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COMPOUND III KINETICS AND CHEMILUMINESCENCE IN OSCILLATORY OXIDATION REACTIONS CATALYZED BY HORSERADISH PEROXIDASE

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SUMMARY

1. The peroxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) catalyzed oxidation of reduced nicotinamide adenine dinucleotides, dihydroxyfumaric acid or indole-3-acetic acid, in a reaction system open to O_2, exhibit damped oscillations in the reaction rate.

2. All known aerobic oxidation reactions catalyzed by peroxidase alone are autocatalytic. The damped oscillation is a consequence of the autocatalysis.

3. Compound III is observed spectroscopically in the NADH and the dihydroxyfumaric acid oxidation but not in the indole-3-acetic acid oxidation. Large differences exist in the Compound III kinetics in the NADH and the dihydroxyfumaric acid oxidation. Compound III is not essential in the mechanism of the oscillation.

4. The oxidation of the three donors is accompanied by chemiluminescence whose intensity increases in the order NADH < dihydroxyfumaric acid < indole-3-acetic acid. Except in the NADH oxidation the chemiluminescence is strong enough to be used for kinetic measurements and possibly also for spectroscopic identification of intermediates.

INTRODUCTION

The classical enzyme activity of peroxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) is the oxidation of donors using H_2O_2 as the acceptor. However, peroxidase is also known to catalyze oxidation reactions where O_2 is the acceptor. The first such reaction to be described was the oxidation of dihydroxyfumaric acid, investigated by SWEDIN AND THEORELL[1]. In subsequent studies of the oxidase activity of peroxidase, it was found that Mn^{2+} and certain phenols stimulate the reaction, and in some cases these factors were considered essential for the oxidase activity[2,3]. It is now established that at pH lower than 6 a few donors are oxidized rapidly in the presence of peroxidase without any additional factors. These donors are dihydroxyfumaric acid[1,2,4,5], triose reductone[6], indole-3-acetic acid[7–12] and reduced nicotinamide adenine dinucleotide (NADH and NADPH)[3,13]. The two types of oxidation reactions catalyzed by peroxidase, using H_2O_2 or O_2 as the acceptor, are in the following referred to as peroxidation and aerobic oxidation, respectively. The work before 1961 on the aerobic oxidase activity of peroxidase has been reviewed by NICHOLLS[14].

Neither of the three components involved in a peroxidase catalyzed aerobic

oxidation, namely O_2, donor and peroxidase, are capable of reacting with each other. It is currently assumed that the mechanism of the oxidation reaction involves the formation of H_2O_2 from O_2 and donor, mediated by free radicals. H_2O_2 is then used in the peroxidation of donor. This assumption is supported by the stimulating effect of added H_2O_2 and the inhibitory effect of catalase.

The mechanism of peroxidation is assumed to be[15,16]

$$\text{Ferriperoxidase} + H_2O_2 \rightarrow \text{Compound I} \quad (1)$$

$$\text{Compound I} + YH_2 \rightarrow \text{Compound II} + YH^{\bullet} + H_2O \quad (2)$$

$$\text{Compound II} + YH_2 \rightarrow \text{Ferriperoxidase} + YH^{\bullet} + H_2O \quad (3)$$

where YH_2 is the donor and $YH^{\bullet}$ is donor free radical. The utilization of O_2 as acceptor is due to a reaction between O_2 and the donor free radical formed in the peroxidation Reactions 2 and 3 (ref. 4).

$$YH^{\bullet} + O_2 \rightarrow Y + O_2^- + H^+ \quad (4)$$

The dismutation of O_2^-

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \quad (5)$$

gives 1 molecule of H_2O_2 for 1 molecule of H_2O_2 used in Reaction 1. Reactions 1–5, therefore, constitute a chain reaction. Chain termination would be effected by dismutation of $YH^{\bullet}$.

$$2YH^{\bullet} \rightarrow Y + YH_2 \quad (6)$$

A reaction scheme composed of Reactions 1–6 would require an addition of H_2O_2 or free radical initiator. However, such an addition is not required in the peroxidase catalyzed aerobic oxidation of any of the four donors mentioned above. If an initiator is not added the initial reaction rate may be low, but the rate increases with time indicating an autocatalytic reaction. It must, therefore, be assumed that a reaction step which causes the multiplication of the active intermediates is involved. This reaction could be[14]

$$O_2^- + YH_2 + H^+ \rightarrow YH^{\bullet} + H_2O_2 \quad (7)$$

Under suitable conditions, 1 $YH^{\bullet}$ molecule can during its lifetime generate many molecules of H_2O_2 in the cycle composed of Reactions 4 and 7. Under such circumstances more than 1 molecule of H_2O_2 is produced for every one consumed in Reaction 1. The Reactions 1–4 and 7, therefore, constitute a branched chain reaction, *i.e.* an autocatalytic reaction.

Support for the above free radical reaction scheme is the finding by electron paramagnetic resonance spectroscopy, of free radicals originating from donor during different peroxidation and aerobic oxidation reactions catalyzed by peroxidase[4,17,18]. However, measurable radical concentrations have not been found in connection with the aerobic oxidation of any of the donors mentioned above. The occurrence of chemiluminescence during the peroxidation of different donors[19,20] may also be taken as an indication of a free radical mechanism. In the present paper the occurrence of chemiluminescence during aerobic oxidations catalyzed by horseradish peroxidase is reported.

YAMAZAKI *et al.*[21] and YAMAZAKI AND YOKOTA[22] have found that a damped oscillation of the O_2 concentration in the solution may occur when the peroxidase catalyzed aerobic oxidation of NADH takes place in an open system where O_2 is continuously supplied by bubbling with an N_2–O_2 mixture. They also found a synchronous damped oscillation of the concentration of Compound III. They concluded that Compound III acts as a regulator of the enzyme activity, and is thereby responsible for the oscillations. The present paper reports damped oscillations in the oxidation of dihydroxyfumaric acid and indole-3-acetic acid similar to the oscillations in the NADH oxidation reported by YAMAZAKI *et al.*[21] and YAMAZAKI AND YOKOTA[22]. In view of the important role ascribed to Compound III in the theory of YAMAZAKI of the oscillation mechanism, a comparison is made between the Compound III kinetics in the oxidation of the three donors known to give oscillations. Triose reductone was not tested for oscillatory oxidation, but there is reason to believe that it would work also.

Autocatalysis generally presents a severe obstacle to quantitative kinetic measurements because small unavoidable variations in the initial conditions are amplified so as to cause a very poor reproducibility of the kinetic curves. For this reason only qualitative deductions from the kinetic curves obtained in the present experiments were attempted. A preliminary report of a part of the present work has been published[23].

EXPERIMENTAL

The experiments were carried out in a 1 cm × 1 cm × 4 cm glass cuvette mounted in a brass block which was thermostated by a relay-less electronic device, without the intervention of a water bath. The thermostat worked without temperature oscillations. All experiments were carried out at 25°. A mixture of N_2 and O_2 whose composition could be regulated by means of flow meters was blown on the surface of the solution at a rate of 10 ml/sec. The gas was not bubbled into the solution. The O_2 concentration in the solution was measured polarographically by means of a vibrating platinum electrode which also stirred the solution efficiently. The cuvette had a side arm with a porous plug separating the reaction solution and a concentrated KCl solution, serving as a bridge to a calomel electrode.

The thermostated cuvette was mounted in a dual wavelength spectrophotometer (Johnson Foundation design). The increase in light absorption was measured at 418 mμ with 615 mμ as a reference wavelength. The chemiluminescence was measured in the same setting except that the light was off and a different circuit to measure the photocurrent was used. When measuring chemiluminescence the photomultiplier tube (E.M.I. 9592B) was cooled by solid CO_2 filled into a wooden box surrounding the holder of the tube. To avoid condensing water the socket of the photomultiplier tube was wrapped in a plastic bag containing silica. A Keithley 242 stabilized high voltage source was used for the chemiluminescence measurements. In most measurements the voltage on the photomultiplier was 1450 V, and the photocurrent was measured as a potential across a $10^8\ \Omega$ resistance. 1 unit on the chemiluminescence scale in the figures corresponds to approx. $2.5 \cdot 10^4$ photons/sec hitting the photocathode. The window of the phototube was situated 1 cm from the cuvette, and separated from it by a plexiglass window. Dry N_2 was blown on the plexiglass window to prevent water condensation.

The enzyme used in the experiments was horseradish peroxidase purchased from Boehringer und Soehne. The purity number was 2.8.

Interpretation of O_2 concentration curves in the open system

The interpretation of O_2 concentration measurements in a system open to O_2 is not as straightforward as the interpretation of measurements in the accustomed closed system, and will, therefore, be discussed briefly. Because of the rapid flow of the gas mixture above the solution, the O_2 uptake of the solution has a negligible influence on the composition of the gas mixture and, because of the stirring, the liquid is practically homogeneous with respect to O_2 concentration. Under these circumstances the rate of transport of O_2 from gas to liquid, v_t, is proportional to the difference between the equilibrium concentration of O_2 in the solution, $[O_2]_e$, and the actual O_2 concentration in the solution, $[O_2]$ (ref. 24).

$$v_t = k([O_2]_e - [O_2]) \tag{8}$$

where k is a constant depending on the surface area of the solution and the rate of stirring.

If during an O_2 consuming reaction, the O_2 concentration remains at practically zero, the transport of O_2 from the gas to the liquid is the rate limiting process. The reaction rate is then equal to the constant $k[O_2]_e$, as can be seen from Eqn. 8. This is the maximal possible steady state rate of the O_2 consuming reaction, but a higher reaction rate can occur for a short time until the dissolved O_2 is depleted. Obviously, the maximal steady state rate of reaction does not depend on the chemical composition of the reaction solution. In chemiluminescence measurements the open system, therefore, offers an easy way to compare the intensity of chemiluminescence of different reaction mixtures at the same reaction rate.

The observed rate of increase of the O_2 concentration, v_o, the rate of transport of O_2 from gas to liquid, v_t, and the rate of consumption of O_2 by the chemical reaction, v_r, are connected by the equation:

$$v_o = v_t - v_r \tag{9}$$

By substituting Eqn. 8 into Eqn. 9 and differentiating we obtain

$$dv_r/dt = -kv_o - dv_o/dt \tag{10}$$

If the O_2 concentration is observed to decrease ($v_0 < 0$) at an increasing rate ($dv_0/dt < 0$) it follows from Eqn. 10 that $dv_r/dt > 0$, *i.e.* the O_2 consuming reaction is accelerating. In a closed system an inflection point on a negative slope indicates the maximal reaction rate. For an inflection point on a negative slope of the O_2 concentration curve in the open system we find from Eqn. 10 that $dv_r/dt > 0$. This means that the O_2 consuming reaction is still accelerating when the observed fall of O_2 concentration has ceased to accelerate. With other words, the maximal rate of the chemical reaction occurs later than the maximal rate of decrease of the O_2 concentration. Even if decreasing rates of decrease of the O_2 concentration is observed throughout the reaction, there may still be acceleration in the O_2 consumption if only $|dv_0/dt| < |kv_0|$.

RESULTS AND DISCUSSION

Aerobic oxidation of NADH

When NADH was oxidized aerobically in the horseradish peroxidase catalyzed reaction a very weak chemiluminescence was found. At the high sensitivity required to measure the light the response time of the measurement was so long that meaningful kinetic curves could not be obtained. Therefore, only light absorption studies were done on the NADH oxidation. The wavelength used in these studies was 418 mμ where both Compound II and Compound III have absorption maxima of nearly equal intensity. Therefore, the measurements cannot distinguish between these two compounds. However, by scanning the visible absorption spectrum during the aerobic oxidation of NADH, YAMAZAKI AND YOKOTA[13,25] found that the intermediate form of the enzyme present during this reaction is Compound III.

Fig. 1A shows the simultaneous measurement of the increase in light absorption at 418 mμ and the O_2 concentration in the solution in an experiment where NADH was added at time zero to the enzyme–buffer solution which was initially in O_2 equilibrium with the gas phase. For practical reasons the curves are squeezed a little by omitting two monotonous sections. It is seen that the light absorption ascribed to Compound III increases rapidly after NADH is introduced. It then decreases slowly during the reaction. A more rapid final decrease occurs after 43 min. At that time a small minimum in the O_2 concentration is observed, followed by an increase to the

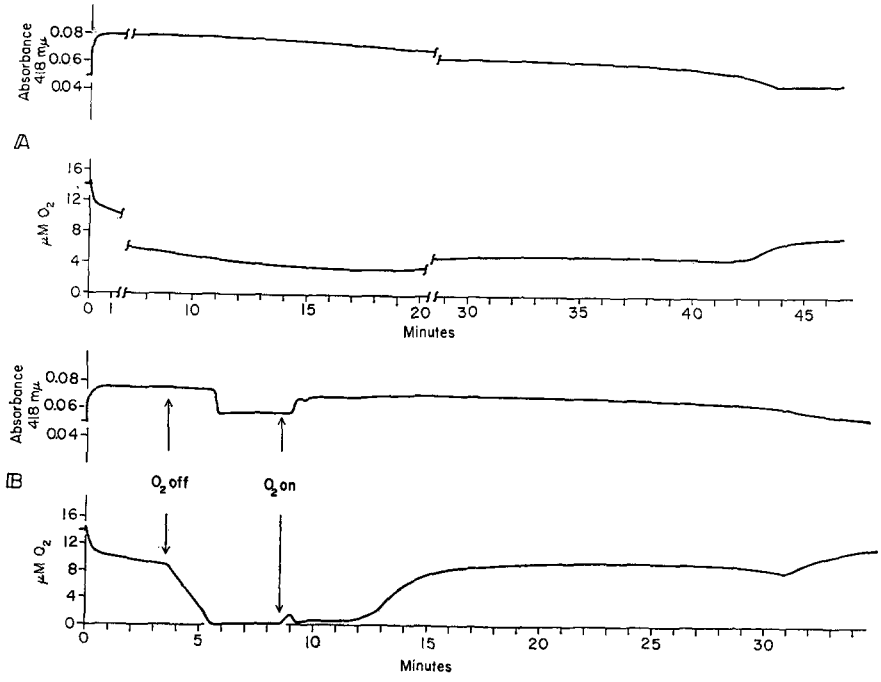

Fig. 1. A, simultaneous measurement of light absorption at 418 mμ (absorption maximum of Compound III) and O_2 concentration in the solution during the aerobic oxidation of NADH (0.88 mM) catalyzed by horseradish peroxidase (0.8 μM) in phosphate buffer (0.1 M, pH 5.1). NADH dissolved in water is added at time zero to solution in equilibrium with N_2–O_2 gas mixture. B, the same as A except that the O_2 in the gas is turned off and on during the experiment.

initial level. If NADH is now added again the reaction will proceed in the same way as before. This shows that the O_2 consuming reaction stops due to depletion of donor. Fig. 1B shows an identical experiment except that the O_2 in the gas phase is turned off and on. When the O_2 in the gas is turned off, the O_2 concentration in the solution falls nearly linearly with time but the concentration of Compound III is unchanged until the O_2 concentration, after a final acceleration, falls to zero. Coincident with the final accelerated phase of disappearance of O_2, Compound III disappears rapidly. When the O_2 in the gas is turned on to its previous value, Compound III is formed again after a lag time. An overshoot followed by a strongly damped oscillation is observed in the O_2 concentration of the solution. For a few minutes the O_2 concentration is low, indicating a higher reaction rate than before the O_2 was turned off. As long as the reaction rate is high the Compound III concentration does not reach the level it had when the reaction rate was low.

Discussion of NADH results

This experiment reiterates the previously reported finding that two different stable steady states can be realized in the open system at the same composition of the reaction solution and the gas mixture[26]. From the analysis of simple reaction schemes, it is known that bistability may occur in an enzyme reaction where the enzyme is reversibly converted to an inactive form by a reaction between the substrate and the enzyme–substrate complex[27–29]. Because 2 molecules of the substrate are required to form the inactive complex this is a second order forward inhibition. The bistability found in the present experimental system is caused by a forward inhibition exerted by one of the substrates, O_2. The mechanism of inhibition involves the formation of an inactive form of the enzyme, Compound III, in a way which is much more complicated than in the examples which have been analyzed.

Peroxidase does not react directly with O_2 to form Compound III. The formation of this compound may take place by one or more of the following reactions[30–32].

$$\text{Compound II} + H_2O_2 \rightarrow \text{Compound III} \quad (11)$$

$$\text{Ferriperoxidase} + O_2^- \rightarrow \text{Compound III} \quad (12)$$

$$\text{Ferroperoxidase} + O_2 \rightarrow \text{Compound III} \quad (13)$$

Ferroperoxidase is presumably formed during aerobic oxidations by the reaction[13]

$$\text{Ferriperoxidase} + YH^{\cdot} \rightarrow \text{Ferroperoxidase} + Y \quad (14)$$

All three reactions which may give rise to Compound III involve as reactants O_2 or species which are only present during the aerobic oxidation, and whose concentrations will increase with the O_2 concentration. As a result, the rate of formation of the inactive Compound III depends on the O_2 concentration and an indirect inhibitory effect of O_2 is observed.

Simultaneously with the formation of Compound III a decomposition of this substance is also taking place. This may happen by one or both of the following reactions[13,31].

$$\text{Compound III} + NADH \rightarrow \text{Ferriperoxidase} + H_2O_2 + NAD^{\cdot} \quad (15)$$

$$\text{Compound III} + NAD^{\cdot} + H^+ \rightarrow \text{Ferriperoxidase} + NAD^+ + H_2O_2 \quad (16)$$

The first of these reactions, Reaction 15, cannot be very fast as concluded from the observed low rate of NADH oxidation when the enzyme is converted to Compound III. The second, Reaction 16, is compatible with the observed inhibition by conversion to Compound III because there is no NAD· present when the oxidation reaction is inhibited.

In their experiments with horseradish peroxidase catalyzed aerobic oxidation of NADH, YOKOTA AND YAMAZAKI[13] and YAMAZAKI *et al.*[21] found that the rate of O_2 consumption was increasing with an increasing degree of conversion of the enzyme to Compound III, and the highest rate of oxidation occurred at the highest Compound III concentration. The large decrease of the reaction rate at very high degrees of conversion to Compound III probably escaped these investigators because their experimental conditions did not favor a very high degree of conversion. However, their suggestion that Compound III is a prerequisite for a high reaction rate is not necessarily erroneous. If two different forms of the enzyme are both required for the reaction to take place, the activity is zero if the enzyme is fully converted to either of the two forms, but there is at present no evidence that Compound III is involved in the chain reacton leading to rapid aerobic oxidation of NADH. The positive correlation between Compound III concentration and reaction rate which exists at lower degrees of conversion to Compound III is probably accidental and not causal as interpreted by YOKOTA AND YAMAZAKI[13] and YAMAZAKI *et al.*[21].

In the experiment of Fig. 1B, it was observed that after the O_2 in the gas was turned off, the O_2 concentration in the liquid fell nearly linearly with time which reveals a weak acceleration of the O_2 consuming reaction. This acceleration increased markedly just before the O_2 concentration reached zero. An acceleration in the disappearance of a reactant in a chemical reaction may be caused by two different effects, namely product activation (autocatalysis) or reactant inhibition. As it was found that the enzyme is inhibited by O_2, the observed acceleration may be ascribed to a relief of this inhibition when the O_2 concentration falls. However, the overshoot in O_2 concentration after the O_2 in the gas is turned on again is also an indication of an acceleration, and in this case the substrate inhibition effect can be excluded because it cannot lead to an overshoot. It is concluded that, at least in the latter case, the acceleration of the O_2 consuming reaction is due to autocatalysis. The autocatalysis in the O_2 consuming reaction is adequately accounted for by the reaction scheme for aerobic oxidation discussed in the INTRODUCTION.

The rapid decomposition of Compound III at the same time as the O_2 concentration falls to zero after the O_2 in the gas is turned off (Fig. 1B) is probably caused by a reaction between Compound III and NAD· according to Reaction 6. Depletion of O_2 interrupts the reaction cycle of aerobic oxidation in such a point that a temporary accumulation of NAD· above its previous level may take place. Furthermore, there is a possibility for autocatalysis in the decomposition reaction because the H_2O_2 formed in Reaction 16 by the expenditure of an NAD· may give rise to two NAD· through peroxidation of NADH. When O_2 is present, this autocatalytic decomposition reaction is prevented from accelerating due to chain termination caused by Reaction 4.

Many years ago, LOTKA[33] investigated a theoretical reaction system where a reactant enters at a constant rate and is consumed by an autocatalytic reaction. He found that damped oscillations may occur if the system is perturbed. The present experimental system conforms approximately with Lotka's model, and the damped

oscillations observed in the system may be considered as a verification of his prediction. The mechanism of the oscillation in the aerobic oxidations catalyzed by peroxidase is discussed on the basis of the Lotka model in the following paper[34].

Aerobic oxidation of dihydroxyfumaric acid

When dihydroxyfumaric acid was oxidized the chemiluminescence was strong enough to allow the measurement of kinetic curves. Because light absorption and chemiluminescence could not be measured at the same time all experiments were performed twice using the O_2 measurement as a common reference.

Fig. 2 shows experiments where increasing amounts of dihydroxyfumaric acid were added to solutions of horseradish peroxidase in phosphate buffer at pH 5.1, initially in equilibrium with the N_2–O_2 gas mixture. In all cases the O_2 concentration in the beginning falls with an increasing rate indicating that the O_2 consuming reaction is autocatalytic. At a certain initial concentration of dihydroxyfumaric acid a damped oscillation of the O_2 concentration is observed (Fig. 2C). The chemiluminescence measurements show a maximum about the same time as the O_2 curve has its steepest negative slope, which is approximately when the reaction rate is maximal. The damped oscillation of O_2 concentration in Fig. 2C is accompanied by a damped oscillation in the chemiluminescence. The maxima in this oscillation seem to correspond to maxima in the rate of O_2 consumption. In all the chemiluminescence curves there is observed a final peak which occurs when the O_2 concentration has nearly returned to its initial value. This maximum in chemiluminescence does not coincide with a high rate of O_2 consumption as in the previous phases of the reaction. A very small upward inflection of the O_2 concentration curve can sometimes be seen at the time when the final peak

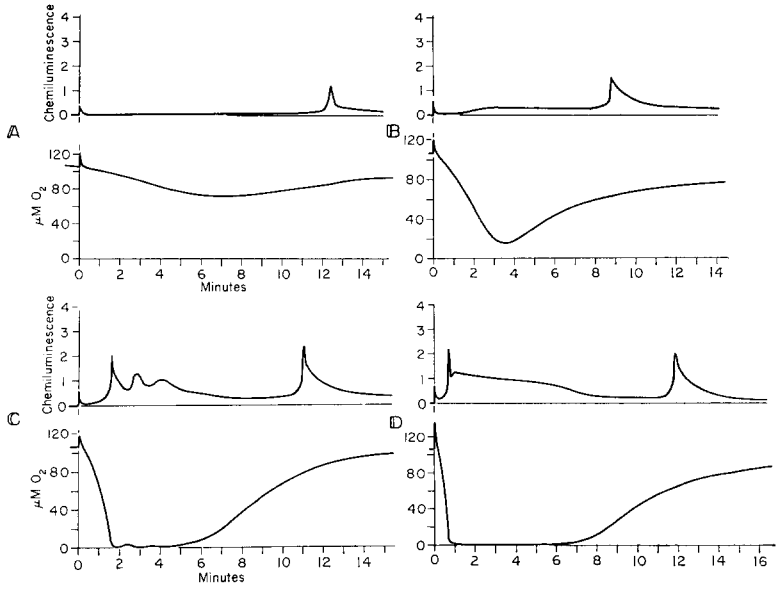

Fig. 2. Chemiluminescence and O_2 concentration during the oxidation of dihydroxyfumaric acid (A, 0.43 mM; B, 0.85 mM; C, 1.28 mM; D, 1.7 mM) catalyzed by horseradish peroxidase (0.8 μM) in phosphate buffer (0.1 M, pH 5.1). Dihydroxyfumaric acid dissolved in ethanol was added at time zero.

of chemiluminescence is observed. This indicates that the rate of O_2 consumption decreases when the peak appears.

The identity of the intermediate form of the enzyme which predominates during the aerobic oxidation of dihydroxyfumaric acid was established by CHANCE[2] who scanned the visible absorption spectrum during the reaction. In the absence of Mn^{2+} the increase in light absorption at 418 mμ was found to be due to Compound III. In Fig. 3 the experiments from Fig. 2 are repeated except that the increase in light absorption at 418 mμ is measured instead of the chemiluminescence. It is seen that the addition of dihydroxyfumaric acid to the O_2 equilibrated enzyme solution causes a very rapid formation of Compound III. Later on when the O_2 concentration begins to return to its original value, the concentration of Compound III decreases slowly. At a certain time, when the O_2 concentration is close to its initial value, the remaining Compound III suddenly disappears rapidly. By comparing Figs. 2 and 3, it is seen that the final peak of chemiluminescence occurs at the time when the rapid disappearance of Compound III takes place. When dihydroxyfumaric acid was added once more to the reaction solution after the O_2 concentration had returned to its original value, the course of reaction described above was found to repeat itself. This indicates that the oxidation reaction stops because of depletion of dihydroxyfumaric acid.

The effect of turning the O_2 in the gas off and on during the dihydroxyfumaric acid oxidation was studied in experiments (Fig. 4) where the conditions were the same as in the experiments of Figs. 2C and 3C. It is seen that turning off the O_2 in the gas is followed by a rapid disappearance of the chemiluminescence whereas the Compound III concentration decreases very slowly. When the O_2 is turned on again, a lag time before the reappearance of the Compound III and the chemiluminescence is

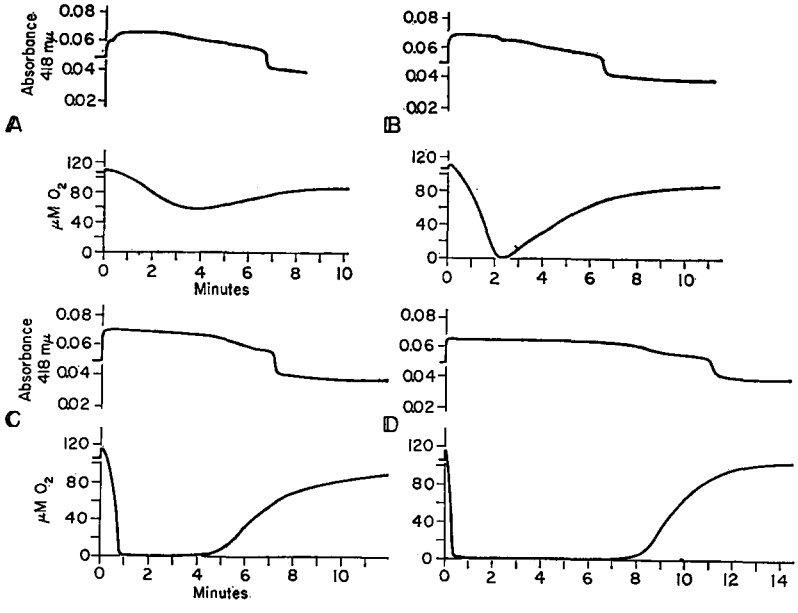

Fig. 3. Light absorption change at 418 mμ and O_2 concentration during the oxidation of dihydroxyfumaric acid. Concentrations are as in Fig. 2.

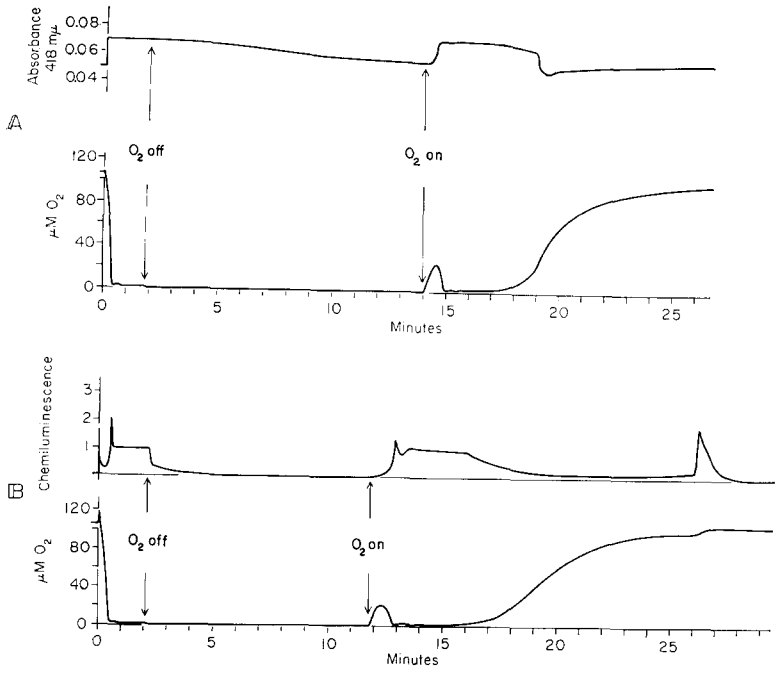

Fig. 4. Light absorption at 418 mμ (A) and chemiluminescence (B) in oxidation of dihydroxyfumaric acid. Conditions as in Figs. 2C and 3C except that the O_2 in the gas is turned off and on during the experiment.

observed, and the O_2 concentration shows an overshoot followed by a strongly damped oscillation. Bistability was not observed when dihydroxyfumaric acid was used as the donor.

The experiments shown in Figs. 2 and 3 were repeated with 2,4-dichlorophenol added. The results are shown in Figs. 5 and 6. By comparing with Figs. 2 and 3 it is seen that the most significant effect of adding 2,4-dichlorophenol is that the final disappearance of Compound III occurs at a lower O_2 concentration and is much faster than when 2,4-dichlorophenol is not added. It is accompanied by a sharp break in the O_2 concentration curve indicating a sudden cessation of the O_2 consumption. Whereas the intensity of the chemiluminescence during the steady state phase of the oxidation reaction is not significantly influenced by the addition of 2,4-dichlorophenol, the final peak is much narrower and more intense than when 2,4-dichlorophenol is not added.

Figs. 7 and 8 show the influence of Mn^{2+} in experiments similar to those of Figs. 2 and 3. The O_2 concentration falls much more rapidly than in the previous experiments and there is a high peak of chemiluminescence immediately when the dihydroxyfumaric acid is added. However, there is no chemiluminescence during the steady state phase of the reaction. When the O_2 concentration begins to increase again, a low and broad final peak of chemiluminescence is seen. In the light absorption measurements in Fig. 8, it is seen that the increase in light absorption at 418 mμ is lower than when Mn^{2+} was not added (Fig. 3). The final value of light absorption is significantly lower than the initial value. When Mn^{2+} is present the light absorption

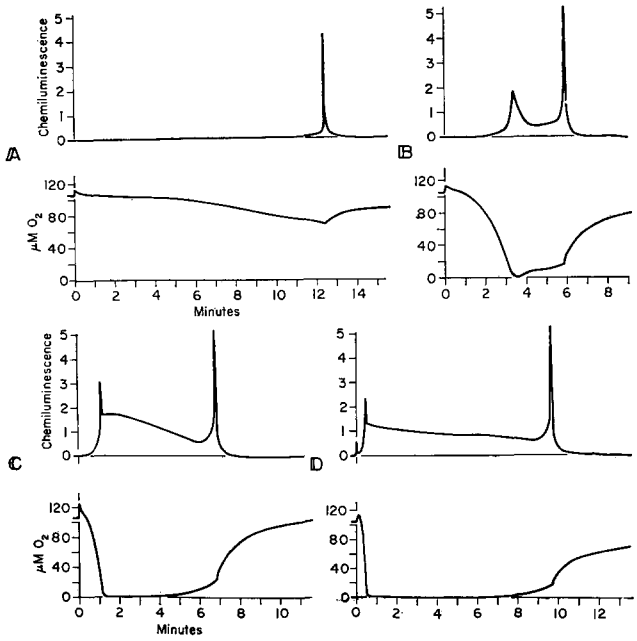

Fig. 5. Chemiluminescence and O_2 concentration in oxidation of dihydroxyfumaric acid in the presence of 2,4-dichlorophenol. Concentrations as in Fig. 2 except that 2,4-dichlorophenol (A, 7.7 μM; B, 15.4 μM; C, 23.1 μM; D, 30.8 μM) was added together with dihydroxyfumaric acid at time zero.

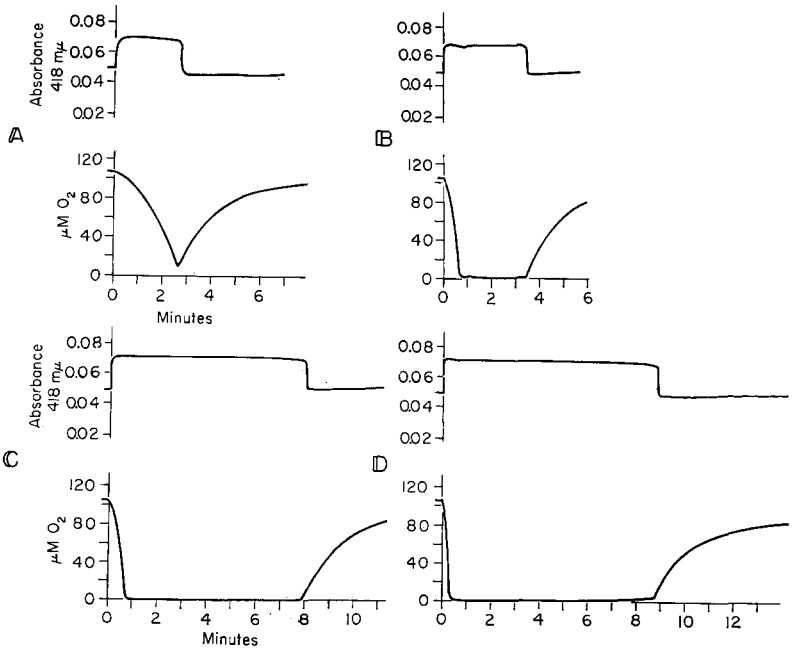

Fig. 6. Light absorption at 418 mμ and O_2 concentration in oxidation of dihydroxyfumaric acid in the presence of 2,4-dichlorophenol. Concentrations as in Fig. 5.

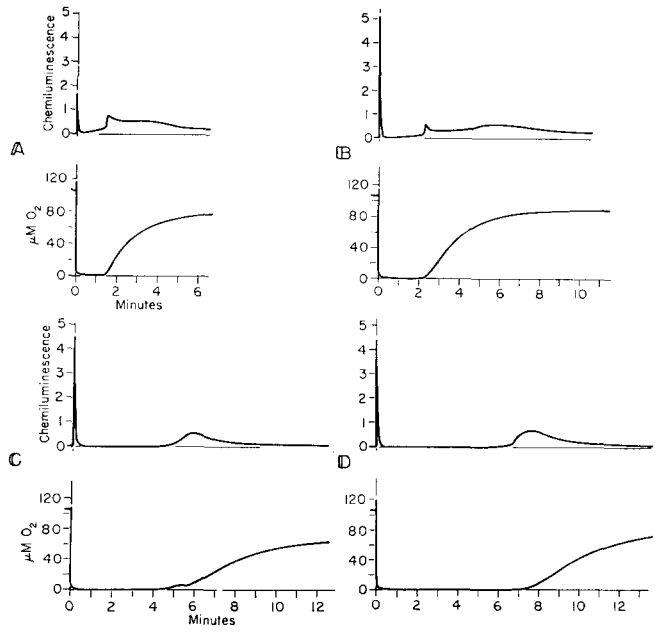

Fig. 7. Chemiluminescence and O_2 concentration in oxidation of dihydroxyfumaric acid in the presence of Mn^{2+} (A, B, C, and D, 2.50 μM). Other concentrations as in Fig. 2.

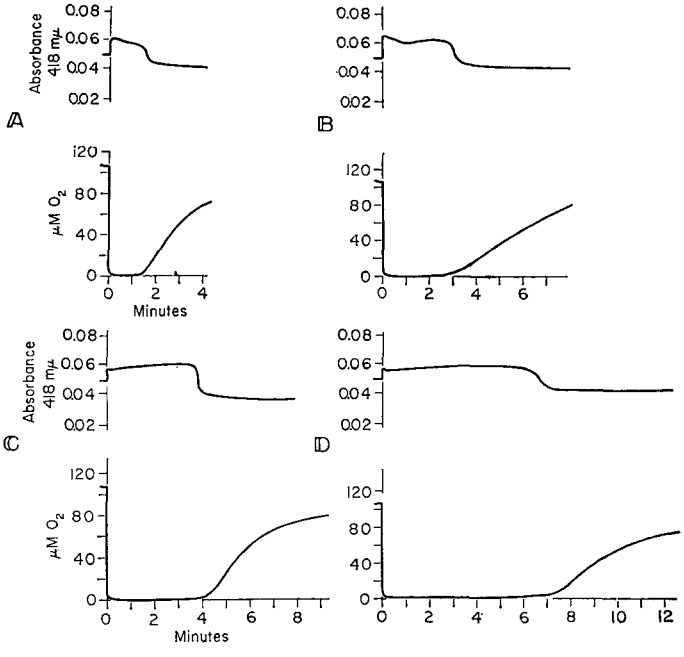

Fig. 8. Light absorption at 418 mμ (ascribed to Compound II) in oxidation of dihydroxyfumaric acid in the presence of Mn^{2+}. Concentrations as in Fig. 7.

at 418 mμ is, according to CHANCE[2], due to Compound II and not to Compound III.

The O_2 content of the gas used in the previous experiments corresponded to an equilibrium concentration of 105 μM O_2 in the solution. This concentration was close to the upper limit of O_2 concentration where oscillations could be obtained under the present conditions. It was chosen because it gave a convenient intensity of chemiluminescence. The optimal equilibrium concentration of O_2 for oscillations was about one third of the concentration used in the above experiments. Fig. 9 shows chemiluminescence and light absorption measurements in experiments similar to those of Figs. 2C and 3C except that the O_2 concentration of the gas corresponds to an equilibrium concentration in the gas of 35 μM O_2 in the solution. It is noted that the light absorption at 418 mμ shows an oscillation of a very small amplitude synchronous with the oscillation of the O_2 concentration.

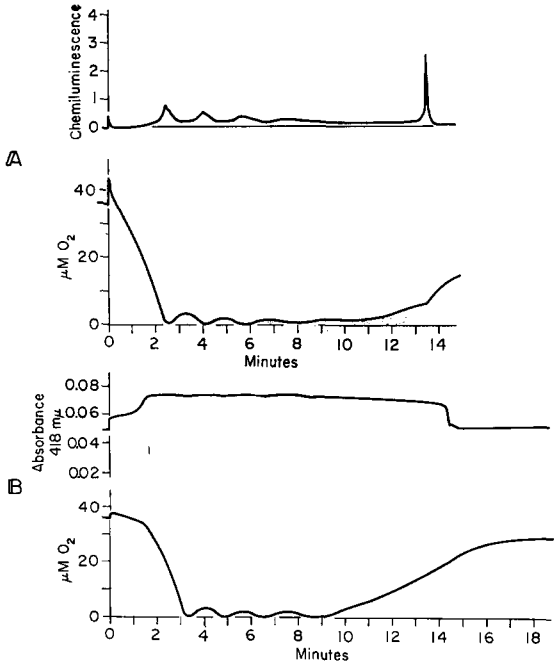

Fig. 9. Typical examples of oscillations obtained at optimal O_2 concentration of the gas mixture during the oxidation of dihydroxyfumaric acid (A, 0.85 mM; B, 0.68 mM) catalyzed by horseradish peroxidase (0.8 μM) in phosphate buffer (0.1 mM, pH 5.1).

Discussion of dihydroxyfumaric acid results

It is usually assumed that, in a chemiluminescent reaction, the intensity of the light emission is proportional to the reaction rate[35]. This assumption is based on the idea that the light originates from a short-lived excited intermediate whose concentration is proportional to the reaction rate. In the experiments of Figs. 2 and 5, there is observed a qualitative correlation between the intensity of the chemiluminescence and the rate of O_2 consumption during the first phases of the reaction. However, the intense final peak of chemiluminescence takes place when the O_2 consumption rate is almost zero and when 2,4-dichlorophenol is present, the final peak is accompanied by

an abrupt cessation of the O_2 consumption. Therefore, if the reaction rate is defined as the rate of O_2 consumption, the chemiluminescence in the present reaction is not proportional to the reaction rate.

The chemiluminescence does not necessarily have the same origin in the phase of the reaction where the light intensity is correlated to the rate of O_2 consumption as it has in the phase of the reaction where no such correlation exists. If the light has different origins in the two cases, the spectrum will probably be different. Unfortunately, sufficiently sensitive equipment to measure the spectrum of the chemiluminescence was not at hand.

If the final peak of chemiluminescence accompanying the disappearance of Compound III is due to an intermediate in the Compound III decomposition reaction, these experiments reveal that this decomposition reaction is slow during the rapid steady state phase of the aerobic oxidation but accelerates strongly at a time when the donor is being depleted. The donor enters at two different points in the mechanism of aerobic oxidation. The interruption of the reaction cycle at one of these points will cause the accumulation of H_2O_2 and at the other point of O_2^-. However, O_2^- dismutates rapidly to H_2O_2 and O_2. Only if the oxidation reaction proceeds rapidly and is suddenly interrupted will there be a significant temporary accumulation of H_2O_2 or O_2^-. In the experiments of Fig. 3, the depletion of dihydroxyfumaric acid causes a very gradual decrease of the rate of the oxidation reaction and the final rapid decomposition of Compound III takes place when the O_2 consumption has already been slow for a while. Therefore, the decomposition of Compound III during depletion of dihydroxyfumaric acid is probably not caused by a temporary accumulation above the previous concentrations of reaction intermediates which can decompose Compound III but, rather, it is caused by the acceleration of a branched chain reaction which suffers chain termination by a reaction between dihydroxyfumaric acid and an active intermediate.

The experiment where the O_2 is turned off (Fig. 4) shows that Compound III has a half life of about 4 min when there is dihydroxyfumaric acid but no O_2 present. The same half life was reported by WITTENBERG *et al.*[32] for Compound III in a solution containing no donor. The half life in their experiments did not depend on the O_2 concentration. These observations show that Compound III reacts very slowly, if at all, with dihydroxyfumaric acid which was also reported by others[37]. It is concluded that the rapid decomposition of Compound III which occurs during the depletion of dihydroxyfumaric acid is a branched chain reaction requiring both dihydroxyfumaric acid and O_2. However, high concentrations of dihydroxyfumaric acid inhibit the reactions.

As in the case of the NADH oxidation the depletion of O_2 is presumably followed by a temporary accumulation of donor free radical. The stability of Compound III during the depletion of O_2 indicates that Compound III does not react with dihydroxyfumaric acid free radical. According to YAMAZAKI *et al.*[31], Compound III may be decomposed in either oxidative or reductive reactions. In the NADH oxidation, the decomposition by Reaction 16 is a reductive reaction. As Compound III does not react with dihydroxyfumaric acid or dihydroxyfumaric acid free radical there seems to be no reductive way of decomposition of Compound III in the dihydroxyfumaric acid oxidation. It may, therefore, be assumed that the rapid decomposition of Compound III observed during the depletion of dihydroxyfumaric acid is an oxidative

reaction. If the oxidant in this reaction is H_2O_2 the stoichiometry would call for an HO radical as a product.

$$\text{Compound III} + H_2O_2 \rightarrow \text{Ferriperoxidase} + HO^{\cdot} + O_2 + H_2O \quad (17)$$

The HO radical could react with dihydroxyfumaric acid to form dihydroxyfumaric acid free radical which could generate several H_2O_2 molecules for every OH radical used by Reactions 4 and 7. The decomposition of Compound III according to Reaction 17 would, therefore, be autocatalytic.

Another possible way of oxidative decomposition of Compound III involves a reaction between Compound II and Compound III (refs. 31, 36). The Compound II formed in Reaction 2 will normally react with dihydroxyfumaric acid according to Reaction 3 but at a low concentration of dihydroxyfumaric acid the reaction

$$\text{Compound II} + \text{Compound III} \rightarrow 2\ \text{Ferriperoxidase} + O_2 + H_2O \quad (18)$$

may be predominant. The dihydroxyfumaric acid radical formed in Reaction 2 will generate H_2O_2 as before. Reaction 18 will cause an autocatalytic decomposition of Compound III because 1 molecule of peroxidase enters in Reaction 1 and 2 molecules of peroxidase appear as products in Reaction 18.

It is commonly believed that the catalytic effect of certain phenols in aerobic oxidation reactions is due to the ability of the phenols to transport electrons between donors and acceptors which for steric reasons cannot easily react with each other. According to this hypothesis the phenols would be particularly useful in interenzyme reactions such as Reaction 18. The assumption that Reaction 18 takes part in the decomposition of Compound III is, therefore, supported by the finding that 2,4-dichlorophenol strongly increases the rate of decomposition of Compound III.

The effect of Mn^{2+} is quite different from that of 2,4-dichlorophenol. Most remarkable is the absence of chemiluminescence during the steady state phase of the reaction. This finding may indicate that a different reaction mechanism which does not cause the accumulation of free radicals is responsible for the aerobic oxidation when Mn^{2+} is present. The absence of Compound III (ref. 2) also supports this assumption, because the most plausible reactions forming Compound III (Reactions 12 and 13) involve free radical reactants directly or indirectly.

No evidence was found that the enzyme activity is lowered when the enzyme is converted to Compound III in the dihydroxyfumaric acid oxidation, and it cannot be excluded that Compound III is, in some unknown way, involved in the rapid oxidation of dihydroxyfumaric acid. The observation that the O_2 consumption stops abruptly simultaneously with the decomposition of Compound III might be taken as an indication that Compound III is an active intermediate. However, the final peak of chemiluminescence may indicate that there is little turnover of Compound III during the rapid steady state phase of the oxidation reaction.

The curve in Fig. 9B resembles closely curves obtained by YAMAZAKI *et al.*[21] and YAMAZAKI AND YOKOTA[22] using NADH as the donor. One important difference is that the high amplitude oscillations of Compound III concentration found in the NADH case is not found in the dihydroxyfumaric acid case. The close resemblance of the oscillation in the O_2 concentration in the aerobic oxidation of NADH and dihydroxyfumaric acid leads to the assumption that the mechanism of the oscillation is the same in the two cases. The absence of a significant oscillation in the Compound

III concentration during the oscillatory oxidation of dihydroxyfumaric acid contradicts the hypothesis by YAMAZAKI AND YOKOTA[22] that Compound III has an essential regulatory role in the mechanism of the oscillation.

Aerobic oxidation of indole-3-acetic acid

Figs. 10 and 11 show experiments similar to those of Figs. 2 and 3 except that indole-3-acetic acid is used as a donor instead of dihydroxyfumaric acid. In these

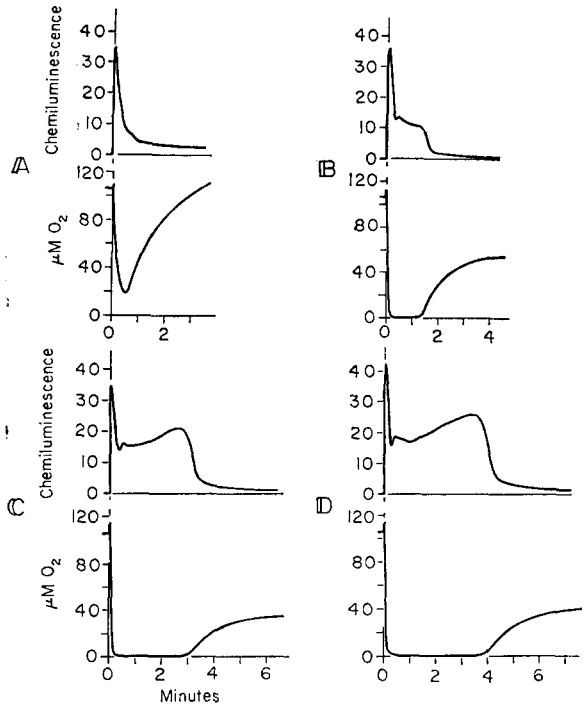

Fig. 10. Chemiluminescence and O_2 concentration in oxidation of indole-3-acetic acid (A, 0.2 mM; B, 0.4 mM; C, 0.6 mM; D, 0.8 mM) catalyzed by horseradish peroxidase (0.8 μM) in phosphate buffer (0.1 M, pH 5.1). Indole-3-acetic acid dissolved in ethanol is added at time zero.

experiments the O_2 reading does not return to its original value after the reaction has stopped except in the experiments with the lowest initial concentration of indole-3-acetic acid. This is because of a poisoning of the platinum electrode. The chemiluminescence when indole-3-acetic acid is used is about ten times stronger than when dihydroxyfumaric acid is used. The chemiluminescence shows a high peak immediately when indole-3-acetic acid is added. During the steady state phase of the reaction the chemiluminescence increases a little at the higher initial concentrations of indole-3-acetic acid. When the O_2 concentration begins to increase again, indicating that the reaction has stopped, the chemiluminescence falls rapidly. There is no final peak of chemiluminescence. At a low initial concentration of indole-3-acetic acid a decrease in the light absorption at 418 mμ is observed immediately after indole-3-acetic acid is introduced (Fig. 10A). At higher initial concentrations of indole-3-acetic acid, the light absorption also falls but increases again first rapidly and then more slowly. The

slow increase stops when the O_2 begins to increase again. In the cases where the light absorption increases again after the initial fall a transient resembling a damped oscillation is observed.

When the O_2 in the gas mixture was turned off and on again a little later there occurred an overshoot in the O_2 concentration followed by a damped oscillation as it was also found when NADH or dihydroxyfumaric acid was used as the substrate. When a high concentration of indole-3-acetic acid is used and the turning on and off

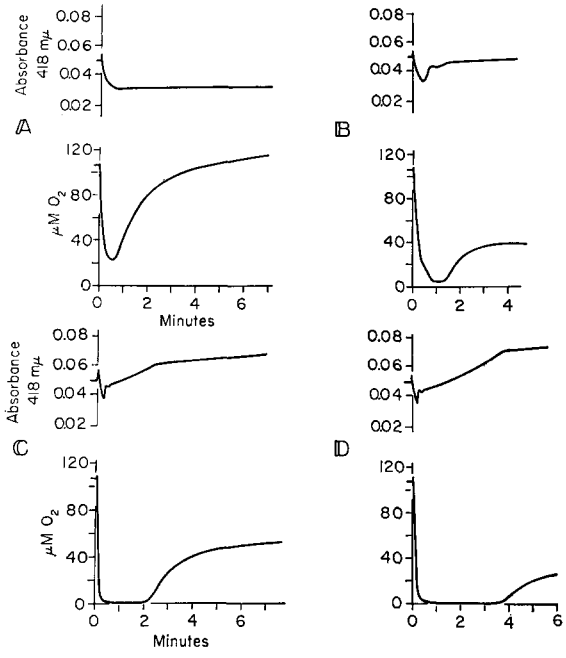

Fig. 11. Light absorption at 418 mμ and O_2 concentration in oxidation of indole-3-acetic acid. Concentrations as in Fig. 10.

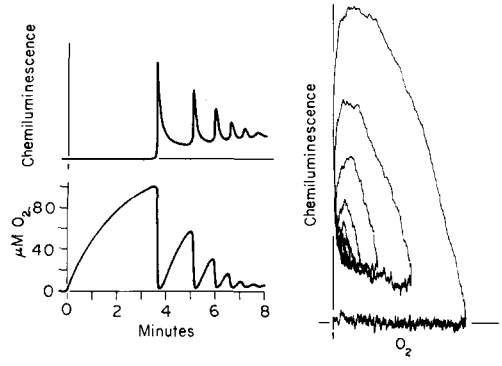

Fig. 12. Damped oscillation in chemiluminescence and O_2 concentration during the oxidation of indole-3-acetic acid (2.9 mM) catalyzed by horseradish peroxidase (0.8 μM) in phosphate buffer (0.1 M, pH 6.1). The O_2 in the gas is turned on at time zero. The curve to the right is a phase trajectory obtained by recording the chemiluminescence against the O_2 concentration on an x–y recorder.

of the O_2 is repeated several times, the overshoot becomes successively higher and the oscillation less damped. Fig. 12 shows a damped oscillation of O_2 concentration and chemiluminescence in such an experiment the fifth time the O_2 was turned on. At the same time as the two variables were recorded as functions of time, they were also recorded on an x–y recorder yielding an inward spiralling trajectory.

Discussion of indole-3-acetic acid results

The light absorption measurements give no indication of the formation and decomposition of Compound III as it was found in the NADH and dihydroxyfumaric acid oxidation reactions. Compound III is known to react very rapidly with indole-3-acetic acid[37,38]. It can, therefore, not be excluded that the aerobic oxidation of indole-3-acetic acid involves Compound III but there is no evidence in favor of such an assumption. Although the waveform of the damped oscillation in the indole-3-acetic acid case is somewhat different from that of the NADH and dihydroxyfumaric acid cases, it seems reasonable to assume that the mechanism leading to oscillations is essentially the same. The absence of measurable concentrations of Compound III during the aerobic oxidation of indole-3-acetic acid further weakens YAMAZAKI's theory that Compound III has a regulatory role in the mechanism of the oscillation. Unfortunately, it was not practicable to measure the light absorption changes when the oscillation was obtained, because the reaction solution was made intransparent by reaction products.

The phase trajectory representation of the oscillation shows the phase relationship between the O_2 concentration and the chemiluminescence more clearly than does the time-function representation. It is seen that the chemiluminescence has its maximum at a low O_2 concentration. This is expected if the chemiluminescence originates from indole-3-acetic acid free radical because when the O_2 concentration is lowered there is less O_2 available to remove indole-3-acetic acid free radical by Reaction 4 and it may accumulate temporarily.

Conclusion

The three components in a peroxidase catalyzed aerobic oxidation reaction, namely O_2, donor and peroxidase, cannot react with each other. The oxidation reaction is mediated by reactive substances such as H_2O_2,O_2^- and donor free radical, which are regenerated and multiplied. Therefore, aerobic oxidation reactions catalyzed by peroxidase alone are autocatalytic.

The occurrence of damped oscillations in an open system where a reactant is entering at a constant rate and consumed by an autocatalytic reaction was predicted by LOTKA[33]. Except for minor deviations, the present open reaction system is a realization of Lotka's theoretical model. All donors capable of peroxidase catalyzed aerobic oxidation which have been tested in the open system show damped oscillations in the oxidation rate in accordance with Lotka's theory.

YAMAZAKI *et al.*[21] and YAMAZAKI AND YOKOTA[22] who discovered the damped oscillation in the NADH oxidation have postulated that Compound III has a regulatory effect which is essential in the mechanism of the oscillation. For this reason the light absorption at 418 mμ was surveyed in the three peroxidase catalyzed aerobic oxidation reactions which are known to exhibit damped oscillations. Although the light absorption measurement at 418 mμ does not distinguish between Compound II

and Compound III, the increase in light absorption at this wavelength in the aerobic oxidation of NADH and dihydroxyfumaric acid is ascribed to Compound III as indicated by the visible absorption spectra of similar reaction solutions recorded by YAMAZAKI AND YOKOTA[13,25] and CHANCE[2].

Compound III is not found spectrophotometrically in the indole-3-acetic acid oxidation and large differences exist between the Compound III kinetics in the NADH and the dihydroxyfumaric acid oxidation. In the NADH oxidation O_2 depletion causes Compound III to decompose in an autocatalytic reaction involving a reduction of Compound III by NAD free radical. In the dihydroxyfumaric acid oxidation O_2 depletion does not cause decomposition of Compound III, because Compound III does not react with dihydroxyfumaric acid free radical. Only an oxidative decomposition of Compound III is possible in the dihydroxyfumaric acid oxidation. This takes place in an autocatalytic reaction when dihydroxyfumaric acid is being depleted, but not when dihydroxyfumaric acid is absent. H_2O_2 or Compound II are proposed as possible acceptors in the autocatalytic oxidative decomposition of Compound III. Because of the large differences between the Compound III kinetics in three oscillatory oxidation reactions, it is concluded that Compound III is not essential to the mechanism of the oscillation.

The occurrence of bistability in an open homogeneous system, where a substrate is entering by diffusion and consumed by a substrate inhibited enzyme reaction, was predicted by SEL'KOV[27] and HIGGINS[28,29]. The aerobic oxidation of NADH catalyzed by peroxidase is indirectly inhibited by O_2 due to the formation of Compound III. Bistability was observed in the NADH oxidation in the open system in accordance with the theory. Bistability was not found in the dihydroxyfumaric acid or indole-3-acetic acid oxidation.

The aerobic oxidation of the three donors employed in the present study is accompanied by a chemiluminescence whose intensity increases strongly in the order NADH < dihydroxyfumaric acid < indole-3-acetic acid. Because of the large differences in the light intensity in the different oxidation reactions at the same reaction rate, it is believed that the light is emitted from intermediates originating from donor. The spectrum of the light may, therefore, be used for the identification of such intermediates. The chemiluminescence can also give information about the kinetics of the oxidation reaction. The most important finding in the present chemiluminescence measurements is the intense peak of chemiluminescence which accompanies the decomposition of Compound III during the depletion of dihydroxyfumaric acid. This phenomenon reveals an accumulation of excited intermediates during the Compound III decomposition which is evidence for an autocatalytic character of this reaction.

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