BBA 45767

ONE-ELECTRON-TRANSFER REACTIONS IN BIOCHEMICAL SYSTEMS

II. THE REACTION OF FREE RADICALS FORMED IN THE ENZYMIC OXIDATION

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(Received September 24th, 1968)

SUMMARY

Free radicals formed in the enzymic oxidation are extremely reactive and act as strong reductants or oxidants. The rate constants of the reaction of free radicals with cytochromes are measured directly using an electron spin resonance (ESR) spectrometer and a sensitive spectrophotometer, both equipped with a flow apparatus. 2-Methyl-1,4-naphthosemiquinone (MKH), for instance, is a very active reductant and reduces cytochrome *c* and cytochrome b_5. The rate constants are about $3 \cdot 10^8$ $M^{-1} \cdot sec^{-1}$. MKH is also found to react with molecular oxygen at a rate constant of roughly $5 \cdot 10^6$ $M^{-1} \cdot sec^{-1}$. The rate constants of cytochrome *c* reduction by monodehydroascorbate and *p*-benzosemiquinone are measured directly as $4.0 \cdot 10^4$ and $2.5 \cdot 10^6$ $M^{-1} \cdot sec^{-1}$, respectively.

On the other hand, free radicals derived from *p*-cresol and chlorpromazine act as effective one-electron oxidants. The one-electron transfer from donor molecules to these free radicals is confirmed by means of ESR spectroscopy.

INTRODUCTION

HABER AND WILLSTÄTTER[1] suggested long ago that many oxidation–reduction reactions occur by way of one-electron transfer. Almost at the same time MICHAELIS[2] also proposed the hypothesis of the reversible two-step oxidation of organic molecules. The concept of compulsory one-electron steps in the oxidation of organic molecules clearly implies that the reactions are of a free-radical type. It may be generally difficult, however, to make a sharp distinction between one-electron and two-electron processes, as has been discussed by WESTHEIMER[3].

Abbreviations: ESR, electron spin resonance; MK, MKH and MKH_2 are 2-methyl-1,4-naphthoquinone (menaquinone-*o*), its semiquinone and its fully reduced form (quinol), respectively; H_2A, HA· and A are an organic molecule, its monodehydro-form and its oxidized (2 equivalents) form, respectively.

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In several enzymic oxidation reactions a one-electron process has been established by means of electron spin resonance (ESR) spectroscopy. The formation of free radicals associated with the substrates has been identified by the characteristic hyperfine structure[4–6]. Free radicals formed in the enzymic oxidation appear to be slow substrates for the enzymes and decay principally by dismutation or dimerization[7]. In the presence of suitable electron donors or acceptors, electron transfers occur effectively between the free radicals and the added molecules. Direct measurement of the absolute values of the rate constants of these reactions seems to be important to obtain a general concept of one-electron-transfer reaction in biochemical systems. Most of the reactions are very fast and the direct estimation of the rate constants can generally be achieved by means of a spectrophotometer and an ESR spectrometer, both equipped with a flow apparatus. The results will be reported in this paper.

MATERIALS AND METHODS

The equipment used for the stopped-flow and continuous-flow experiments was a sensitive spectrophotometer combined with a flow apparatus which had been designed by CHANCE[8]. The details of the equipment were described in the previous paper by NAKAMURA[9].

The ESR spectrometer was a Varian V-450 X-band instrument, utilizing 100 kcycles field modulation and Fieldial control of the magnetic field. The flow apparatus used and the determination of free radical concentrations were described in the previous paper[10]. In the continuous-flow experiments, a 4-jet mixing chamber was used, and the volume from the mixer to the center of the cavity was 0.12 ml.

Horseradish peroxidase (EC 1.11.1.7) was prepared from wild horseradish by a slight modification of the method of KENTEN AND MANN[11]. The enzyme was passed through a DEAE-cellulose column at pH 7.0 and then adsorbed to CM-cellulose at pH 5.0. The ratio of $A_{403\ m\mu}$ to $A_{278\ m\mu}$ of the enzyme was between 3.0 and 3.3.

Ascorbate oxidase (EC 1.10.3.3) was prepared from Japanese cucumber by the method of NAKAMURA, MAKINO AND OGURA[12].

p-Benzoquinone and MK were purified from commercial supplies by sublimation. Benzohydroquinone was recrystallized from ethanol–benzene solution. MKH_2 was prepared from MK by $Na_2S_2O_4$ reduction and crystallized from ethanol–light petroleum solution.

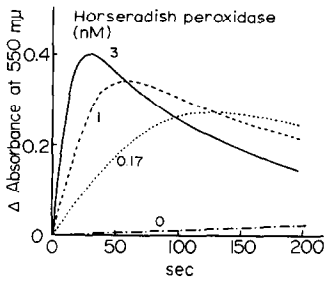
Cytochrome b_5 was solubilized from pig liver microsomes by the method of OMURA, SIEKEVITZ AND PALADE[23] and purified by the procedure of KAJIHARA AND HAGIHARA[24] with some modification. Cytochrome *c* and NADH were obtained from Boehringer, and cytochrome *c* was purified by column chromatography with Amberlite XE-64. The rates of cytochrome reduction were calculated assuming that $\Delta\varepsilon_{mM}$ between the oxidized and reduced states was 18.7 at 550 mμ for cytochrome *c* and 19.0 at 556 mμ for cytochrome b_5.

All experiments were carried out at 25°.

RESULTS

The accelerating effect of *p*-benzoquinone in the reduction of cytochrome *c* by benzohydroquinone was reported by WILLIAMS[13]. He suggested an involvement of

the *p*-benzosemiquinone in the reaction. YAMAZAKI AND OHNISHI[10] confirmed the assumption by kinetic analysis using the ESR spectroscopy. The participation of the monodehydro-molecules in the cytochrome *c* reduction has been suggested by YAMAZAKI in the enzymic reaction systems, such as peroxidase[14] and ascorbic acid oxidase[15].



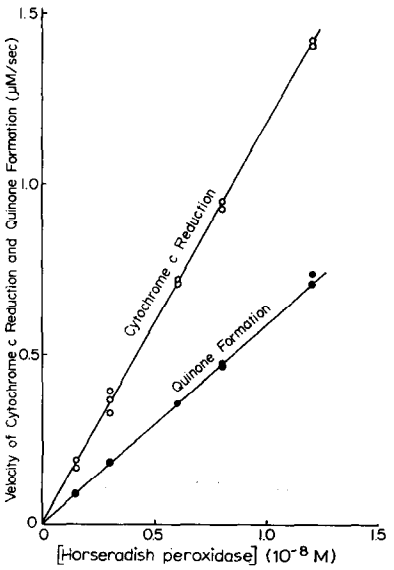


Fig. 1. Reduction of cytochrome *c* during peroxidatic oxidation of benzohydroquinone. The reaction was started by the addition of H_2O_2. 50 μM H_2O_2, 100 μM benzohydroquinone, 33 μM cytochrome *c* and 0.05 M phosphate (pH 6.5). Horseradish peroxidase concentrations are given in the figure.

Fig. 2. Relationship between the rates of *p*-benzoquinone formation and cytochrome *c* reduction in the presence of a small amount of horseradish peroxidase. The rate of cytochrome *c* reduction was measured in the reaction system containing horseradish peroxidase (variable), 50 μM H_2O_2, 100 μM benzohydroquinone, 35 μM cytochrome *c* and 0.05 M acetate (pH 5.0). The rate of *p*-benzoquinone formation was measured under the same conditions as above except that cytochrome *c* was omitted.

Cytochrome *c* is reduced when it is present in the reaction mixture of peroxidatic oxidation of benzohydroquinone (Fig. 1). When the rate of *p*-benzosemiquinone supply is slow and in the presence of a large amount of cytochrome *c*, there is a stoichiometric relationship between the rates of benzohydroquinone oxidation and cytochrome *c* reduction. The result depicted in Fig. 2 suggests the following overall reaction:

$$2\ \text{Benzohydroquinone} + 2\ \text{cytochrome } c^{3+} + H_2O_2 \xrightarrow{\text{peroxidase}} 2\ p\text{-benzoquinone} + 2\ \text{cytochrome } c^{2+} + 2\ H_2O + 2\ H^+ \quad (1)$$

Horseradish peroxidase is known to catalyze the one-electron oxidation of donor molecules (H_2A), forming their monodehydro-forms (HA·) (ref. 7).

$$2\ H_2A + H_2O_2 \xrightarrow{\text{peroxidase}} 2\ HA\cdot + 2\ H_2O \quad (2)$$

$$2\ HA\cdot \longrightarrow H_2A + A \quad (2')$$

Consequently, the stoichiometry of Reaction 1 shows that HA· formed in the peroxidase reaction decays by the reaction with cytochrome *c* but not by dismutation (Reaction 2') under the conditions described in Fig. 2. Under these experimental conditions the steady-state concentration of *p*-benzosemiquinone is too low to be measured by the present ESR spectrometer, and it is impossible to estimate the rate constant of the reaction of *p*-benzosemiquinone with the cytochrome.

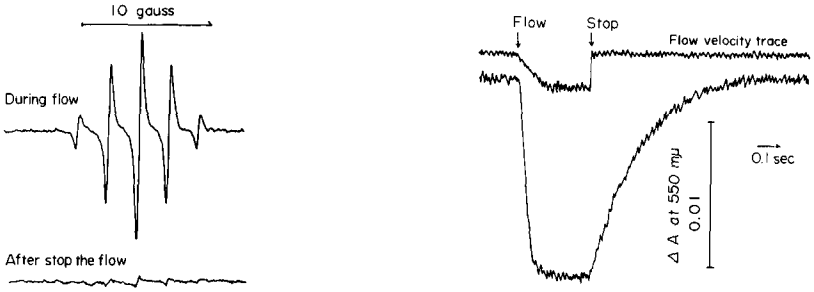


Fig. 3. The ESR spectrum of *p*-benzosemiquinone formed in the steady state of peroxidase reaction during a continuous slow flow. The flow rate was 2 ml/sec. The spectrum was not affected by the minor change in the flow rate. Final concentrations: 0.1 μM horseradish peroxidase, 0.5 mM H_2O_2, 1 mM benzohydroquinone and 0.05 M phosphate (pH 6.5). The concentration of *p*-benzosemiquinone reached a steady-state level (1.2 μM) within 40 msec and was not affected by the addition of 1 μM cytochrome *c* to this reaction system. A trace amount of *p*-benzosemiquinone remained after the flow stopped, which was an equilibrium amount of *p*-benzosemiquinone that appeared in the presence of 0.5 mM benzohydroquinone and 0.5 mM *p*-benzoquinone at pH 6.5. The modulation amplitude of the magnetic field was 0.1 gauss.

Fig. 4. Measurement of the rate of cytochrome *c* reduction using a stopped flow method. Experimental conditions were the same as those described in Fig. 3. The steady-state concentration of *p*-benzosemiquinone was 1.2 μM and the initial rate of cytochrome *c* reduction was 3.0 μM/sec. k_r was estimated to be $2.5 \cdot 10^6$ $M^{-1} \cdot sec^{-1}$ according to Eqn. 3 in the text.

In the presence of 0.1 μM horseradish peroxidase, a considerable amount of *p*-benzosemiquinone accumulates during flow, approx. 60 msec after mixing the solutions, and its concentration can be measured using an ESR spectrometer (see Fig. 3). The addition of 1 μM cytochrome *c* to this reaction mixture does not change the steady-state concentration of *p*-benzosemiquinone, though a very rapid reduction of cytochrome *c* occurs. The reduction is so rapid and the change in absorbance is so small that only a sensitive spectrophotometer combined with a flow apparatus enables one to measure the rate of the cytochrome *c* reduction, as illustrated in Fig. 4. As the reduction of cytochrome *c* by benzohydroquinone is very slow at pH 6.5, the rate of the cytochrome *c* reduction can be given by Eqn. 3.

$$\frac{\text{d cytochrome } c^{2+}}{dt} = k_r \,(p\text{-benzosemiquinone})\,(\text{cytochrome } c^{3+}) \qquad (3)$$

The value of $2.5 \cdot 10^6$ $M^{-1} \cdot sec^{-1}$ thus obtained for k_r is fairly consistent with the value obtained by the indirect methods in the nonenzymic system, which has been reported previously[10].

MKH_2 is also a very fast substrate for horseradish peroxidase. The redox potential of MKH_2 is much lower than that of benzohydroquinone and the MKH free radical is expected to have powerful reducing activity. Fig. 5 shows the reduction

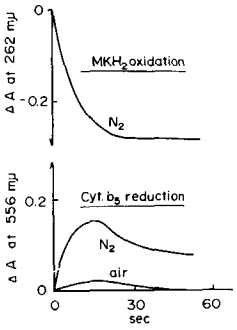


Fig. 5. Horseradish peroxidase-induced reduction of cytochrome b_5 in the presence of MKH_2 and H_2O_2. 0.01 μM horseradish peroxidase, 20 μM H_2O_2, 20 μM MKH_2, 17 μM cytochrome b_5 and 0.1 M phosphate (pH 6.5). The reduction of cytochrome b_5 and the oxidation of MKH_2 were measured at 557 mμ and 262 mμ, respectively. The experiments were carried out under acrobic (air) or anaerobic (N_2) conditions. The oxidation of MKH_2 was measured under the same conditions except that cytochrome b_5 was omitted.

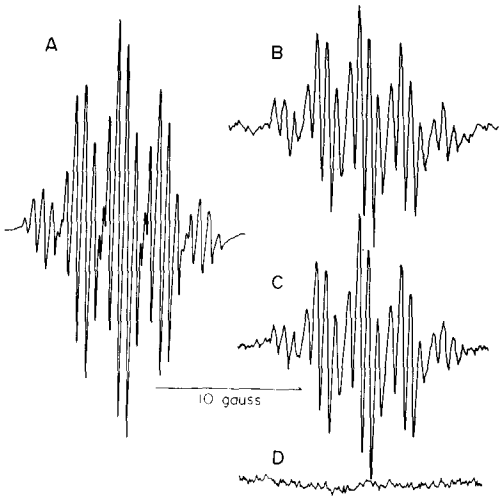


Fig. 6. ESR spectra of MKH free radical present at equilibrium (A and B) and formed in the steady state of peroxidase reaction (C). 2.5 mM MKH_2 and 1 mM MK were present in the 0.05 M carbonate (20% ethanol), pH 9.7 (A), and in the 0.05 M phosphate (10% ethanol), pH 8.5 (B). In C, the spectrum was taken in the steady state of peroxidatic oxidation MKH_2 under anaerobic conditions. Final concentrations: 1.6 μM horseradish peroxidase, 1 mM H_2O_2, 2 mM MKH_2 and 0.05 M phosphate (pH 7.5). The same magnetic field was scanned in D immediately after the flow stopped. The amplitude of field modulation was 0.02 gauss in A and 0.2 gauss in B, C and D. Ten times lower gain was used in A than in the others.

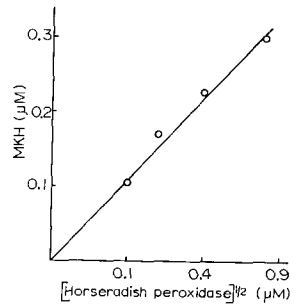


Fig. 7. Dependence of the steady-state concentration of MKH upon the horseradish peroxidase concentrations. The abscissa is the square root of the horseradish peroxidase concentrations. 0.5 mM H_2O_2, 0.25 mM MKH_2 and 0.05 M phosphate (pH 7.5).

of cytochrome b_5 which occurs during the oxidation of MKH_2 in the presence of horseradish peroxidase and H_2O_2. The reduction is strongly inhibited by the presence of molecular oxygen. MKH seems to be less stable than *p*-benzosemiquinone, and its hyperfine structure can be observed only in the presence of a large amount of horseradish peroxidase, as shown in Fig. 6. Fig. 7 shows that the steady-state concentration of MKH is proportional to the square root of the enzyme concentration. The result suggests that MKH concentrations in the steady state are formulated as Eqn. 4, which has been discussed previously[7].

$$(HA\cdot)_s = \left(\frac{\kappa \cdot v}{2\, k_d}\right)^{1/2} \tag{4}$$

Where k_d is a dismutation constant, v is the velocity of the formation of A or the disappearance of H_2A and κ is a constant defined by the equation, $v_f = \kappa \cdot v$, where v_f is the velocity of HA· formation. Measuring the velocity of MKH_2 oxidation and assuming that κ is 2, one can estimate approximate values of $8 \cdot 10^9\ M^{-1} \cdot sec^{-1}$ for k_d of MKH (see Table I).

TABLE I

EFFECT OF O_2 CONCENTRATION UPON THE STEADY-STATE CONCENTRATION OF MKH IN THE HORSERADISH PEROXIDASE REACTION AND APPROXIMATE VALUES OF THE RATE CONSTANT (k) OF THE REACTION OF MKH WITH O_2

Final concentrations: 250 μM MKH_2, 250 μM H_2O_2 and 0.1 M phosphate. The initial velocity (v) of MK formation was obtained by extrapolation from those measured at lower horseradish peroxidase concentrations. k_d was calculated from Eqn. 4 assuming that κ is 2. k was calculated from the following equation: $2v = 2\, k_d\, (HA\cdot)^2 + k\, (HA\cdot)\, (O_2)$.

pH	*Horseradish peroxidase* (μM)	O_2 (mM)	*Steady-state concn. of MKH* (μM)	v ($mM \cdot sec^{-1}$)	$k_d \times 10^{-9}$ ($M^{-1} \cdot sec^{-1}$)	$k \times 10^{-6}$ ($M^{-1} \cdot sec^{-1}$)
6.5	1.0	0	0.31	0.87	9	5.6
		0.6	0.23			
7.5	0.5	0	0.22	0.36	7.5	5.2
		0.6	0.14			

When cytochrome b_5 is present in the reaction mixture where ESR-detectable MKH is accumulated, the cytochrome is reduced almost instantaneously. The reduction is so fast that a direct measurement of the rate can be made only under very specific conditions. In the experiment depicted in Fig. 8, the steady-state concentration of MKH is close to the limit of ESR measurement, and the half-time of cytochrome reduction is less than 20 msec. The value thus obtained for k_r is $3.0 \cdot 10^8\ M^{-1} \cdot sec^{-1}$.

In the case of nonenzymic reduction of cytochrome b_5 by MKH_2, the MK-accelerating reduction ascribed to MKH at equilibrium between MKH_2 and MK is observed. This reaction can also be used for the direct measurement of k_r, as shown in Fig. 9. The measurements of the velocity of cytochrome b_5 reduction are made at different cytochrome b_5 concentrations using different wavelengths: 556 mμ for 1 μM and 424 mμ for 0.1 μM. The values thus obtained for k_r are almost the same and

are also very close to that obtained by the enzymic method. The second-order rate constant of $3 \cdot 10^8$ $M^{-1} \cdot sec^{-1}$ is fairly high and is almost the upper limit that can be measured by the method described here.

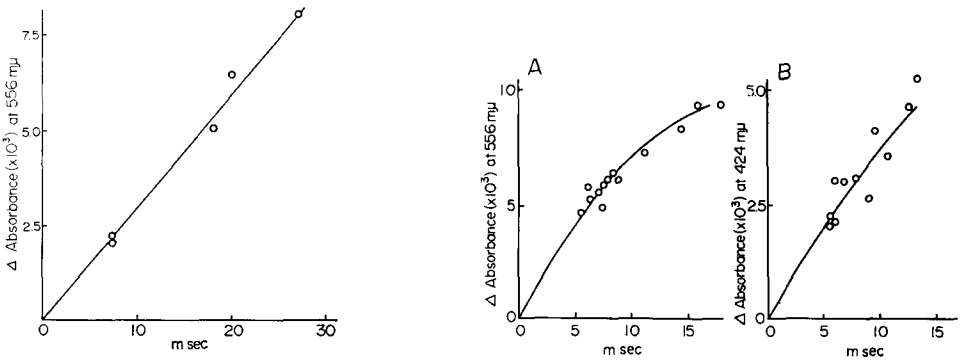


Fig. 8. Measurement of the rate of cytochrome b_5 reduction in the horseradish peroxidase reaction system using a continuous-flow method. The solution of horseradish peroxidase and cytochrome b_5 was mixed with the solution of H_2O_2 and MKH_2. Final concentrations: 0.1 μM horseradish peroxidase, 100 μM H_2O_2, 100 μM MKH_2, 1 μM cytochrome b_5 and 0.05 M phosphate (pH 6.5). The steady-state concentration of MKH free radical was 0.063 μM. This concentration was almost at the lower limit which could be measured with the present ESR spectrometer, and a reliable value could be obtained by extrapolating the horseradish peroxidase concentration in the same manner as shown in Fig. 7

Fig. 9. Measurement of the rate of cytochrome b_5 reduction in the nonenzymic system using a continuous-flow method. Cytochrome b_5 solution was mixed with the solution of MKH_2 and MK. Final concentrations: 1 μM (A) and 0.1 μM (B) cytochrome b_5, 1 mM MKH_2, 0.25 mM MK, 0.05 M phosphate (pH 7.5). The reduction of cytochrome b_5 was observed at 556 mμ in A and at 424 mμ in B. The steady-state concentration of MKH was 0.17 μM in both cases.

The oxidase function of microsome in the presence of a catalytic amount of MK was explained by NISHIBAYASHI, OMURA AND SATO[16] on the basis of an involvement of MKH. The same conclusion may be deduced from the finding of KLAPPER AND HACKETT[17,18] that MKH_2 is a substrate for the peroxidase–oxidase reaction in which the involvement of monodehydro substrate has been suggested by YAMAZAKI[14]. The measurement of the rate constant of oxygen reduction by MKH is difficult but can be made indirectly using ESR spectroscopy. The steady-state concentration of MKH

TABLE II

RATE CONSTANTS ($M^{-1} \cdot sec^{-1}$) OF THE REACTION OF MONODEHYDRO MOLECULES (HA·) WITH CYTOCHROMES OXIDIZED AND O_2

Electron acceptor	*HA·*		
	MKH	*p-Benzosemiquinone*	*Monodehydroascorbate*
Cytochrome *c*	$2 \cdot 10^8$	$2.5 \cdot 10^6$	$4 \cdot 10^4$
Cytochrome b_5	$3 \cdot 10^8$	—*	—*
O_2	$5 \cdot 10^6$ (approx.)	—*	—*

* — sign means that a positive reaction has not yet been observed.

formed in the peroxidase reaction decreases in the presence of molecular oxygen (see Table I). This can be explained by assuming that MKH gives one electron to molecular oxygen and the perhydroxyl radical thus formed decays rapidly by dismutation. This simple assumption gives an approximate value of $5 \cdot 10^6$ $M^{-1} \cdot sec^{-1}$ for the rate constant of the reaction of MKH with molecular oxygen (see Table I).

Ascorbate oxidase is an enzyme which catalyzes one-electron oxidation of ascorbate and reductate[7]. Fig. 10 shows ESR spectra of monodehydroascorbate observed during continuous flow. The hyperfine pattern of Spectrum C is different from Spectrum A reported in the enzymic system[4,7] and is identical with that reported by FOESTER, WEIS AND STAUDINGER[19] in the nonenzymic equilibrium system. This discrepancy is due to the difference in the modulation amplitudes of the magnetic field. The resolution in Spectrum C is still somewhat lower than that reported by FOESTER, WEIS AND STAUDINGER[19], but this seems to be due to the use of the flow method in the experiment of Fig. 10. Monodehydroascorbate thus formed can reduce cytochrome *c* but is not as active as MKH or *p*-benzosemiquinone. The rate constant of $4 \cdot 10^4$ $M^{-1} \cdot sec^{-1}$ is given for the reaction of monodehydroascorbate with cytochrome *c* (Fig. 11). This is very close to the value obtained by the ordinary photometric method without the flow technique[15].

NADH and indoleacetate are also substrates for the peroxidase–oxidase reaction, and it has been suggested that the monodehydro forms of these molecules are active intermediates. No ESR signal, however, has been observed during the peroxidatic oxidation of NADH and indoleacetate. This may be due mainly to the fast dismutation of these monodehydro molecules and partly to the slow reaction of NADH and indoleacetate with peroxidase–H_2O_2 complexes. The rate constants of the reaction of horseradish peroxidase Compound II with NADH and indoleacetate

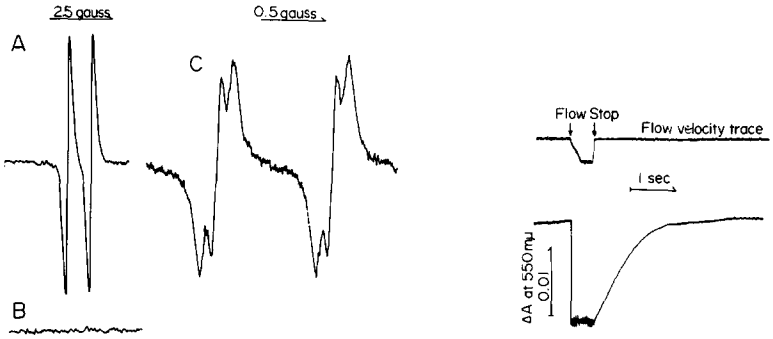


Fig. 10. ESR spectra of monodehydroascorbate formed in the steady state of ascorbate oxidase reaction during a continuous flow. The ascorbate solution was mixed with the solution of ascorbate oxidase. Final concentrations: 1 μM ascorbate oxidase (on the basis of copper), 1 mM ascorbate and 0.05 M citrate (pH 5.5). The spectra were not affected by the addition of 10 μM cytochrome *c* to this reaction system. A and C were taken during the flow and B after the flow was stopped. The amplitude of field modulation was 0.1 gauss in A and B and 0.04 gauss in C. The concentration of monodehydroascorbate in A and C was 2.0 μM. The gain used in C was twice that in A and B.

Fig. 11. Measurement of the rate of cytochrome *c* reduction in the ascorbate oxidase system using a stopped-flow method. The reaction conditions were the same as those of Fig. 10 except that cytochrome *c* (final concentration, 10 μM) was added. The steady-state concentration of monodehydroascorbate was 2.0 μM. Only 0.8 μM cytochrome *c* was reduced during the course of the reaction.

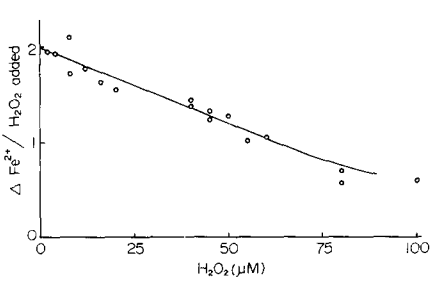
are known to be on the order of $10^4\ M^{-1}\cdot sec^{-1}$, which is much less than that with benzohydroquinone. Though monodehydro-NADH was not detected by ESR during enzymic oxidation, the stoichiometric formation of such intermediates is strongly supported by the experiments illustrated in Fig. 12. Horseradish peroxidase catalyzes the reduction of iron by NADH in the presence of H_2O_2. The molar ratio of iron reduced to H_2O_2 added becomes almost 2 when H_2O_2 concentration is extrapolated to zero. The same type of result has been obtained for the reaction system containing indoleacetate instead of NADH[20]. The direct reduction of iron by NADH is negligible, and the results suggest the following mechanism:

$$2\ NADH + H_2O_2 \xrightarrow{\text{peroxidase}} 2\ NAD\cdot + 2\ H_2O \quad (5)$$

$$2\ NAD\cdot + 2\ Fe^{3+} \longrightarrow 2\ NAD^+ + 2\ Fe^{2+} \quad (6)$$

NAD· formed in the peroxidatic oxidation can also reduce cytochromes b_5 and c, metmyoglobin and peroxidase as well as molecular oxygen.

Monodehydro forms of oxidogenic substrates[14] for peroxidase are very unstable in general except for the chlorpromazine radical. Of various oxidogenic substrates, *p*-cresol is a very fast substrate for horseradish peroxidase, and its free radical is relatively stable. The hyperfine pattern of monodehydro-*p*-cresol can be clearly observed in the presence of a large amount of horseradish peroxidase, as shown in Fig. 13. Monodehydro-*p*-cresol dimerizes in the ordinary peroxidase reaction but can remove an electron from the hydrogen donors which are slow substrates for horseradish peroxidase. This is why the addition of *p*-cresol accelerates the peroxidatic oxidation of sluggish substrates for horseradish peroxidase. When ascorbate is added to the system containing horseradish peroxidase, *p*-cresol and H_2O_2, the ESR signal of monodehydro-*p*-cresol (Fig. 13, A) is completely replaced by that of monodehydro-



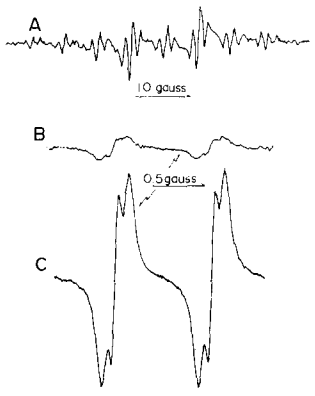


Fig. 12. Stoichiometry of the iron reduction during the peroxidatic oxidation of NADH. 2 μM horseradish peroxidase, 1 mM NADH, 0.4 mM $FeCl_3$, 1.2 mM *o*-phenanthroline and 0.05 M phosphate (pH 6.0). The molar ratio of reduced iron to added H_2O_2 was plotted against the concentration of added H_2O_2.

Fig. 13. ESR spectra of monodehydro-*p*-cresol (A) and monodehydroascorbate (B and C) formed in the steady state of peroxidase reaction. Final concentrations: 2 μM horseradish peroxidase, 0.5 mM H_2O_2 and 0.05 M phosphate (pH 6.2). (A) 1 mM *p*-cresol; (B) 2 mM ascorbate; (C) 1 mM *p*-cresol and 2 mM ascorbate. The amplitude of field modulation was 0.4 gauss in A and 0.02 gauss in B and C. The gain used in A was twice that in B and C.

ascorbate (Fig. 13, C), though ascorbate is a much slower substrate for peroxidase than *p*-cresol. Fig. 14 shows that the addition of 1 mM *p*-cresol causes a 16-fold increase in the rate of ascorbate oxidation and a 4-fold increase in the steady-state concentration of monodehydroascorbate.

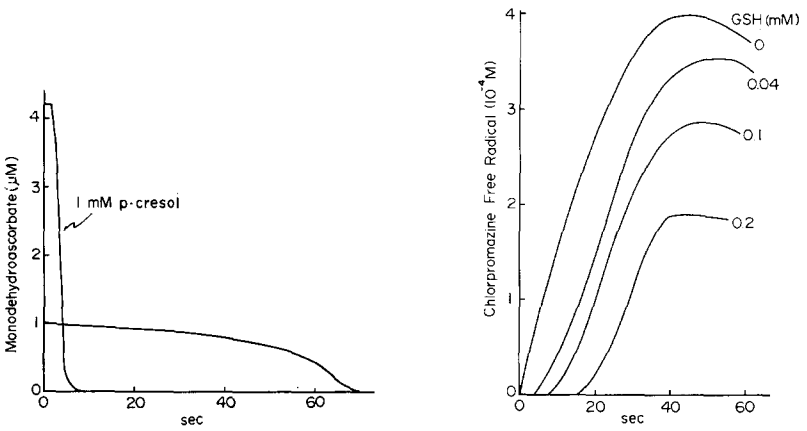


Fig. 14. Time courses of monodehydroascorbate in the presence and absence of *p*-cresol in the peroxidase reaction. The magnetic field was adjusted so as to obtain the maximum of the derivative curve. Final concentrations: 1 μM horseradish peroxidase, 0.5 mM H_2O_2, 1 mM ascorbate and 0.05 M phosphate (pH 6.2).

Fig. 15. Effect of the glutathione concentration upon the accumulation of chlorpromazine free radical in the peroxidatic oxidation of chlorpromazine. Final concentrations: 0.028 μM horseradish peroxidase, 0.2 mM H_2O_2, 1 mM chlorpromazine and 0.05 M acetate (pH 4.8). Glutathione concentrations are indicated in the figure.

It has been reported by PIETTE, BULOW AND YAMAZAKI[21] that 2 moles of chlorpromazine radical are accumulated by the consumption of 1 mole of H_2O_2 in the presence of horseradish peroxidase. The ESR signal of chlorpromazine radical is replaced by the monodehydroascorbate signal when ascorbate is mixed with the solution containing the chlorpromazine radicals. The addition of a limited amount of glutathione to the reaction system of peroxidatic oxidation of chlorpromazine causes a lag phase in the formation of chlorpromazine radicals, as shown in Fig. 15. This indicates that the chlorpromazine radical returns to the original chlorpromazine molecule by oxidizing glutathione to the monodehydro form. Monodehydroglutathione appears to dimerize very rapidly into oxidized glutathione. An ESR signal has never been observed from glutathione as well as NADH and indoleacetate in the enzymic oxidation.

DISCUSSION

Although free radicals are known to be involved in many chemical reactions, a direct method of measurement of the reaction rate has not yet been established. This may be due to the nonspecific high reactivity and the low steady-state concentration of the free radicals present in the reactions. Many biological processes including respiration and photosynthesis also involve radical intermediates which seem to be in the activated state. The quantitative measurements of the radical activity are very

important in order to clarify the activation nature of these biochemical processes.

For the direct measurements of free radical reactions enzymic systems have the advantage of their high specificity and efficiency. The observed kinetics and stoichiometry of the reaction support the mechanism, given in Fig. 16, in which the

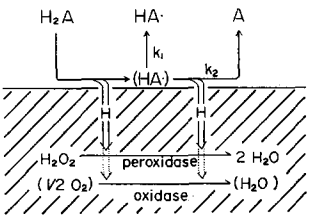


Fig. 16. Schematic representation of apparent one-electron process ($\kappa = 2$) and two-electron process ($\kappa = 0$) on the surface of oxidative enzymes. κ is defined in Eqn. 7.

one-electron transfer from electron donors is catalyzed by the enzymes and the main path of radical disappearance is dismutation or dimerization[7]. The value of κ defined in Eqn. 4 is now given by

$$\kappa = \frac{k_1}{\frac{1}{2}k_1 + k_2} \tag{7}$$

When κ is close to 2, as in the case of peroxidase and ascorbate oxidase reactions, the reaction can be said to consist of compulsory one-electron steps. In the case of catechol oxidation by tyrosinase[22], on the contrary, κ is almost zero, and it can be said for the time being that the mechanism involves a two-electron step.

When cytochromes are present in the enzymic oxidation of redogenic molecules in which $\kappa = 2$, the rapid reduction of the cytochromes will generally occur. Under the conditions where the cytochromes are reduced only by the monodehydro molecules, the rate constant, k_r, in Eqn. 3 can be measured by simultaneous measurements of the concentration of monodehydro molecules with an ESR spectrometer and the velocity of the cytochrome reduction with a spectrophotometer. Simultaneous measurements in the same reaction solution are possible in principle but very difficult in practice[21]. In the present study the two measurements are carried out separately under the same experimental conditions.

The steady-state concentrations of the monodehydro molecules are usually measured at a concentration around 0.1 and 1 μM. Eqn. 8 shows the relationship between the half-time of the cytochrome reduction ($t_{1/2}$) and the second-order rate constant (k_r).

$$t_{1/2} = \frac{0.693}{k_r\,(\mathrm{HA}\cdot)} \tag{8}$$

It can be seen from this equation that the rate constant of $10^9\ \mathrm{M^{-1}\cdot sec^{-1}}$ is almost the upper limit which can be measured by the present method.

Fig. 17 shows selectivity and exothermicity of reactions. Electron-transfer reactions from donor molecules to enzymes and from monodehydro molecules to cytochromes seem to be exothermic ones which require a small activation energy. Fig. 17 also shows that a selective one-electron oxidation of donor molecule by the enzyme system produces a strong one-electron reductant. Stoichiometric reduction of cyto-

chromes (as shown in Fig. 2) is easily obtained under suitable conditions. This selectivity of the reaction observed even in these free radical reactions seems to be characteristic of biochemical reactions. Monodehydro molecules may act not only as reductants, but also as oxidants. A possible participation of monodehydroascorbate as an oxidant in the microsome-catalyzed oxidation of NADH[25] and in the oxidation of cytochrome b_5 has been suggested[26]. The details will be reported in the following paper[27].

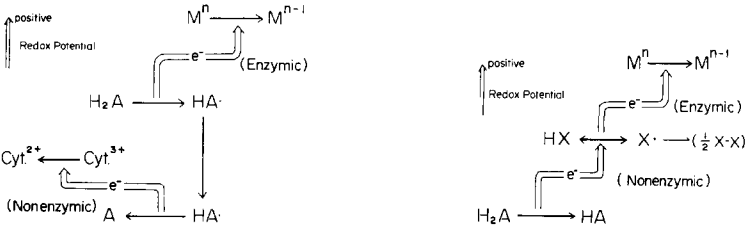


Fig. 17. Effective reduction of cytochromes catalyzed by specific one-electron oxidants, such as oxidative enzymes (M). n indicates the effective valence in the catalytic site of the enzyme. The ordinate position indicates the level of redox potential, and consequently the exothermicity of the reaction corresponds to the upward electron flow. The efficiency of the electron-transfer reaction from HA· to cytochromes will be discussed in the following paper of this series[27].

Fig. 18. HX-mediated one-electron transfer from H_2A to the enzyme. HX indicates an oxidogenic molecule[14]. The other symbols are the same as in Fig. 17.

A strong one-electron oxidation by monodehydro molecules can usually be seen when oxidogenic substrates are used as electron donors in the peroxidase system. The role of oxidogenic substrates in the electron-transfer reaction is schematized in Fig. 18. Here, the oxidogenic substrate plays the role of a positive-hole carrier. In the presence of sluggish substrates for peroxidase the removal of an electron from such molecules is sometimes very stimulated by the presence of an oxidogenic substrate, such as monohydroxybenzene derivatives[14]. This type of electron transfer has been demonstrated in Figs. 13, 14 and 15, using the ESR spectroscopy.

The monodehydro compounds of *p*-cresol and chlorpromazine oxidize both ascorbate and NADH. Glutathione is oxidized by the chlorpromazine free radical but not by the monodehydro-*p*-cresol. The quantitative measurements of the reaction of free radicals derived from oxidogenic substrates with the biological redox compounds have not yet been successful.

ACKNOWLEDGEMENT

This investigation was supported by research grants from the U.S. National Institutes of Health (AM 06518).

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