120). Cytochrome  $b_5$  reductase contains only FAD, but the FADH $\cdot$  form is stabilized in the presence of pyridine nucleotide (121). Therefore, in the NADH-quinone reductase reaction the reduced enzyme (FADH $_2$ ) is reoxidized by a quinone via FADH $\cdot$  and the semiquinone is released. Of course, *in vivo*, the electron acceptor is cytochrome in either case, and the reaction with quinones is artificial. At any rate, it is likely that the formation of stable flavin semiquinone is prerequisite to the one-electron transfer mechanism.

## Possible Roles of Free-Radical Reactions

A molecule in biological systems is activated and undergoes an ordered reaction at an active site of an enzyme. Active sites of oxido-reductive enzymes consist of prosthetic groups and functional aminoacid residues. There are two major groups of enzymes which catalyze the formation of free radicals. One is flavin-containing enzymes present in electron transport systems and the other is heme-containing enzymes, peroxidases, and peroxidase-like enzymes. The physiological meanings of free radical formation appear to be quite different between these two groups of enzymes.

## Energy Conservation

Flavoproteins present in electron transport systems catalyze one-electron transfer to cytochromes or quinones. The one-electron flux is 100% even when qui-

Table 4. Factors which increase the one-electron flux in some enzyme catalyses.

Enzyme	Reactions <sup>a</sup>	Condition	Reference
Xanthine oxidase	Xanthine-O $_2$	$\Delta[O_2]$ (5 to 50%) <sup>b</sup>	(24,27)
		$\Delta pH$ (15 to 7%)	(24,27)
	Xanthine-Q	$\Delta[Q]$ (0 to 100%) Deflavo (100%) Dehydrogenase type (100%) <sup>c</sup>	(23,117) (117) (118)
Lipamide dehydrogenase	NADH-Q	$\Delta pH$ (15 to 80%) <sup>d</sup> Copper treatment <sup>e</sup> (15 to 100%)	(117) (25)
Peroxidase	Sulfite-H $_2$ O $_2$	$\Delta pH$ (0 to 100%)	(69)

<sup>a</sup> One-electron oxidation and reduction of substrate are measured.

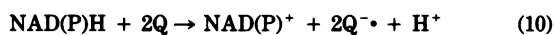
<sup>b</sup> This means that the one-electron flux increases from 5 to 50% with the increase of  $[O_2]$ .

<sup>c</sup> Milk xanthine oxidase can be prepared as a xanthine dehydrogenase type (118).

<sup>d</sup> The one-electron flux does not depend on  $[Q]$  in this case.

<sup>e</sup> Sulfhydryl groups of the enzyme are oxidized without changing the NADH-Q reductase activity.

ones are used as acceptor.



The free energy is fairly conserved in the above reaction as compared with the two-electron transfer reaction:



The standard free energy to be saved can be calculated by

$$\Delta E_o' = -1.36 \log K_s \quad (12)$$

where  $K_s$  is a free-radical formation constant, represented as

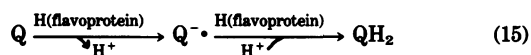
$$K_s = \frac{[\text{Q}^{\cdot-}]}{[\text{QH}_2][\text{Q}]} \quad (13)$$

the redox potential ( $E_2$ ) for  $\text{Q}/\text{Q}^{\cdot-}$  is

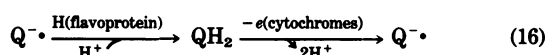
$$E_2 = E_m + (RT/2F) \ln K_s \quad (14)$$

where,  $E_m$  is mean redox potential for  $\text{QH}_2/\text{Q}$  (122).  $\text{Q}^{\cdot-}$  is a strong reductant and can reduce cytochromes and molecular oxygen at considerably high rates. Therefore, if quinones in respiratory chains are exposed to molecular oxygen, electrons will leak out to the oxygen to form superoxide radicals.

The quinone *in vivo* is a component of respiratory chains and embedded in membranes. It is reasonable to assume that the quinone is reduced to the semiquinone in membrane as well as in solution. In mitochondria, ubiquinone may act as translocator of protons (123-125). There may be striking difference in the kinetics of electron transfer from flavin to quinone between in solution and in membrane. In solution, the semiquinone formed in the first one-electron transfer from flavin is freed from the enzyme and the second electron of the flavo-protein is transferred to a fresh quinone molecule (41,42). In contrast, it is possible that the same quinone molecule accepts two electrons successively from the enzyme. The reduction of quinone in respiratory chains can be formulated as shown in Eq. (15):



As the quinone is mobile in membrane, it will be possible that the first and the second reduction of quinone at opposite sites of membrane, causes proton translocation. The translocation of two protons per electron may also be possible as shown in Eq. (16):



if the semiquinone-hydroquinone cycle shuttles between

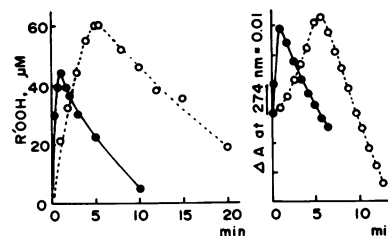


FIGURE 7. Formation and decay of hydroperoxide ( $\text{R}'\text{OOH}$ ) derived from indole-3-acetate. The amount of  $\text{R}'\text{OOH}$  can be measured either from its reaction with peroxidase (left) or from the increase of absorbance at 274 nm (right). The solid and dotted lines show time courses for horseradish peroxidases C and A, respectively (136).

the opposite site of membrane.

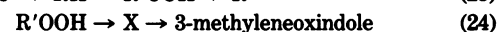
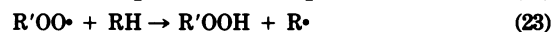
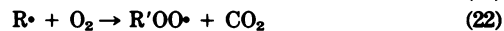
## Metabolism

Hydroperoxides are found to be intermediates in metabolic paths. The peroxidase is a typical enzyme to be involved in the metabolism of hydroperoxides. Therefore, it is of interest to look at the role of peroxidase reactions involved in hydroperoxide metabolism

**Hydroperoxide Derived from Indole-3-acetic Acid (IAA).** The mechanism of oxidation of a plant hormone, IAA has been a subject of investigation by many plant physiologists (126-135). It is now accepted that free radical species which are formed through catalysis of peroxidase are intermediates in the degradation of IAA (128-134). The stoichiometry of the peroxidase-catalyzed formation of free radicals has been confirmed by using ferric *o*-phenanthroline complex as an electron acceptor for the free radical (40).



It is also found (136) that a hydroperoxide derived from IAA is a good substrate for peroxidase. There is, more or less, a lag in the oxidase reaction and the oxidase reaction in the steady state can be formulated as shown in Eqs. (19)-(24) (136):



where  $\text{RH}$  and  $\text{R}'\text{OH}$  are IAA and indole-3-methanol, respectively. The hydroperoxide ( $\text{R}'\text{OOH}$ ) accumulates during the reaction (Fig. 7) and reacts with 3 very fast. Therefore, the formation of 5 becomes no more rate-limiting shortly after the reaction starts. Then, like ordinary peroxidase reactions, the reduction of 4 by  $\text{RH}$  is rate-limiting, and most of the peroxidase exists at 4

during the reaction (136).

It may be emphasized from the physiological point of view that in the presence of oxygen the peroxidase-IAA system yields strong oxidizing species. Since it has been reported that the formation of ethylene (plant hormone) is promoted by the addition of IAA (137-141), there is a possibility that the ethylene formation is promoted by such oxidizing species.

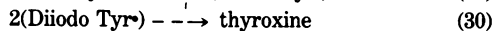
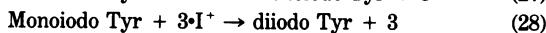
It should be noted also that peroxidases catalyze oxygenation of various compounds in plant (142-144). The reaction may be accelerated in the presence of IAA.

**Prostaglandin Synthesis.** Prostaglandins are produced from arachidonic acid by a cascade of enzymes. The first step is the conversion of arachidonic acid to prostaglandin G (a hydroperoxide form) by cyclooxygenase. The spin-trapping method has shown the formation of a carbon centered free radical of arachidonic acid in the reaction by ram seminal vesicle microsomes (145). The next step is the conversion from prostaglandin G to H catalyzed by a peroxidaselike enzyme. However, a purified enzyme preparation that catalyzes the above two reactions has not been resolved yet (146,147) and is termed prostaglandin endoperoxide synthetase. The formation of free radicals has been demonstrated during the peroxidatic oxidation of various electron donors by the enzyme (35-37).

The enzyme requires heme for the activities and the mechanism of the reactions can be compared with that of IAA oxidase reaction catalyzed by horseradish peroxidase. On the basis of the assumption that the two reactions are essentially of the same type, it will be suggested for the prostaglandin synthesis that the reactions of cyclooxygenase and peroxidase are catalyzed by the same enzyme; that the peroxidase reaction causes dehydrogenation of arachidonic acid to form its free radical; that the free radical reacts with molecular oxygen to form prostaglandin G via a chain reaction, and that a trace amount of hydroperoxide initiates the peroxidase reaction. This mechanism is the same as that proposed by O'Brien and Rahimtula (148).

Although the mechanism of prostaglandin endoperoxide synthetase may not be explained by simple chain reactions involving the hydroperoxy radical, it is of interest to note that a somewhat similar problem has long been discussed also in the mechanism of IAA oxidase reaction.

**Thyroxine Synthesis.** Thyroid peroxidase catalyzes iodination of tyrosine and oxidative coupling of two diiodotyrosines. It is confirmed that the oxidation of iodide occurs in a two-electron process and that of diiodotyrosine occurs by way of one-electron transfer.



The two-electron oxidation of halide ions has been con-

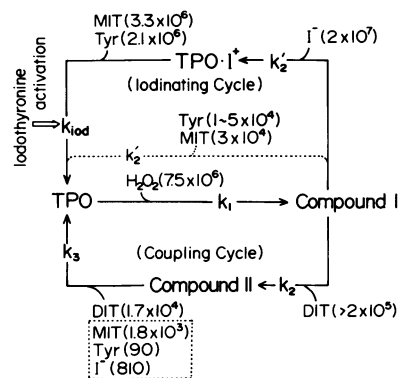


FIGURE 8. One-electron (bottom) and two-electron (upper) oxidation pathways in thyroxine synthesis by thyroid peroxidase. Numerals in parentheses denote second-order rate constants. The mechanism is converted from the upper to the bottom cycle as diiodotyrosine (DIT) accumulates. Reactions shown by dotted lines are not involved in the main path of thyroxine synthesis (150).

firmed with horseradish peroxidase (70,71), lactoperoxidase (149) and thyroid peroxidase (149,150). It is in general believed that peroxidases catalyze one-electron oxidation of phenolic compounds. We have recently found a curious fact that thyroid peroxidase catalyzes one-electron oxidation of diiodotyrosine and two-electron oxidations of monoiodotyrosine and tyrosine (149,151). Horseradish peroxidase and lactoperoxidase both catalyze one-electron oxidations of the above three molecules (151). The ability of thyroid peroxidase of distinguishing the one- and two-electron processes is so peculiar that biochemists will tend to consider its physiological meaning. We have concluded (150) that the switch-over of the mechanism from the two-electron to the one-electron type with the increase of the iodide content in tyrosine derivatives is a kind of regulation so that the enzyme synthesizes preferentially thyroxine at the expense of a limited amount of substrates. Thyroxine must be synthesized, *in vivo*, in the presence of limited amounts of hydrogen peroxide, iodide or tyrosine residues of thyroglobulin. For economy, thyroxine should be formed without accumulation of monoiodotyrosine and diiodotyrosine.

As shown in Figure 8, tyrosine and monoiodotyrosine compete with iodide but inhibition of iodination by tyrosine and monoiodotyrosine is not serious because of high rate of reaction of iodide with 5. As diiodotyrosine accumulates, the reaction of 5 with diiodotyrosine cannot be neglected, and, in this case, the enzyme is converted into 4, which reacts with iodide very slowly. The values of rate constant tell us that as the concentration of diiodotyrosine increases the iodination is inhibited and the coupling of diiodotyrosine is accelerated.

## Miscellaneous

The redox property of the ascorbate system is still mysterious in many points, in spite of numerous experimental data. The difficulty in studying this redox



property is partly due to the instability of dehydroascorbate. The redox property of semidehydroascorbate appears to differ from that of semiquinones.  $E_o'$  for the dehydroascorbate/semidehydroascorbate couple is calculated from Eq. (14) to be  $-200$  mV (152). Semiquinones having this  $E_o'$  value can reduce molecular oxygen (153,153), but semidehydroascorbate cannot. Dismutation of semidehydroascorbate is very fast, but the reduction of cytochrome c by semidehydroascorbate is relatively slow (155). There is a widely distributed enzyme system that reduces semidehydroascorbate to ascorbate at the expense of NADH (6-13,156). The bicyclic structure of semidehydroascorbate (157,158) might be related to its abnormal reactivity and the characteristic feature of ascorbate redox system.

The generation of superoxide anions during phagocytosis and action of antitumor quinones is an interesting subject which is dealt with in other chapters. Some peculiar enzyme catalyses in which the free-radical mechanism is involved have been recently reported (159-161). A tyrosine radical is involved in the ribonucleotide reductase reaction (159,160) and pyrrole-quinoline semiquinone is involved in the methanol dehydrogenase reaction (161). Since quinoproteins are widely distributed in bacteria (162), the free radical mechanism will actually be more popular in enzyme catalyses.

This research was supported by a Japanese grant-in-aid for science and culture, No. 57430029.

#### REFERENCES

- McCord, J. M., and Fridovich, I. Superoxide dismutase. An enzymic function for erythrocyte hemocuprein (hemocuprein). *J. Biol. Chem.* 244: 6049-6055 (1969).
- Fridovich, I. The biology of oxygen radicals. *Science* 201: 875-880 (1978).
- Bors, W., Saran, M., Lengfelder, E., Michel, C., Fuchs, C., and Frenzel, C. Detection of oxygen radicals in biological reactions. *Photochem. Photobiol.* 28: 629-638 (1978).
- Autor, A. P., Ed. *Pathology of Oxygen*. Academic Press, New York, 1982.
- Halliwell, B., and Gutteridge, J. M. C. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* 219: 1-14 (1984).
- Marre, E., and Arrigoni, O. Ascorbic acid and photosynthesis I. "Monodehydroascorbic acid" reductase of chloroplast. *Biochim. Biophys. Acta* 30: 453-457 (1958).
- Schneider, W., and Staudinger, H. Reduced nicotinamide-adenine dinucleotide-dependent reduction of semidehydroascorbic acid. *Biochim. Biophys. Acta* 96: 157-159 (1965).
- Iyanagi, T., and Yamazaki, I. One-electron-transfer reactions in biochemical systems III One-electron reduction of quinones by microsomal flavin enzymes. *Biochim. Biophys. Acta* 172: 370-381 (1969).
- Schulze, H. U., Schott, H. H., and Staudinger, H. Isolierung und Charakterisierung einer NADH: Semidehydroascorbinsäure-Oxidoreductase aus *Neurospora crassa*. *Z. Physiol. Chem.* 353: 1931-1942 (1972).
- Arrigoni, O., Dipierro, S., and Borraccino, G. Ascorbate free radical reductase, a key enzyme of the ascorbic acid system. *FEBS Letters* 125: 242-244 (1981).
- Diliberto, E. J., Jr., Dean, G., Carter, C., and Allen, P. L. Tissue, subcellular, and submitochondrial distributions of semidehydroascorbate reductase: possible role of semidehydroascorbate reductase in cofactor regeneration. *J. Neurochem.* 39: 563-568 (1982).
- Goldenberg, H., Grebing, C., and Löw, H. NADH-monodehydroascorbate reductase in human erythrocyte membranes. *Biochem. Intern.* 6: 1-10 (1983).
- Hossain, M. A., Nakano, Y., and Asada, K. Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. *Plant Cell Physiol.* 25: 385-395 (1984).
- Mason, H. S. Oxygenases and oxidases: hypothesis. In: 34th Colloquium Mosbach, Biological Oxidations, Springer-Verlag Berlin, Heidelberg, 1983, pp. 151-169.
- Yamazaki, I., and Piette, L. H. Mechanism of free radical formation and disappearance during the ascorbic acid oxidase and peroxidase reactions. *Biochim. Biophys. Acta* 50: 62-69 (1961).
- Yamazaki, I., Mason, H. S., and Piette, L. H. Identification, by electron paramagnetic resonance spectroscopy, of free radicals generated from substrates by peroxidase. *J. Biol. Chem.* 235: 2444-2449 (1960).
- Ohnishi, T., Yamazaki, H., Iyanagi, T., Nakamura, T., and Yamazaki, I. One-electron-transfer reactions in biochemical systems. II. The reaction of free radicals formed in the enzymic oxidation. *Biochim. Biophys. Acta* 172: 357-569 (1969).
- Skotland, T., and Ljones, T. Direct spectrophotometric detection of ascorbate free radical formed by dopamine  $\beta$ -monooxygenase and by ascorbate oxidase. *Biochim. Biophys. Acta* 630: 30-35 (1980).
- Nakamura, T. On the process of enzymatic oxidation of hydroquinone. *Biochem. Biophys. Res. Commun.* 2: 111-113 (1960).
- Kalyanaram, B., Felix, C. C., and Sealy, R. C. Electron spin resonance-spin stabilization of semiquinones produced during oxidation of epinephrine and its analogues. *J. Biol. Chem.* 259: 354-358 (1984).
- Kalyanaram, B., Felix, C. C., and Sealy, R. C. Peroxidatic oxidation of catecholamines. *J. Biol. Chem.* 259: 7584-7589 (1984).
- Iyanagi, T., and Yamazaki, I. One-electron-transfer reactions in biochemical systems. V. Differences in the mechanism of quinone reduction by the NADH dehydrogenase and the NAD(P)H dehydrogenase (DT-diaphorase). *Biochim. Biophys. Acta* 216: 282-294 (1970).
- Nakamura, S., and Yamazaki, I. One-electron transfer reactions in biochemical systems. IV. A mixed mechanism in the reaction of milk xanthine oxidase with electron acceptors. *Biochim. Biophys. Acta* 189: 29-37 (1969).
- Nakamura, M., Kurebayashi, H., and Yamazaki, I. One-electron and two-electron reduction of acceptors by xanthine oxidase and xanthine dehydrogenase. *J. Biochem.* 83: 9-17 (1978).
- Nakamura, M., and Yamazaki, I. One-electron transfer reactions in biochemical systems. VI. Changes in electron transfer mechanism of lipoamide dehydrogenase by modification of sulfhydryl groups. *Biochim. Biophys. Acta* 267: 249-257 (1972).
- Knowles, P. F., Gibson, J. F., Pick, F. M., and Bray, R. C. Electron-spin-resonance evidence for enzymic reduction of oxygen to a free radical, the superoxide ion. *Biochem. J.* 111: 53-58 (1969).
- Fridovich, I. Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. *J. Biol. Chem.* 245: 4053-4057 (1970).
- Shiga, T., and Imaizumi, K. Electron spin resonance study on peroxidase- and oxidase-reactions of horseradish peroxidase and methemoglobin. *Arch. Biochem. Biophys.* 167: 469-479 (1975).
- Perez-Reyes, E., and Mason, R. P. Characterization of the structure and reactions of free radicals from serotonin and related indoles. *J. Biol. Chem.* 256: 2427-2432 (1981).
- Caldwell, E. S., and Steelink, C. Phenoxy radical intermediates in the enzymatic degradation of lignin model compounds. *Biochim. Biophys. Acta* 184: 420-431 (1969).
- Griffin, B. W., and Ting, P. L. Mechanism of N-demethylation of aminopyrine by hydrogen peroxide catalyzed by horseradish peroxidase, metmyoglobin, and protohemin. *Biochemistry* 17: 2206-2211 (1978).
- Galliani, G., Rindone, B., and Marchesini, A. Horseradish peroxidase-catalyzed oxidation of aromatic tertiary amines with hydrogen peroxide. *J. Chem. Soc. Perkin I*: 456-460 (1978).

33. Josephy, P. D., Eling, T., and Mason, R. P. The horseradish peroxidase-catalyzed oxidation of 3,5,3',5'-tetramethylbenzidine. *J. Biol. Chem.* 257: 3669-3675 (1982).
34. Lasker, J. M., Sivarajah, K., Mason, R. P., Kalyanaraman, B., Abou-Donia, M. B., and Eling, T. E. A free radical mechanism of prostaglandin synthetase-dependent aminopyrine demethylation. *J. Biol. Chem.* 256: 7764-7767 (1981).
35. Egan, R. W., Gale, P. H., Baptista, E. M., Kenmicott, K. L., Vanden Henvel, W. J. A., Walker, R. W., Fagerness, P. E., and Kuehl, F. A. Jr. Oxidation reactions by prostaglandin cyclooxygenase-hydroperoxidase. *J. Biol. Chem.* 256: 7352-7361 (1981).
36. Josephy, P. D., Mason, R. P., and Eling, T. Cooxidation of the clinical reagent 3,5,3',5'-tetramethylbenzidine by prostaglandin synthetase. *Cancer Res.* 42: 2567-2570 (1982).
37. Marnett, L. J., Siedlik, P. H., and Fung, L. W. M. Oxidation of phenidone and BW755C by prostaglandin endoperoxide synthetase. *J. Biol. Chem.* 257: 6957-6964 (1982).
38. Griffin, B. W., Marth, C., Yasukochi, Y., and Masters, B. S. S. Radical mechanism of purified liver microsomal cytochrome P-450. *Arch. Biochem. Biophys.* 205: 543-553 (1980).
39. Piette, L. H., Bulow, G., and Yamazaki, I. Electron-paramagnetic-resonance studies of the chlorpromazine free radical formed during enzymic oxidation by peroxidase-hydrogen peroxide. *Biochim. Biophys. Acta* 88: 120-129 (1964).
40. Yamazaki, I., and Souza, H. The mechanism of indoleacetic acid oxidase reaction catalyzed by turnip peroxidases. *Arch. Biochem. Biophys.* 86: 294-301 (1960).
41. Yamazaki, I. One-electron and two-electron transfer mechanisms in enzymic oxidation-reduction reactions. *Adv. Biophys.* 2: 33-76 (1971).
42. Yamazaki, I. Free radical in enzyme-substrate reactions. In: *Free Radicals in Biology*, Vol. III (W. A. Pryor, Ed.), Academic Press, New York, 1977, pp. 183-218.
43. Walling, C. *Free Radicals in Solution*. John Wiley, New York, 1957.
44. Ballou, D., Palmer, G., and Massey, V. Direct demonstration of superoxide anion production during the oxidation of reduced flavin and of its catalytic decomposition by erythrocyte. *Biochem. Biophys. Res. Commun.* 36: 898-904 (1969).
45. Misra, H. P., and Fridovich, I. The univalent reduction of oxygen by reduced flavins and quinones. *J. Biol. Chem.* 247: 188-192 (1972).
46. Marklund, S., and Marklund, G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.* 47: 469-474 (1974).
47. Dupré, S., Federici, G., Santoro, L., Rossi Fanelli, M. R., and Cavallini, D. The involvement of superoxide anions in the autoxidation of various cofactors of cystamine-oxygenase. *Mol. Cell. Biochem.* 9: 149-154 (1975).
48. Nishikimi, M., Rao, N. A., and Yagi, K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochem. Biophys. Res. Commun.* 46: 849-854 (1972).
49. Bors, W., Michel, C., Saran, M., and Lengfelder, E. The involvement of oxygen radicals during the autoxidation of adrenalin. *Biochim. Biophys. Acta* 540: 162-172 (1978).
50. Bros, W., Michel, C., Saran, M., and Lengfelder, E. Kinetic investigations of the autoxidation of adrenalin. *Z. Naturforsch.* 33: 891-896 (1978).
51. Scarpa, M., Stevanato, R., Viglino, P., and Rigo, A. Superoxide ion as active intermediate in the autoxidation of ascorbate by molecular oxygen. *J. Biol. Chem.* 258: 6695-6697 (1983).
52. Iyanagi, T., Yamazaki, I., and Anan, K. One-electron oxidation-reduction properties of ascorbic acid. *Biochem. Biophys. Acta* 806: 255-261 (1985).
53. Foerster, G. V., Weis, W., and Staudinger, H. Kinetik der Entstehung von Semidehydroascorbinsäure. *Z. Physiol. Chem.* 344: 217-222 (1966).
54. Bielski, B. H. J., Comstock, D. A., and Bowen, R. A. Ascorbic acid free radicals. I. Pulse radiolysis study of optical absorption and kinetic properties. *J. Am. Chem. Soc.* 93: 5624-5629 (1971).
55. Bielski, B. H. J., Allen, A. O., and Schwarz, H. A. Mechanism of disproportionation of ascorbate radicals. *J. Am. Chem. Soc.* 103: 3516-3518 (1981).
56. Mason, H. S., Spencer, E., and Yamazaki, I. Identification by electron spin resonance spectroscopy of the primary product of tyrosinase-catalyzed catechol oxidation. *Biochem. Biophys. Res. Commun.* 4: 236-238 (1961).
57. Saunders, B. C., Holmes-Siedle, A. G., and Stark, B. P. *Peroxidase*. Butterworths, London, 1964.
58. Gaspar, T., Penel, C., Thorpe, T., and Greppin, H. *Peroxidases*. University of Geneva Press, Geneva, 1982.
59. Yamada, H., and Yamazaki, I. Proton balance in conversions between five oxidation reduction states of horseradish peroxidase. *Arch. Biochem. Biophys.* 165: 728-738 (1974).
60. Noble, R. W., and Gibson, Q. H. The reaction of ferrous horseradish peroxidase with hydrogen peroxide. *J. Biol. Chem.* 245: 2409-2413 (1970).
61. Yamazaki, I., Yokota, K., and Tamura, M. Horseradish peroxidase compound III. In: *Hemes and Homoproteins* (B. Chance, R. W. Estabrook, and T. Yonetani, Eds.), Academic Press, New York, 1966, pp. 319-325.
62. Wittenberg, J. B., Noble, R. W., Wittenberg, B. A., Antonini, E., Brunori, M., and Wyman, J. Studies on the equilibria and kinetics of the reactions of peroxidase with ligands II. The reaction of ferropoxidase with oxygen. *J. Biol. Chem.* 242: 626-634 (1967).
63. Hayashi, Y., and Yamazaki, I. The oxidation-reduction potential of Compound I/Compound II and Compound II/ferric couples of horseradish peroxidases A<sub>2</sub> and C. *J. Biol. Chem.* 254: 9101-9106 (1979).
64. Schonbaum, G. R., and Lo, S. Interaction of peroxidases with aromatic peracids and alkyl peroxides. Product analysis. *J. Biol. Chem.* 247: 3353-3360 (1972).
65. Marklund, S., Ohlsson, P. I., Opara, A., and Paul, K. G. The substrate profiles of the acid and slightly basic horseradish peroxidases. *Biochim. Biophys. Acta* 350: 304-313 (1974).
66. Yamazaki, I., and Piette, L. H. The mechanism of aerobic oxidase reaction catalyzed by peroxidase. *Biochim. Biophys. Acta* 77: 47-64 (1963).
67. Odajima, T., and Yamazaki, I. Myeloperoxidase of the leucocyte of normal blood. III. The reaction of ferric myeloperoxidase with superoxide anion. *Biochim. Biophys. Acta* 284: 355-359 (1972).
68. George, P. The third intermediate compound of horseradish peroxidase and hydrogen peroxide. *J. Biol. Chem.* 201: 427-434 (1953).
69. Araiso, T., Miyoshi, K., and Yamazaki, I. Mechanism of electron transfer from sulfite to horseradish peroxidase-hydroperoxide compounds. *Biochemistry* 15: 3059-3063 (1976).
70. Björkstén, F. The horseradish peroxidase-catalyzed oxidation of iodide. Outline of the mechanism. *Biochim. Biophys. Acta* 212: 396-406 (1970).
71. Roman, R., and Dunford, H. B. pH Dependence of the oxidation of iodide by Compound I of horseradish peroxidase. *Biochemistry* 11: 2076-2082 (1972).
72. Kobayashi, K., and Hayashi, K. One-electron reduction of oxyform of homoproteins. *J. Biol. Chem.* 256: 12350-12354 (1981).
73. Tamura, M., and Yamazaki, I. Reactions of the oxyform of horseradish peroxidase. *J. Biochem.* 71: 311-319 (1972).
74. Yamazaki, I. Peroxidase. In: *Molecular Mechanisms of Oxygen Activation* (O. Hayaishi, Ed.), Academic Press, New York, 1974, pp. 535-558.
75. Poulos, T. L., and Kraut, J. The stereochemistry of peroxidase catalysis. *J. Biol. Chem.* 255: 8199-8205 (1980).
76. Dolphin, D., Forman, A., Borg, D. C., Fajer, J., and Felton, R. H. Compound I of catalase and horse radish peroxidase:  $\pi$ -cation radicals. *Proc. Natl. Acad. Sci. (U.S.)* 68: 614-618 (1971).
77. Kaneko, Y., Tamura, M., and Yamazaki, I. Formation of porphyrin  $\pi$ -cation radical in zinc-substituted horseradish peroxidase. *Biochemistry* 19: 5795-5799 (1980).
78. Kuwahara, Y., Tamura, M., and Yamazaki, I. The reactivity of Mg-substituted horseradish peroxidases. *J. Biol. Chem.* 257: 11517-11522 (1982).
79. Yamazaki, I., Nakajima, R., Miyoshi, K., Makino, R., and Ta-

- mura, M. The functional relationship between horseradish peroxidase and other hemoproteins. In: *Oxidases and Related Redox Systems* (T. E. King, H. S. Mason and M. Morrison, Eds.), University Park Press, Baltimore, 1973, pp. 407-419.
80. Dunford, H. B., and Stillman, J. S. On the function and mechanism of action of peroxidases. *Coord. Chem. Revs.* 19: 187-251 (1976).
  81. Mottley, C., Trice, T. B., and Mason, R. P. Direct detection of the sulfur trioxide radical anion during the horseradish peroxidase-hydrogen peroxide oxidation of sulfite (aqueous sulfur dioxide). *Mol. Pharmacol.* 22: 732-737 (1982).
  82. Sinha, B. K. Irreversible binding of quinacrine to nucleic acids during horseradish peroxidase- and prostaglandin synthetase-catalyzed oxidation. *Biochem. Pharmacol.* 32: 2604-2607 (1983).
  83. Hirata, F., and Hayashi, O. Possible participation of superoxide anion in the intestinal tryptophan 2,3-dioxygenase reaction. *J. Biol. Chem.* 246: 7825-7826 (1971).
  84. Hayashi, K., Lindenau, D., and Tamura, M. *Adv. Exptl. Med. Biol.* 94: 353-359 (1978).
  85. Shimizu, N., Kobayashi, K., and Hayashi, K. The reaction of superoxide radical with catalase. Mechanism of the inhibition of catalase by superoxide radical. *J. Biol. Chem.* 259: 4414-4418 (1984).
  86. Sawada, Y., and Yamazaki, I. One-electron transfer reactions in biochemical systems. VIII. Kinetic study of superoxide dismutase. *Biochim. Biophys. Acta.* 327: 257-265 (1973).
  87. Taniguchi, T., Sono, M., Hirata, F., Hayaishi, O., Tamura, M., Hayashi, K., Iizuka, T., and Ishimura, Y. Indoleamine 2,3-dioxygenase. Kinetic studies on the binding of superoxide anion and molecular oxygen to enzyme. *J. Biol. Chem.* 254: 3288-3294 (1979).
  88. Bernhardt, F. H., and Kuthan, H. Dioxigen activation by putidamonoxin. The oxygen species formed and released under uncoupling conditions. *Eur. J. Biochem.* 120: 547-555 (1981).
  89. Hrycaj, E. G., and O'Brien, P. J. Cytochrome P-450 as a microsomal peroxidase utilizing a lipid peroxidase substrate. *Arch. Biochem. Biophys.* 147: 14-27 (1971).
  90. Hollenberg, P. F., and Hager, L. P. The P-450 nature of the carbon monoxide complex of ferrous chloroperoxidase. *J. Biol. Chem.* 248: 2630-2633 (1973).
  91. Loew, G. H., Kert, C. J., Hjelmeland, L. M., and Kircher, R. F. Active site models of horseradish peroxidase Compound I and a cytochrome P-450 analogue: electronic structure and electric field gradients. *J. Am. Chem. Soc.* 99: 3334-3336 (1977).
  92. Renneberg, R., Scheller, F., Ruckpaul, K., Pirwitz, J., and Mohr, P. NADPH and H<sub>2</sub>O<sub>2</sub>-dependent reactions of cytochrome P-450<sub>LM</sub> compared with peroxidase catalysis. *FEBS Letters* 96: 349-353 (1978).
  93. McCarthy, M. B., and White, R. E. Functional differences between peroxidase compound I and the cytochrome P-450 reactive oxygen intermediate. *J. Biol. Chem.* 258: 9153-9158 (1983).
  94. Yonetani, T., and Schleyer, H. Studies on cytochrome c peroxidase VII. Electron paramagnetic resonance absorptions of the enzyme and complex ES in dissolved and crystalline forms. *J. Biol. Chem.* 241: 3240-3243 (1966).
  95. Rahimtulala, A. D., O'Brien, P. J., Hrycaj, E. G., Peterson, J. A., and Estabrook, R. W. Possible higher valence states of cytochrome P-450 during oxidative reactions. *Biochem. Biophys. Res. Commun.* 60: 695-702 (1974).
  96. White, R. E., Sliger, S. G., and Coon, M. J. Evidence for a homolytic mechanism of peroxide oxygen-oxygen bond cleavage during substrate hydroxylation by cytochrome P-450. *J. Biol. Chem.* 255: 11108-11111 (1980).
  97. Blake, R. C. II, and Coon, M. J. On the mechanism of action of cytochrome P-450. *J. Biol. Chem.* 256: 12127-12133 (1981).
  98. McCarthy, M. B., and White, R. E. Competing models of peroxyacid flux through cytochrome P-450. *J. Biol. Chem.* 258: 11610-11616 (1983).
  99. Ashley, P. L., and Griffin, B. W. Involvement of radical species in the oxidation of aminopyrene and 4-aminoantipyrene by cumen hydroperoxide in rat liver microsomes. *Mol. Pharmacol.* 19: 146-152 (1981).
  100. Renneberg, R., Damerau, W., Ebert, J. B., and Scheller, F. Study of H<sub>2</sub>O<sub>2</sub>-supported N-demethylations catalyzed by cytochrome P-450 and horseradish peroxidase. *Biochem. Biophys. Res. Commun.* 113: 332-339 (1983).
  101. Griffin, B. W. Use of spin traps to elucidate radical mechanisms of oxidations by hydroperoxides catalyzed by hemoproteins. *Can. J. Chem.* 60: 1463-1473 (1982).
  102. Ortiz de Montellano, P. R., Kunze, K. L., Beilan, H. S., and Wheeler, C. Destruction of cytochrome P-450 by vinyl fluoride, fluorene, and acetylene. Evidence for a radical intermediate in olefin oxidation. *Biochemistry* 21: 1331-1339 (1982).
  103. Augusto, O., Beilan, H. S., and Ortiz de Montellano, P. R. The catalytic mechanism of cytochrome P-450. *J. Biol. Chem.* 257: 11288-11295 (1982).
  104. Cederbaum, A. I., Dicker, E., and Cohen, G. Effect of hydroxyl radical scavengers on microsomal oxidation of alcohols and on associated microsomal reactions. *Biochemistry* 17: 3058-3064 (1978).
  105. Winston, G. W., and Cederbaum, A. I. NADPH-dependent production of oxy radicals by purified components of the rat liver mixed function oxidase system. II. Role in microsomal oxidation of ethanol. *J. Biol. Chem.* 258: 1514-1519 (1983).
  106. Ingelman-Sundberg, M., and Ekstrom, G. Aniline is hydroxylated by the cytochrome P-450-dependent hydroxyl radical-mediated oxygenation mechanism. *Biochem. Biophys. Res. Commun.* 106: 625-631 (1982).
  107. Johansson, I., and Ingelman-Sundberg, M. Hydroxyl radical-mediated, cytochrome P-450-dependent metabolic activation of benzene in microsomes and reconstituted enzyme systems from rabbit liver. *J. Biol. Chem.* 258: 7311-7316 (1983).
  108. Cohen, G., and Cederbaum, A. I. Microsomal metabolism of hydroxyl radical scavenging agents: relationship to the microsomal oxidation of alcohols. *Arch. Biochem. Biophys.* 199: 438-447 (1980).
  109. Feierman, D. E., and Cederbaum, A. I. The effect of EDTA and iron on the oxidation of hydroxyl radical scavenging agents and ethanol by rat liver microsomes. *Biochem. Biophys. Res. Commun.* 116: 765-770 (1983).
  110. Winston, G. W., and Cederbaum, A. I. NADPH-dependent production of oxy radicals by purified components of the rat liver mixed function oxidase system. 1. Oxidation of hydroxyl radical scavenging agents. *J. Biol. Chem.* 258: 1508-1513 (1983).
  111. Ingelman-Sundberg, M., and Johansson, I. Mechanism of hydroxyl radical formation and ethanol oxidation by ethanol-inducible and other forms of rabbit liver microsomal cytochromes P-450. *J. Biol. Chem.* 259: 6447-6458 (1984).
  112. Kuthan, H., and Ullrich, V. Oxidase and oxygenase function of the microsomal cytochrome P-450 monooxygenase system. *Eur. J. Biochem.* 126: 583-588 (1982).
  113. Lichtenberger, F., Nastainczyk, W., and Ullrich, V. Cytochrome P-450 as an oxene transferase. *Biochem. Biophys. Res. Commun.* 70: 939-946 (1976).
  114. Hrycaj, E. G., Gustafsson, J. A., Ingelman, Sundberg, M., and Ernster, L. The involvement of cytochrome P-450 in hepatic microsomal steroid hydroxylation reactions supported by sodium periodate, sodium chlorite, and organic hydroperoxides. *Eur. J. Biochem.* 61: 43-52 (1976).
  115. Hlavica, P., Golly, I., and Mietaschk, J. Comparative studies on the Cumene hydroperoxide- and NADPH-supported N-oxidation of 4-chloroaniline by cytochrome P-450. *Biochem. J.* 212: 539-547 (1983).
  116. Blankenhorn, G. Nicotinamide-dependent one-electron and two-electron (flavin) oxidoreduction: thermodynamics, kinetics, and mechanism. *Eur. J. Biochem.* 67: 67-80 (1976).
  117. Nakamura, M., and Yamazaki, I. One-electron transfer reactions in biochemical systems. VII. Two types of electron outlets in milk xanthine oxidase. *Biochim. Biophys. Acta* 327: 247-256 (1973).
  118. Nakamura, M., and Yamazaki, I. Preparation of bovine milk xanthine oxidase as a dehydrogenase form. *J. Biochem.* 92: 1279-1286 (1982).
  119. Iyanagi, T., and Mason, H. S. Some properties of hepatic reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase. *Biochemistry* 12: 2297-2308 (1973).

120. Iyanagi, T., Makino, N., and Mason, H. S. Redox properties of the reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 and reduced nicotinamide adenine dinucleotide-cytochrome  $b_5$  reductases. *Biochemistry* 13: 1701-1710 (1974).
121. Iyanagi, T. Redox properties of microsomal reduced nicotinamide adenine dinucleotide-cytochrome  $b_5$  reductase and cytochrome  $b_5$ . *Biochemistry* 16: 2725-2730 (1977).
122. Michaelis, L. Theory of oxidation-reduction. In: *The Enzyme*, Vol. 2, Part I (J. B. Sumner and K. Myrback, Eds.), Academic Press, New York, 1951, pp. 1-54.
123. Mitchell, P. Some comments on the promotive Q cycle. In: *Oxidases and Related Redox Systems* (T. E. King, H. S. Mason, and M. Morrison, Eds.), Pergamon Press, Oxford, 1980, pp. 1225-1246.
124. Gutman, M. Electron flux through the mitochondria ubiquinone. *Biochim. Biophys. Acta* 594: 53-84 (1980).
125. DeVries, S., Albracht, S. P. J., Berden, J. A., and Slater, E. C. The pathway of electrons through  $QH_2$ : cytochrome c oxidoreductase studies by pre-steady-state kinetics. *Biochim. Biophys. Acta* 681: 41-53 (1982).
126. Ray, P. Destruction of auxin. *Ann. Rev. Plant Physiol.* 9: 81-118 (1958).
127. Morita, Y., Kameda, K., Mizuno, M. Studies on phyto-peroxidase. Part XVI. Aerobic destruction of indole-3-acetic acid catalyzed by crystalline Japanese-radish peroxidase a and c. *Agr. Biol. Chem.* 26: 442-446 (1962).
128. Hinman, R. L., and Lang, J. Peroxidase-catalyzed oxidation of indole-3-acetic acid. *Biochemistry* 4: 144-153 (1965).
129. Fox, L. R., Purves, W. K., and Nakada, H. I. The role of horseradish peroxidase in indole-3-acetic acid oxidation. *Biochemistry* 4: 2754-2763 (1965).
130. Gelinas, D. A. Proposed model for the peroxidase-catalyzed oxidation of indole-3-acetic acid in the presence of the inhibitor ferulic acid. *Plant Physiol.* 51: 967-972 (1973).
131. Ricard, J., and Job, D. Reaction mechanism of indole-3-acetate degradation by peroxidases. A stopped-flow and low-temperature spectroscopic study. *Eur. J. Biochem.* 44: 359-374 (1974).
132. Kokkinakis, D. M., and Brooks, J. L. Hydrogen peroxide-mediated oxidation of indole-3-acetic acid by tomato peroxidases and molecular oxygen. *Plant Physiol.* 64: 220-223 (1979).
133. Grambow, H. J. On the role hydrogen peroxide in peroxidase catalyzed metabolism of indole-3-acetic acid. *Z. Naturforsch.* 37c: 884-888 (1982).
134. Smith, A. M., Morrison, W. L., and Milham, P. J. Oxidation of indole-3-acetic acid by peroxidase: involvement of reduced peroxidase and Compound III with superoxide as a product. *Biochemistry* 21: 4414-4419 (1982).
135. Sabater, F., Acosta, M., Sanchez-Bravo, J., Cuello, J., and del Rio, J. A. Indole-3-methanol as an intermediate in the oxidation of indole-3-acetic acid by peroxidase. *Physiol. Plant* 57: 75-78 (1983).
136. Nakajima, R., and Yamazaki, I. The mechanism of indole-3-acetic acid oxidation by horseradish peroxidases. *J. Biol. Chem.* 254: 872-878 (1979).
137. Aharoni, H., and Yang, S. F. Auxin-induced ethylene production as related to auxin metabolism in leaf discs of tobacco and sugar beet. *Plant Physiol.* 73: 598-604 (1983).
138. Blake, T. J., Reid, D. M., and Rood, S. B. Ethylene, indoleacetic acid and apical dominance in peas: a reappraisal. *Physiol. Plant* 59: 481-487 (1983).
139. Shimokawa, D. An ethylene-forming enzyme in *Citrus unshiu* fruits. *Phytochemistry* 22: 1903-1908 (1983).
140. Kang, B. G., Lee, J. S., Oh, S. E., Horiuchi, Y., and Imaseki, H. Inhibition of auxin-induced ethylene production by lycoridinol. *Plant Cell Physiol.* 25: 249-256 (1984).
141. Parups, E. V. Free radical and free radical scavenger effects on indole-3-acetic acid levels and ethylene production. *Physiol. Plant* 60: 149-153 (1984).
142. Rathmell, W. G., and Bendall, D. S. The peroxidase-catalyzed oxidation of a chalcone and its possible physiological significance. *Biochem. J.* 127: 125-132 (1972).
143. Wilson, J. M., and Wong, E. Peroxidase catalyzed oxygenation of 4,2',4'-trihydroxychalcone. *Phytochemistry* 15: 1333-1341 (1976).
144. Liljegren, D. Peroxidase-mediated hydroxylation of p-hydroxylacetoneitrile, an intermediate in dhurrin synthesis in sorghum. *Phytochemistry* 17: 1695-1699 (1978).
145. Mason, R. P., Kalyanaraman, B., Tainer, B. E., and Eling, T. E. A carbon-centered free radical intermediate in the prostaglandin synthetase oxidation of arachidonic acid. Spin trapping and oxygen uptake studies. *J. Biol. Chem.* 255: 5019-5022 (1980).
146. Ohki, S., Ogino, N., Yamamoto, S., and Hayaishi, O. Prostaglandin hydroperoxidase, an integral part of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J. Biol. Chem.* 254: 829-836 (1979).
147. Boyd, J. A., Harvan, D. J., and Eling, T. E. The oxidation of 2-aminofluorene by prostaglandin endoperoxide synthetase. Comparison with other peroxidases. *J. Biol. Chem.* 258: 8246-8254 (1983).
148. O'Brien, P. J., and Rahimtula, A. The possible involvement of a peroxidase in prostaglandin biosynthesis. *Biochem. Biophys. Res. Commun.* 70: 832-838 (1976).
149. Ohtaki, S., Nakagawa, H., Kimura, S., and Yamazaki, I. Analysis of catalytic intermediates of hog thyroid peroxidase during its iodinating reaction. *J. Biol. Chem.* 256: 805-810 (1981).
150. Nakamura, M., Yamazaki, I., Nakagawa, H., and Ohtaki, S. Steady state kinetics and regulation of thyroid peroxidase-catalyzed iodination. *J. Biol. Chem.* 258: 3837-3842 (1983).
151. Ohtaki, S., Nakagawa, H., Nakamura, M., and Yamazaki, I. One- and two-electron oxidation of tyrosine, monoiodotyrosine, and diiodotyrosine catalyzed by hog thyroid peroxidase. *J. Biol. Chem.* 257: 13398-13403 (1982).
152. Yamazaki, I., Yokota, K., and Nakajima, R. A mechanism and model of peroxidase-oxidase reaction. In: *Oxidases and Related Redox Systems* (T. E. King, H. S. Mason, and M. Morrison, Eds.), Wiley and Sons, New York, 1965, pp. 485-513.
153. Patel, K. B., and Willson, R. L. Semiquinone free radicals and oxygen. Pulse radiolysis study of one electron transfer equilibria. *J. Chem. Soc. Faraday Trans. 1:* 814-825 (1973).
154. Sawada, Y., Iyanagi, T., and Yamazaki, I. Relation between redox potential and rate constants in reactions coupled with the system oxygen-superoxide. *Biochemistry* 14: 3761-3764 (1975).
155. Yamazaki, I. The reduction of cytochrome c by enzyme-generated ascorbic free radical. *J. Biol. Chem.* 237: 224-229 (1962).
156. Nakagawa, S., and Sagisaka, S. Increase in enzyme activities related to ascorbate metabolism during cold acclimation in poplar twigs. *Plant Cell Physiol.* 25: 899-906 (1984).
157. Sapper, H., Pleyer-Weber, A., and Lohmann, W.  $^1H$ -NMR- and ESR-Messungen zur Struktur der Dehydroascorbinsäure und des Semidehydroascorbatradikals. *Z. Naturforsch.* 37c: 129-131 (1982).
158. Sapper, H., Kang, S. O., Paul, H. H., and Lohmann, W. The reversibility of the vitamin C redox system: electrochemical reactions and biological aspects. *Z. Naturforsch.* 37c: 942-946 (1982).
159. Graslund, A., Ehrenberg, A., and Thelander, L. Characterization of the free radical of mammalian ribonucleotide reductase. *J. Biol. Chem.* 257: 5711-5715 (1982).
160. Sjöberg, B. M., Graslund, A., and Eckstein, F. A substrate radical intermediate in the reaction between ribonucleotide reductase from *Escherichia coli* and 2'-azido-2'-deoxynucleoside diphosphates. *J. Biol. Chem.* 258: 8060-8067 (1983).
161. DeBeer, R., Duine, J. A., Frank, J. Jzn., and Westerling, J. The role of pyrrolo-quinoline semiquinone forms in the mechanism of action of methanol dehydrogenase. *Eur. J. Biochem.* 130: 105-109 (1983).
162. Duine, J. A., and Frank, J. J. Quinoproteins: a novel class of dehydrogenases. *Trends Biochem. Sci.* 6: 278-280 (1981).