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OSCILLATORY KINETICS OF THE PEROXIDASE-OXIDASE REACTION IN AN OPEN SYSTEM

EXPERIMENTAL AND THEORETICAL STUDIES

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Summary

1. The oscillations in the peroxidase (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7)-catalyzed reaction between NADH and O_2 are undamped when the reaction is carried out in a system open to both substrates and when 2,4-dichlorophenol and methylene blue are present in the solution.

2. The waveform of the oscillations changes when the concentration of peroxidase is varied.

3. The waveforms obtained experimentally can be simulated by a branched chain reaction model in which the branching is quadratic.

4. A correlation between the present knowledge of the reaction and the model can be made by combining well established and hypothetical reaction steps into a few reaction schemes. A selection among schemes however, is not possible at the present time.

5. Compound III participates in the reaction as an active intermediate. This is possible because dichlorophenol stimulates the break down of compound III.

Introduction

The reaction usually assumed to be the raison d'être of peroxidase (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) is

$$H_2O_2 + YH_2 \rightarrow 2H_2O + Y \quad (1)$$

where YH_2 is one of a very large number of different hydrogen donors. An

Abbreviation: DCP, 2,4-dichlorophenol.

alternative type of reaction also catalyzed by peroxidase is

$$O_2 + 2YH_2 \rightarrow 2H_2O + 2Y \quad (2)$$

where YH_2 is one of a very small number of hydrogen donors, including NAD(P)H [1], dihydroxyfumaric acid [2], indole acetic acid [3] and triose reductone [4]. The donors for the two alternative reactions are different. None of the donors work well in both reactions. Reaction 2 which is the subject of the present work we shall call the peroxidase-oxidase reaction.

Five different forms of the enzyme with characteristic absorption spectra are known to exist. These are ferric peroxidase, ferrous peroxidase, compound I, compound II and compound III. Ferric peroxidase, compound I and compound II have been shown to be intermediates in the catalytic cycle of the peroxidase reaction (Eqn. 1) [5—7]. In the peroxidase-oxidase reaction a considerable amount of the enzyme may be converted into compound III as shown by Yokota and Yamazaki [8]. It is an open question whether compound III is an active intermediate in the peroxidase-oxidase reaction.

The peroxidase-oxidase reaction began to reveal some peculiar properties when Yokota and Yamazaki [8] found that a minimal concentration of NADH is required for the reaction to take place at all. They also found [9] that with NADH as a substrate and a continuous supply of oxygen, a steady-state oxygen concentration was approached through a damped oscillation. From simultaneous measurements of oxygen concentration and light absorption they concluded that compound III played an essential regulatory role in the mechanism causing the oscillation. Degn [10,11] confirmed Yamazakis observation of damped oscillations with NADH and, in addition, he found, that damped oscillations could be obtained with dihydroxyfumaric acid and indole acetic acid and that bistability existed in a system open to oxygen with NADH as a hydrogen donor. He did not support Yamazakis conclusion about a regulatory role of compound III in the oscillations. However, he assigned such a role to compound III in the bistability phenomenon. Degn and Mayer [12] held that autocatalysis is causing the oscillations by a mechanism similar to the classical Lotka scheme. The models analysed by Degn and Mayer [12] only exhibit damped oscillations. However Yamazaki et al. [13,14] have also reported sustained oscillations in a system where glucose-6-phosphate dehydrogenase is used to regenerate NADPH.

It is widely believed that the peroxidase-oxidase reaction is a free radical chain reaction which does not depend on the formation of an enzyme · substrate complex with the hydrogen donor, accounting for the striking dissimilarity between the known reactive hydrogen donors. The intricacies of the chain reaction are not known. Theories have been advanced that it consists of the well known individual reaction steps of the peroxidase reaction supplemented with a number of free radical reactions taking place in the solvent without the help of the enzyme. A bewildering number of seemingly plausible free radical reaction steps has been proposed but experimental falsifications of hypothetical mechanisms are not easily obtained.

In the present work we have studied the sustained oscillations in the peroxidase-oxidase reaction in an open system, where both substrates, NADH and O_2, are continuously supplied to the reaction mixture from external sources. We

have found that the waveform of the oscillation is dependent on the enzyme concentration. A simple model based on a branched chain mechanism involving quadratic branching and linear termination has been studied. This model is capable of reproducing the waveforms of the oscillations of NADH and O_2 observed in the experiments.

Methods and Materials

Experiments were carried out in a cuvette mounted in a dual wavelength spectrophotometer (Perkin-Elmer-Hitachi 356). In some of the experiments the isosbestic point for the ferriperoxidase-compound III couple at 460 nm was used as the reference wavelength and compound III was measured at 418 nm. In other experiments the spectrophotometer was operated in the two wavelenth two phenomena mode with the wavelengths set at 380 (measuring NADH concentration) and 418 nm. The sample in the cuvette was supplied with oxygen by blowing a mixture of nitrogen and oxygen over the surface of the solution. NADH was pumped into the reaction mixture through a capillary using a high precision infusion pump (Harvard Apparatus Co. model 971). Oxygen concentration was measured with a Clark-type electrode (Radiometer, Copenhagen). In the present experiments a 5 ml reaction vessel fitted with a stirrer was used. Efficiency of stirring could be tested by two independent measurements. Firstly if the solution was inhomogeneous one would expect noise to occur in the absorbance due to NADH measured by the spectrophotometer. No such noise was observed. Secondly the rate law stated below for the diffusion of O_2 into the liquid only applies to a fully homogeneous solution. Since this rate law was found to be valid for the present experimental system we conclude that the solution was homogeneous with respect to O_2 and NADH. The open system with a continuous supply of O_2 has been described earlier [10,11]. We shall briefly summarize the theory here. When a gas phase with a fixed oxygen partial pressure is in contact with a stirred solution containing an oxygen-consuming substance the rate of change of the oxygen concentration in the liquid is given by the equation:

$$\frac{d[O_2]}{dt} = v_t - v_r \tag{3}$$

where v_t is the rate of diffusion of O_2 from the gas phase into the solution and v_r is the oxygen consumption rate. v_t is given by

$$v_t = K([O_2]_{eq} - [O_2]) \tag{4}$$

where $[O_2]_{eq}$ is the oxygen concentration in the liquid when the liquid phase is equilibrated with the gas phase. The oxygen transfer constant, K, depends on the volume, the surface area and the temperature. The oxygen transfer constant for the present experimental system was $3.5 \cdot 10^{-3}\ s^{-1}$.

Horseradish peroxidase (RZ = 0.6) and NADH were obtained from Boehringer. Methylene Blue and 2,4-dichlorophenol were obtained from Merck. Dichlorophenol was dissolved in 96% ethanol before use.

Results

Oscillatory behaviour in the peroxidase-oxidase reaction

Using a NADPH-regenerating enzyme system, and dichlorophenol (DCP) and Methylene Blue as modifiers, Yamazaki et al. [13,14] have found sustained oscillations in the oxygen concentration and in the absorption at 418 nm ascribed to compound III. We did the same type of experiments, but instead of using glucose-6-phosphate dehydrogenase to regenerate NADPH, NADH was pumped into the solution at a constant rate through a capillary. Oxygen was introduced by diffusion through a stable surface instead of by bubbling. Fig. 1 shows an experiment in which the concentrations of O_2, NADH and compound III were measured simultaneously. The saw-tooth waveform of the NADH curve is strikingly similar to the I_2 curve in the Bray reaction [15]. The experiment confirms the existence of a critical concentration of NADH below which there is no initiation of the reaction. Moreover it shows

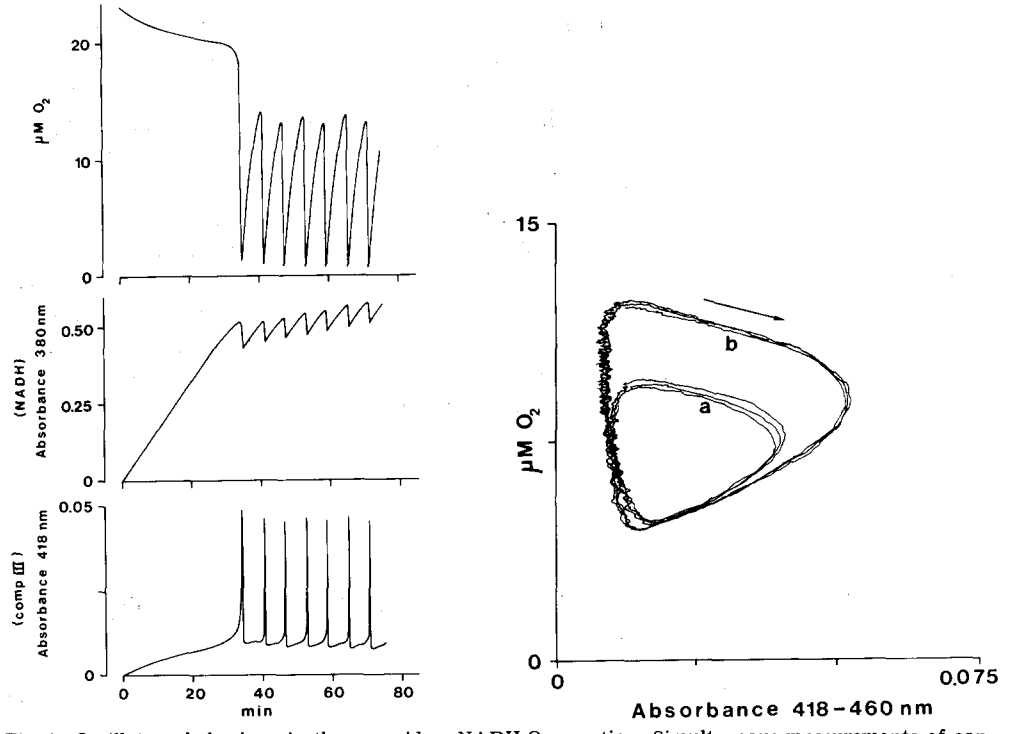

Fig. 1. Oscillatory behaviour in the peroxidase-NADH-O_2 reaction. Simultaneous measurements of concentrations of O_2, NADH and compound III. Experimental conditions: 0.9 μM peroxidase, 0.2 μM Methylene Blue and 10 μM 2,4-dichlorophenol in 0.1 M acetate, pH 5.1. O_2 content in the gas was 1.9% by volume. Temperature 28°C. The experiment was started by an infusion of 0.2 M NADH at a rate of 11 μl/h.

Fig. 2. Phase plot of O_2 concentration against concentration of compound III at two different infusion rates of NADH. Experimental conditions: 1.8 μM peroxidase, 0.6 μM Methylene Blue and 20 μM 2,4-dichlorophenol in 0.1 M acetate, pH 5.1. Temperature 25°C. The oxygen content in the gas is 1.8% by volume. 0.2 M NADH was infused at rates a, 11 μl/h; b, 7 μl/h.

the existence of another, lower critical concentration of NADH at which the reaction turns off. Similar experiments when the liquid was equilibrated with different concentrations of oxygen showed that the critical limit of NADH for initiation of the reaction apparently does not depend on the oxygen concentration. Critical switch-on and switch-off concentration limits also exist in the Bray reaction [15].

Phase diagrams of the oxygen concentration against the concentration of NADH or compound III were obtained by the help of an X,Y-recorder in experiments similar to the one shown in Fig. 1. The oxygen concentration versus NADH curve is a straight line with a positive slope, showing that there is no phase shift between the oscillations in NADH and O_2 concentrations. The phase diagram of O_2 against compound III is shown in Fig. 2. It has a soft triangular form and shows that increase as well as decrease of compound III takes place during the decrease in oxygen concentration. This finding apparently contradicts the suggestion made by Yamazaki et al. [9,13] that an increased level of compound III is a requirement for the onset of the reaction. It is better explained by the assumption of compound III being produced as a consequence of the onset of the reaction.

The waveform of the oscillations depends critically on the enzyme concentration. At decreasing enzyme concentrations the oscillatory waveform changes from a simple periodic mode behaviour (Fig. 1) to an interrupted mode behaviour (Fig. 3). In the latter experiment the system alternates between two states: an irregular spiking state (state I) and a silent state (state II). In the latter state the concentrations of oxygen and compound III are nearly constant while the

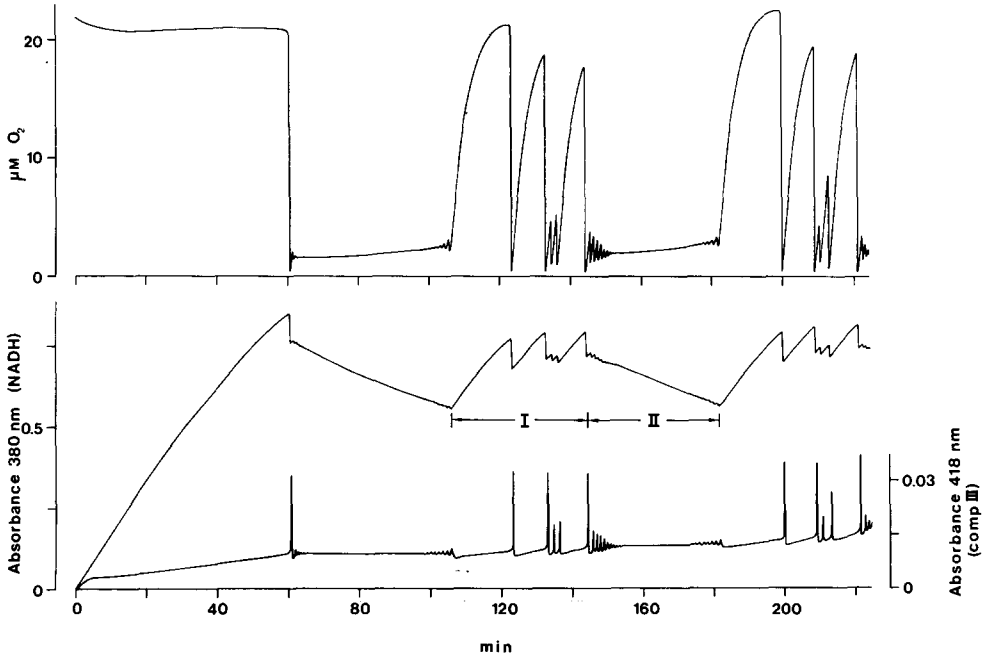

Fig. 3. Effect of a decrease in the peroxidase concentration on the oscillatory waveform. Experimental conditions as in Fig. 1 except for the concentration of peroxidase being 0.45 μM.

NADH concentration is decreasing at a constant rate. At even lower enzyme concentrations than those used in Fig. 3 the irregular spiking state is reduced to a single spike as seen in Fig. 4. At enzyme concentrations at the boundary between those used in Figs. 1 and 3 only irregular aperiodic oscillations (chaos) are seen as described earlier [16].

The role of the modifiers Methylene Blue and dichlorophenol is not clear. It must, however, be catalytic since the total amounts of O_2 and NADH consumed greatly exceed the amounts of Methylene Blue and DCP in the solution. Methylene Blue was not required in order to obtain sustained oscillations. Its presence, however, stabilised the oscillations under the conditions shown in Fig. 1. Increasing the amount of Methylene Blue slightly increases the switch-on level of NADH. At the same time the declining phase of the oscillation becomes less steep. With more than 1 μM Methylene Blue only damped oscillations are obtained. Dichlorophenol on the other hand has a rate-enhancing effect and must be present in order to obtain sustained oscillations. In its absence the concentrations of NADH, O_2 and compound III approach a steady-state level through a damped oscillation (Fig. 5). Addition of DCP causes a rapid decrease in the oxygen concentration and the concentration of compound III. After a short time in a stationary state the system suddenly starts to perform sustained oscillations. However, we found no effect of the

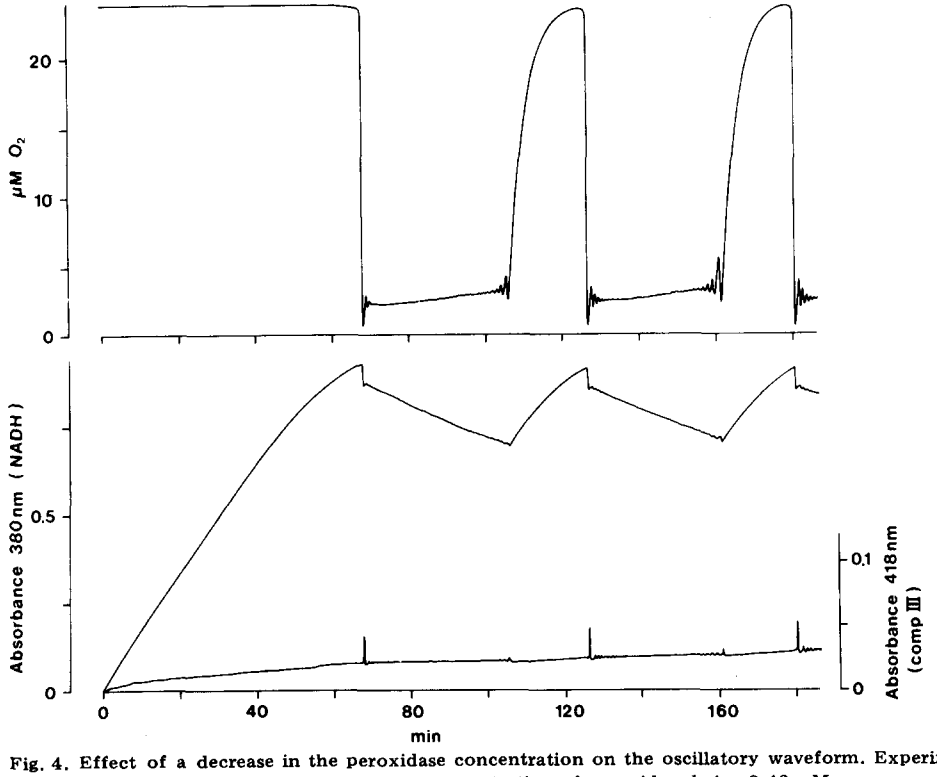

Fig. 4. Effect of a decrease in the peroxidase concentration on the oscillatory waveform. Experimental conditions as in Figs. 1 and 3 except for the concentration of peroxidase being 0.40 μM.

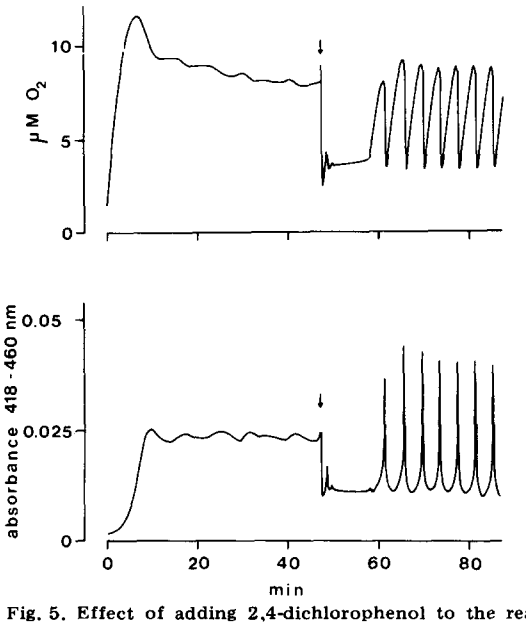

Fig. 5. Effect of adding 2,4-dichlorophenol to the reaction mixture. Experimental conditions: 1.8 μM peroxidase, 0.6 μM Methylene Blue in 0.1 M acetate, pH 5.1. Temperature 25°C. The experiment was started by switching the O_2 content in the gas from 0 to 1.8% by volume and at the same time starting an infusion of 0.2 M NADH at a rate of 11 μl/h. At the indicated arrows 20 μM 2,4-dichlorophenol was added to the solution.

concentration of DCP on the upper critical concentration of NADH. When dichlorophenol is present the bistability phenomenon [10] disappears which means that there is no longer substrate inhibition by O_2.

The experiments shown above were done with solutions of dichlorophenol that had been kept at room temperature for a few days before use. They had a reasonable degree of reproducibility. Solutions of DCP prepared immediately before use gave less reproducible oscillations and at the same time the switch-on level of NADH appeared at lower values. The overall waveform of the oscillations, however, was retained.

Computer simulations of the oscillatory waveform

At the present time the individual steps in the peroxidase reaction are hypothetical. Therefore building a model of this reaction has the limited scope of reproducing the features of the oscillations.

The peroxidase-oxidase reaction has two characteristic properties both of which can cause sustained oscillations in an open system, namely forward inhibition [10] and autocatalysis [11,12]. These two are difficult to distinguish in a reaction where only a few details are known because they give rise to similar kinetic behaviour. Because forward inhibition does not exist in the reaction when DCP is added it follows that autocatalysis must be responsible for the sustained oscillations found in our experiments where DCP was present. Our model is a modified version of the model proposed by Lindblad and Degn [17] to represent Bray's oscillating reaction. This approach was chosen because of the similarity in waveform between the NADH oscillations found in the present

experiments and the I_2 oscillations in Bray's reaction and because of the existence of critical switch-on and switch-off concentrations in both reaction systems. It was pointed out many years ago by Semjonov [18] that quadratic branching in a chain reaction may lead to the existence of two distinct critical concentration limits, one for switch-on and one for switch-off. The inclusion of this effect seems to be necessary for the model to exhibit the characteristic saw-tooth waveform. In our model listed below, the quadratic branching is represented by the interaction of the autocatalytic molecule X to form the more efficient autocatalytic molecule Y.

$$
\begin{aligned}
A + B + X &\xrightarrow{k_1} 2X && (5,1)\\
2X &\xrightarrow{k_2} 2Y && (5,2)\\
A + B + Y &\xrightarrow{k_3} 2X && (5,3)\\
X &\xrightarrow{k_4} P && (5,4)\\
Y &\xrightarrow{k_5} Q && (5,5)\\
&\xrightarrow{k_6} X && (5,6)\\
A_0 &\underset{k_7}{\overset{k_7}{\rightleftharpoons}} A && (5,7)\\
B_0 &\xrightarrow{k_8} B && (5,8)
\end{aligned}
\qquad (5)
$$

A is supposed to be oxygen, B is NADH and X and Y are intermediate free radicals. Reaction [5,6] accounts for the spontaneous formation of intermediate free radicals necessary to initiate the chain reaction. Reactions (5,7) and (5,8) correspond to the diffusion input of O_2 and the constant rate input of NADH, respectively.

The rate equations are

$$
\begin{aligned}
\frac{da}{dt} &= k_7(a_0 - a) - k_1abx - k_3aby\\
\frac{db}{dt} &= k_8b_0 - k_1abx - k_3aby\\
\frac{dx}{dt} &= k_6 + k_1abx - 2k_2x^2 + 2k_3aby - k_4x\\
\frac{dy}{dt} &= 2k_2x^2 - k_3aby - k_5y\,.
\end{aligned}
\qquad (6)
$$

These equations do not follow the stoicheiometry expressed in Eqn. 2. The correct stoicheiometry could be obtained by multiplying the expressions k_1abx and k_3aby in the equation for db/dt by a factor 2. Simulations in which this was done showed the same general waveforms as those presented here. However, the use of this stoicheiometry factor would require a more complex reaction scheme or the inclusion of fourth-order reaction kinetics in the scheme.

Fig. 6 shows one simulation of the above reaction scheme. In order to save computation time, the computation was started near the switch-on level of B

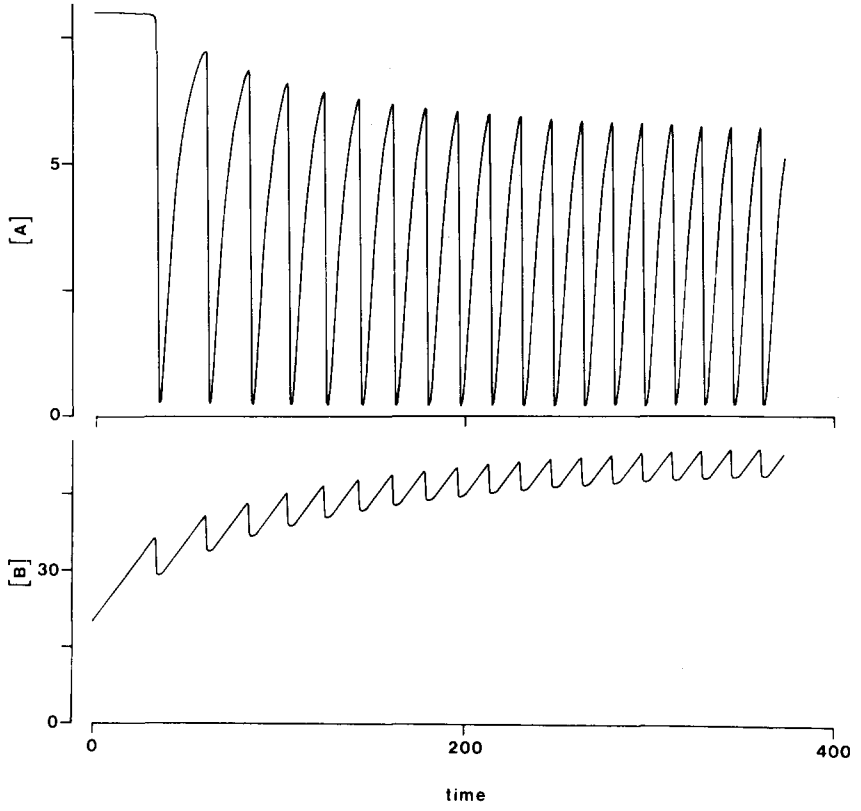

Fig. 6. Numerical integration of Eqn. 6 by a standard Runge-Kutta-Merson method. Parameters: $k_1 = 6.25 \cdot 10^{-2}$, $k_2 = 1.25 \cdot 10^3$, $k_3 = 4.6875 \cdot 10^{-2}$, $k_4 = 20.0$, $k_5 = 1.5$, $k_6 = 10^{-3}$, $k_7 = 0.1$, $k_8 b_0 = 0.5$, $a_0 = 8.0$. Initial conditions: $a = 8.0$, $b = 20.0$, $x = y = 0.0$. Concentration and time are in arbitrary units.

where the gain of X is positive, determined by the inequality (see Degn [15])

$$b > \frac{k_4}{k_1 a} \tag{7}$$

The switch-off level is roughly determined by

$$b < \frac{k_5}{k_3 a} \tag{8}$$

It is seen from the figure that the A and B curves closely resembles those of O_2 and NADH in Fig. 1, respectively. By decreasing the value of k_1 irregular oscillations like those of Figs. 2 and 3 were obtained (Figs. 7 and 8). As one can see, tuning only one parameter appropriately causes the model to exhibit nearly all the characteristic waveforms observed in the experimental system when the enzyme concentration is varied.

The model shown is not the only one which can produce solutions fitting the experimental curves. If reaction (5,2) of the model is replaced by the reaction

$$X + Y \rightarrow 2Y$$

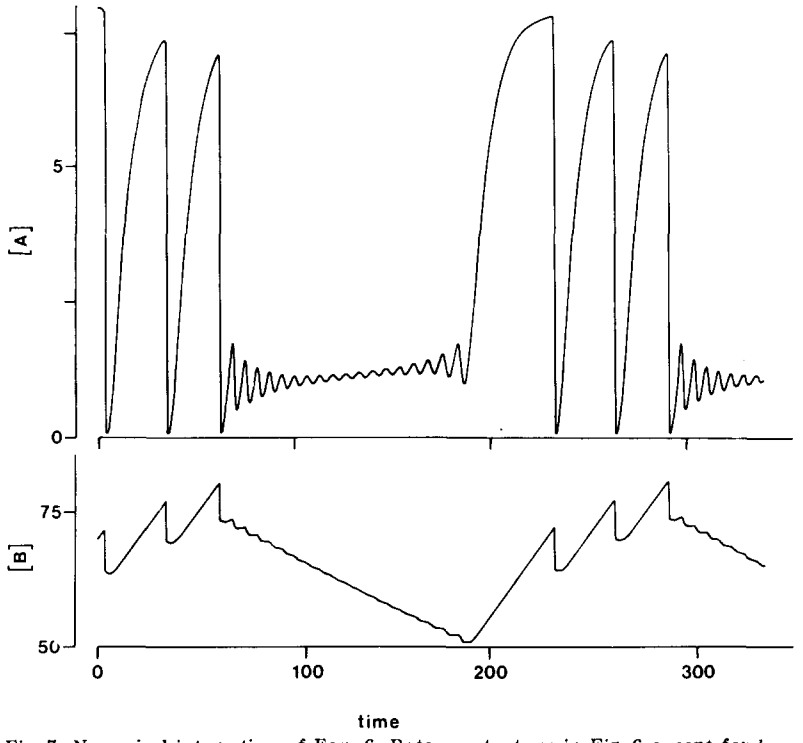

Fig. 7. Numerical integration of Eqn. 6. Rate constants as in Fig. 6 except for $k_1 = 3.125 \cdot 10^{-2}$. Initial conditions: $a = 8.0$, $b = 70.0$, $x = y = 0.0$.

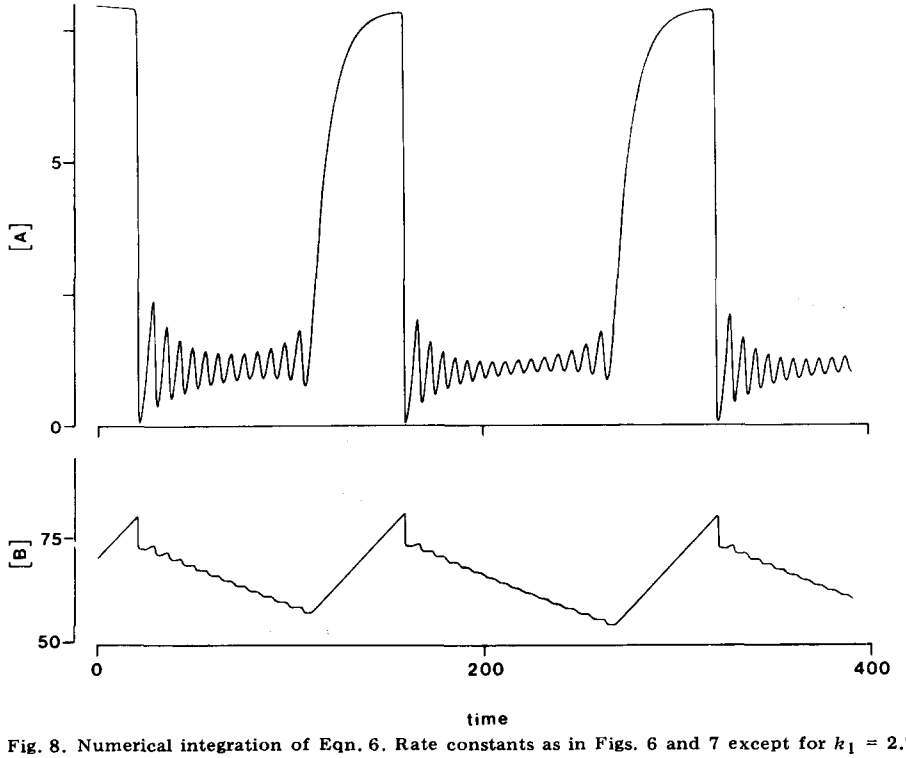

Fig. 8. Numerical integration of Eqn. 6. Rate constants as in Figs. 6 and 7 except for $k_1 = 2.75 \cdot 10^{-2}$. Initial conditions: $a = 8.0$, $b = 70.0$, $x = y = 0.0$.

the model retains the same general properties. We have also tried a model where reaction (5,2) is replaced by

$$X \rightarrow Y$$

This and other similar models failed to reproduce the saw-tooth waveform. From the ability of models with quadratic branching to reproduce the waveform and the failure of models having other types of autocatalysis we conclude that quadratic branching is a characteristic of the peroxidase-oxidase reaction. The fact that models having different kinds of quadratic branching can reproduce the waveform equally well indicates that the waveform does not contain information which can be used to resolve fine detail in the mechanism.

Discussion

It is beyond doubt that the peroxidase-oxidase reaction is a free radical branched chain reaction. Our computer results seem to indicate that this branched chain reaction has a special property called quadratic branching or positive interaction between chain-propagating molecules. This property implies the operation of two different autocatalytic reaction sequences. The first one works at low concentrations of chain-propagating molecules. The second and more efficient one works at high concentrations of chain-propagating molecules. In order to see how the hypothesis of quadratic branching can be reconciled with current knowledge of the mechanism of the peroxidase-oxidase reaction, we have compiled a list of possible reaction steps in the peroxidase-oxidase reaction proposed by different authors. (Table I). Possible free radical intermediates are assumed to be O_2^- (superoxide ion), YH· and YHOO·, excluding OH·. Different combinations of these steps form branched chain reactions, examples of which are given in Fig. 9. The reaction schemes shown are not thought to exhaust the possibilities. Scheme a in Fig. 9 is a simplified form of the one originally proposed by Yokota and Yamazaki [8]. However, in view of the experimental findings by Land and Swallow [19] that O_2^- reacts slowly if at all with NADH we think that this scheme is not tenable. We therefore propose the modified scheme b. This is closely related to scheme a but it does not involve superoxide as an intermediate. Unlike scheme b schemes c and d involve compound III. Scheme d is quite different from the previous ones as it does not have any reaction steps where the enzyme is not involved. Nevertheless it qualifies as a branched chain reaction.

The model studies suggest that not only one but two autocatalytic reactions are involved in the reaction to account for the double threshold characteristics of the reaction. Neither of the schemes a, b, c and d involves more than one autocatalytic reaction sequence. However, by combining two of these schemes we can construct a reaction mechanism with two autocatalytic sequences. For example the combination of scheme b with scheme c or d would result in a mechanism where the initiation of the reaction depends on scheme b because Fe^{3+} peroxidase is the only form of the enzyme present before initiation. After the initiation intermediates permitting the formation of compound III would accumulate and scheme c or scheme d would begin to operate. This idea is supported by the observations that in the presence of dichlorophenol there is

TABLE I

LIST OF INDIVIDUAL REACTION STEPS PROPOSED FOR THE PEROXIDASE-OXIDASE REACTION IN THE EARLIER LITERATURE

	Ref.
Classical peroxidase reactions	
(1) ferric peroxidase + H_2O_2 → compound I	5,6,7
(2) compound I + YH_2 → compound II + YH ·	
(3) compound II + YH_2 → ferric peroxidase + YH ·	
Other reactions involving the enzyme	
(4) compound II + H_2O_2 → compound III	20
(5) ferric peroxidase + YH · → ferrous peroxidase + Y + H^+	8
(6) ferrous peroxidase + O_2 → compound III	8
(7) compound III + YH_2 → compound I + YH · + H^+	21
(8) compound III + YH · → compound I + Y + H^+	14
(9) compound I + O_2^- · → compound II + O_2	23
(10) compound I + HO_2 → compound II + O_2 + H^+	23
(11) ferric peroxidase + O_2^- · → compound III	8,24
(12) ferric peroxidase + YHOO · → compound III + Y + H^+	22
(13) $4H^+$ + compound III + 3 ferrous peroxidase → $2H_2O$ + 4 ferric peroxidase	25
Non-enzymatic reactions	
(14) YH· + O_2 → Y + HO_2 · ($\rightleftharpoons$ H^+ + O_2^- ·)	8
(15) H^+ + O_2^- · + YH_2 → H_2O_2 + YH ·	8
(16) YH · + O_2 → YHO_2 ·	22,26
(17) YHO_2 · + YH_2 → YHOOH + YH ·	22
(18) 2YH · → Y + YH_2	8
(19) $2O_2^-$ · + $2H^+$ → H_2O_2 + O_2	8

no longer substrate inhibition of O_2. Since compound III is an inactive intermediate in the absence of dichlorophenol [10] we conclude that dichlorophenol has a catalytic effect on the breakdown of compound III. Hereby compound III is permitted to participate as an active intermediate in an autocatalytic sequence illustrated by the schemes c and d. There is however one discrepancy between the present model and the experimental system: The experimental results did not show a relationship between the initial oxygen concentration and the switch-on concentration of NADH as expected from inequality [7]. A more detailed investigation of this problem reveals that the initial oxygen concentration and the switch-on concentration of NADH are in fact related (Olsen, L.F., unpublished). The relationship is expressed by the inequality

$$[\mathrm{NADH}] > \alpha\left(\frac{\beta}{[\mathrm{O_2}]} + 1\right)$$

where α and β are constants. A slight modification of the model can easily account for this relationship, namely if the rate expression

$$k_1abx$$

derived from reaction (5,1) is replaced by the expression

$$\frac{k_1abx}{K + a}$$

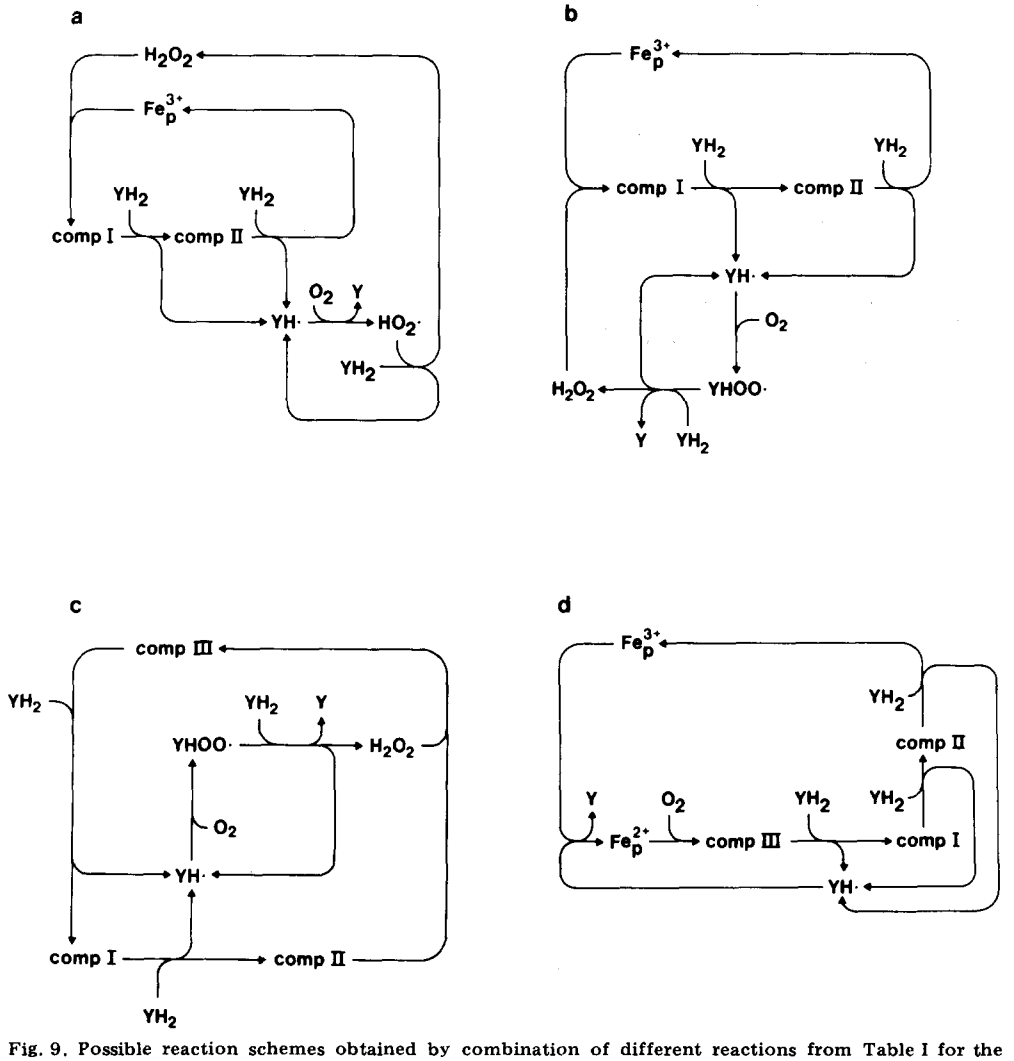

Fig. 9. Possible reaction schemes obtained by combination of different reactions from Table I for the autocatalytic peroxidase-oxidase reaction.

where K is a constant. The latter expression obviously reflects the contribution of the enzyme to the rate equations.

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