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# Hydrogen Peroxide–Sulfite–Ferrocyanide–Horseradish Peroxidase pH Oscillator in a Continuous-Flow Stirred Tank Reactor

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Based on the known  $\text{H}_2\text{O}_2\text{--SO}_3^{2-}\text{--Fe(CN)}_6^{4-}$  pH oscillator, a new pH oscillator with horseradish peroxidase (HRP) has been suggested, in which HRP changes the rate of  $\text{H}^+$  consumption. A simple model is given to simulate experimental results.

## 1. Introduction

A new family of chemical pH oscillators involving more than 10 oscillatory reactions have been discovered during the past decade (see, for example, refs 1 and 2). Among the basic reagents of pH oscillators are simple inorganic substances, such as sulfite, bromate, iodate, ferrocyanide, hydrogen peroxide, etc. Despite the difference between reagents, all pH oscillators follow a unique mechanism involving an autocatalytic production of  $\text{H}^+$  (“positive feedback”) and  $\text{H}^+$  consumption (“negative feedback”).

Besides a unique mechanism and a large amplitude change in the pH, an additional interest in pH oscillators is aroused by a recent discovery in the reaction–diffusion pH system (the ferrocyanide–iodate–sulfite reaction was used<sup>3</sup>) of some stationary structures that look like lamellae<sup>4</sup> and self-replicating spots.<sup>5</sup> And quite lately, the chaotic oscillations were observed in the pH oscillators based on the sulfite–hydrogen peroxide–marble flow system<sup>6a</sup> and on the  $\text{H}_2\text{O}_2\text{--HSO}_3^- \text{--HCO}_3^- \text{--Fe(CN)}_6^{4-}$  flow system.<sup>6b,c</sup> Thus, it can be expected that the whole set of complex dynamic regimes realized in a continuous-flow stirred tank reactor (CSTR) and of spatial structures and autowave regimes realized in spatially distributed systems are typical of the dynamic systems based on pH oscillators.

Compared to the Belousov–Zhabotinsky (BZ) type reactions,<sup>7</sup> pH oscillators show at least one advantage, relative simplicity, i.e., a more or less clear mechanism and easy determination of elementary reaction rate constants. A positive feedback is realized as a result of autocatalytic oxidation of a reductant, for instance, sulfite, which is accompanied by an exponential  $[\text{H}^+]$  increase of several orders. Most importance is the fact that protons are involved in a positive feedback and it is the  $[\text{H}^+]$  increase that causes autocatalysis. A negative feedback, leading to the  $[\text{H}^+]$  decrease, is realized in different pH oscillators differently. In some cases, the rate of the  $[\text{H}^+]$  decrease is proportional to  $[\text{H}^+]$ . In others, it is independent of  $[\text{H}^+]$ . The obviousness and simplicity of the general mechanism for all pH oscillators allow for the modification of the known pH oscillators and a possibility of creating new oscillators with the desired properties.

Many authors forecast the discovery of pH oscillations in enzymatic systems because they truly supposed that in the enzymatic reactions whose rates strongly depended on pH (as

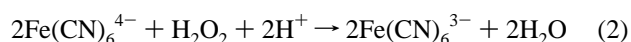
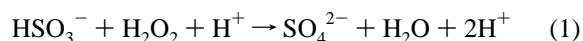
a rule, in a bell-shaped manner) there were all the reasons to get chemical oscillations (see, for example, ref 8). But in reality all the homogeneous pH oscillators currently known contain only inorganic substances. As a whole, the status of enzymatic oscillatory reactions seems to be rather strange. Apart from the complex glycolytic oscillations connected with phosphofructokinase,<sup>9</sup> as well as rather complicated peroxidase–oxidase oscillatory reaction in which peroxidase plays the untypical part,<sup>10,11</sup> in the literature, as far as I know, there are no other reliable data on homogeneous oscillatory reactions with enzymes. And, as a consequence, against to all expectations, there are no enzymatic homogeneous pH oscillators at present.

In this study we make the first small step toward the creation of such enzymatic pH oscillators. This step is in the modification of a well-known hydrogen peroxide–sulfite–ferrocyanide pH oscillator by adding a hemin-containing enzyme horseradish peroxidase (HRP) to it. We consider it to be important to create a pH oscillator with enzymes. First, it gives the strongest possibilities of controlling pH oscillations through the control of enzyme activity. Second, owing to the extremely low diffusion coefficients of enzymes and their practically complete immobility in any matrix (for instance, in gel), there appear new prospects in the formation of dissipative stationary structures. Then the immobilization of enzymes in a matrix according to a given spatial template may lead to the creation of microprocessors on the basis of nonequilibrium dynamic pH systems.

In part 2 we give a brief description of a known hydrogen peroxide–sulfite–ferrocyanide oscillator, of the HRP mechanism, and we shall show which stage of pH oscillations may be modified by adding HRP. In parts 3 and 4, we describe the materials and methods, as well as the results obtained, and in part 5 we discuss them.

## 2. Mechanism of the $\text{H}_2\text{O}_2\text{--SO}_3^{2-}\text{--Fe(CN)}_6^{4-}$ pH Oscillator and HRP Reaction

The mechanism of the  $\text{H}_2\text{O}_2\text{--SO}_3^{2-}\text{--Fe(CN)}_6^{4-}$  pH oscillator is well-known<sup>12,13</sup> and consists of two main stages. These are the oxidation of hydrogen sulfite by hydrogen peroxide (eq 1) and the oxidation of ferrocyanide by hydrogen peroxide (eq 2).



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Reaction 1 is autocatalytic. During pH oscillations, autocatalysis stops as a result of the complete sulfite consumption. The rate of autocatalysis



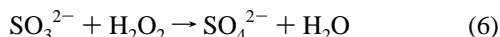
is notably affected by a quick deprotonation equilibrium 4:<sup>14</sup>



with  $k_4 = 3 \times 10^3 \text{ s}^{-1}$ ,  $k_{-4} = 5 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ , and probably by deprotonation equilibrium 5:<sup>15</sup>



with  $K_5 \cong 50 \text{ M}^{-1}$ . This is caused by the fact that the rate of reaction 6



is much smaller than the rate of reaction 1, while the rate of reaction 7



is probably much higher than the rate of reaction 1. Besides, reaction 6 is not autocatalytic. In the first approximation we determine the rate of autocatalysis 3 by the rate of reaction 1.

$$v_1 = (k_1 + k_1'[\text{H}^+])[\text{HSO}_3^-][\text{H}_2\text{O}_2] \quad (8)$$

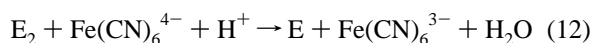
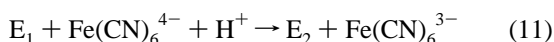
with  $k_1 = 7 \text{ M}^{-1} \text{ s}^{-1}$  and  $k_1' = 1.48 \times 10^7 \text{ M}^{-2} \text{ s}^{-1}$ .<sup>12</sup>

Reaction 2 is rather complicated. It is independent of or hardly dependent on  $[\text{H}^+]$  in the studied range of pH (pH = 4–8). Besides, the rate of reaction 2 is independent of  $[\text{Fe}(\text{CN})_6^{4-}]$  at not very low  $[\text{Fe}(\text{CN})_6^{4-}]$ ,<sup>12</sup> so

$$v_2 = k_2[\text{H}_2\text{O}_2] \quad (9)$$

with  $k_2 = 1.6 \times 10^{-4} \text{ s}^{-1}$ . The independence of the reaction 2 rate on  $[\text{H}^+]$ , or, more generally speaking, the independence of the negative feedback on  $[\text{H}^+]$ , is a distinguishing property of the pH oscillator under study. As a result, the  $\text{H}_2\text{O}_2$ – $\text{SO}_3^{2-}$ – $\text{Fe}(\text{CN})_6^{4-}$  pH oscillator spends much time (about 50% of the oscillation period  $T$ ) at low pH (pH = 4–6). As a rule, the rate of proton consumption in all other pH oscillators is directly proportional to  $[\text{H}^+]$ , and consequently, the time of their stay at low pH is short. Owing to this fact, an introduction of HRP to the  $\text{H}_2\text{O}_2$ – $\text{SO}_3^{2-}$ – $\text{Fe}(\text{CN})_6^{4-}$  pH oscillator should noticeably affect the shape of pH oscillations.

**Peroxidase.** Horseradish peroxidase catalyzes the oxidation of a large group of substrates, ferrocyanide in particular, by hydrogen peroxide.<sup>16,17</sup> The mechanism of ferrocyanide oxidation may be described as follows:



where E is the initial form of HRP with  $\text{Fe}^{3+}$  in the enzyme active center,  $\text{E}_1$  is the so-called compound I of HRP in which the valence of the iron ion is formally +5, and  $\text{E}_2$  is compound II with  $\text{Fe}^{4+}$  in the active center. The rate of processes 10–12 described in general by reaction 2 is given by the following

equation

$$v_{2e} = \frac{E_0}{\frac{1}{k_{10}[\text{H}_2\text{O}_2]} + \frac{1}{k_{11}[\text{Fe}(\text{CN})_6^{4-}]} + \frac{1}{k_{12}[\text{Fe}(\text{CN})_6^{4-}]}} \quad (13)$$

where  $E_0$  is the total concentration of all peroxidase forms,  $E_0 = E + E_1 + E_2$ . The constants  $k_{11}$  and  $k_{12}$  are pH-dependent. The inequality  $k_{11} \gg k_{12}$  holds at all pH. Besides,  $k_{10} \gg k_{11}$ . At 25 °C, pH 7, and ionic strength of 0.1,  $k_{10} = 1.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ ,  $k_{11} = 8.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ,  $k_{12} = 1.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ .<sup>17</sup> Hence, at  $[\text{H}_2\text{O}_2] > [\text{Fe}(\text{CN})_6^{4-}]$ , reaction 2 catalyzed by HRP is limited by reaction 12 and

$$v_{2e} \cong k_{12}E_0[\text{Fe}(\text{CN})_6^{4-}] \quad (14)$$

The constant  $k_{12}$  grows with a pH decrease. At  $3 < \text{pH} < 6$

$$k_{12} \cong k_{12}'[\text{H}^+] \quad (15)$$

with  $k_{12}' = 2 \times 10^{10} \text{ M}^{-2} \text{ s}^{-1}$ .<sup>18</sup> In the range  $6 < \text{pH} < 9$ , the constant  $k_{12}$  depends on  $[\text{H}^+]$  to a smaller degree. For the studied pH range from 4 to 8, it can be approximated that

$$k_{12} \cong k_{12}'[\text{H}^+] + k_{12}'' \quad (15.1)$$

with  $k_{12}'' \cong 10^4 \text{ M}^{-1} \text{ s}^{-1}$ , though a more exact expression for  $k_{12}$  is

$$k_{12} \cong k_{12}'[\text{H}^+] + k_{12}'''[\text{H}^+]^{1/4} \quad (15.2)$$

with  $k_{12}''' \cong 10^6 \text{ M}^{-5/4} \text{ s}^{-1}$ .<sup>18</sup>

Instead of ferrocyanide, HRP may use hydrogen sulfite as a substrate.<sup>17</sup> Though in this case, the rate constants corresponding to reactions 11 and 12 are 2–4 orders of magnitude smaller. Therefore, HRP-catalyzed oxidation of hydrogen sulfite may be neglected in comparison with autocatalytic oxidation of hydrogen sulfite in reaction 1.

Thus, an addition of HRP to the  $\text{H}_2\text{O}_2$ – $\text{SO}_3^{2-}$ – $\text{Fe}(\text{CN})_6^{4-}$  system should not affect the rate of stage 1 but should enlarge the total rate of proton consumption at stage 2,  $v_{2t} = v_2 + v_{2e}$ , so that

$$v_{2t} \cong k_2[\text{H}_2\text{O}_2] + (k_{12}'' + k_{12}'[\text{H}^+])E_0[\text{Fe}(\text{CN})_6^{4-}] \quad (16)$$

A comparison between (16) and (8) shows that if

$$k_{12}'[\text{H}^+]E_0[\text{Fe}(\text{CN})_6^{4-}] > k_1'[\text{H}^+][\text{HSO}_3^-][\text{H}_2\text{O}_2] \quad (17)$$

the autocatalysis will be suppressed. Hence, if we want only to modify oscillations, but not to suppress them completely, the initial concentration of HRP should not exceed  $(E_0)_{\text{max}}$ , where

$$(E_0)_{\text{max}} \cong k_1'[\text{HSO}_3^-]_0[\text{H}_2\text{O}_2]_0 / (k_{12}'[\text{Fe}(\text{CN})_6^{4-}]_0) \quad (18)$$

On the other hand, the rate of the HRP reaction is noticeable (at least at low pH) if the inequality  $v_2 \leq v_{2e}$  holds, or

$$(E_0)_{\text{min}} \cong k_2[\text{H}_2\text{O}_2]_0 / (k_{12}'[\text{H}^+][\text{Fe}(\text{CN})_6^{4-}]_0) \quad (19)$$

For the usually used concentrations  $[\text{H}_2\text{O}_2]_0 = 6 \times 10^{-3} \text{ M}$ ,  $[\text{SO}_3^{2-}]_0 = 4 \times 10^{-3} \text{ M}$ ,  $[\text{Fe}(\text{CN})_6^{4-}]_0 = 2 \times 10^{-3} \text{ M}$ . From

(18) and (19), one finds that  $(E_0)_{\min} \cong 10^{-10}$ – $10^{-9}$  M and  $(E_0)_{\max} = 10^{-5}$  M.

### 3. Experimental Section

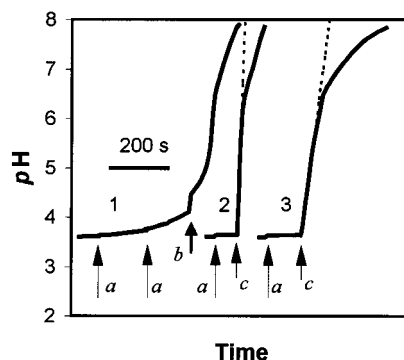
Analytical grade  $K_4[Fe(CN)_6] \cdot 3H_2O$ ,  $Na_2SO_3$  (Aldrich),  $H_2O_2$ , and  $H_2SO_4$  (Reachim) were used without further purification. Hydrogen peroxide was stabilizer-free. Stock solutions of reagents were prepared with distilled water immediately before experiments and kept under argon at ambient temperature. A stock solution of horseradish peroxidase (EC 1.1.3.4) was prepared by dissolving 3 mg of lyophilized commercial powder of HRP (Serva) with  $RZ = A_{403}/A_{278} = 2.44$  in 10 mL of distilled water, purified by centrifugation (5000 g, 10 min), and kept at 4 °C. The concentration of the purified enzyme was determined spectrophotometrically according to  $\epsilon_{403} = 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ .<sup>16</sup> The concentration of  $H_2O_2$  was determined by permanganometric titration,  $[Fe(CN)_6^{4-}]$  was determined spectrophotometrically by  $\epsilon_{420} = 1040 \text{ M}^{-1} \text{ cm}^{-1}$ , and  $[Na_2SO_3]$  was checked by iodometric titration. The activity of HRP, i.e., constants  $k_{10}$  and  $k_{12}$ , was determined by photometrical kinetic curves at  $\lambda = 420 \text{ nm}$  in various buffer solutions (phosphate buffer) with formula 13 and at various ratios of  $[Fe(CN)_6^{4-}]_0$  to  $[H_2O_2]_0$ . The rate of reaction 2 catalyzed by HRP and running in distilled water (without buffer) was also monitored by pH-kinetic curves.

The reactions were conducted both in batch and CSTR-thermostated reactors (both in Teflon and quartz) of 25 mL volume at 25 °C and at 600 rpm. Stirring was done with a magnetically driven Teflon-coated bar 1.3 cm long and 5 mm in diameter, rotating on the bottom of the vessel. The reactions were followed both by continuous measurement of pH and light absorption at  $\lambda = 420$  or 440 nm on a homemade spectrophotometric setup.<sup>19</sup> A pH electrode (ESL-03-47) and a Ag–AgCl reference electrode were inserted into the cell stopper and were connected to a pH-150 pH meter (Russia) or to a I-115M pH meter (Russia). The pH–time curves were recorded by an X–Y (X is the axis time) “Endim 622.01” recorder (Germany) or by an X–Y (X is the axis time) “H-307/1” recorder (Russia) connected to a pH meter. In CSTR experiments, sulfuric acid and sulfite solutions were premixed, and a CSTR was fed by peristaltic pump (“ZALIMP pp 2–15”, Poland) through four inlet tubes entering the reactor bottom. The reactionary mixture flew out of the reactor through its stopper.

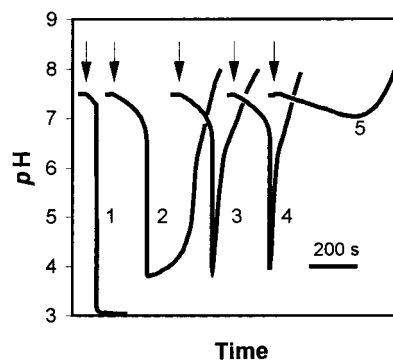
### 4. Experimental Results and Discussion

**HRP Reactions in a Batch Reactor.** To follow the effect of HRP on reactions 1 and 2, we conducted the experiments in a batch reactor. The pH kinetic curves for reaction 2 are shown in Figure 1. It can be seen from Figure 1 that reaction 2 runs rather slowly without HRP, especially at  $pH < 4$  (see curve 1). An addition of HRP, however, results in a quick pH growth with time, showing a linear dependence of pH on time at the initial section of curves 2 and 3 (in the pH range between 3.6 and 5.5). A comparison between the slopes of the straight sections of curves 2 and 3 in Figure 1 shows that the rate of pH growth is directly proportional to  $E_0$ . In accordance with relations 14 and 15, the pH growth in reaction 2 catalyzed by HRP should really be linear in the pH range between 3 and 6. If

$$\frac{d[H^+]}{dt} = -2k_{12}'E_0[Fe(CN)_6^{4-}][H^+] \quad (20)$$



**Figure 1.** Dependence of pH on time for the reaction  $H_2O_2 + Fe(CN)_6^{4-}$ . At the initial moment of time, a 18 mL volume cell contained  $[H_2SO_4]_0 = 0.394 \text{ mM}$  and  $[Fe(CN)_6^{4-}]_0 = 1.44 \text{ mM}$ . The reaction was started by adding 2 mL of stock  $H_2O_2$  solution so that after the addition the cell contained  $[H_2O_2]_0 = 2.86 \text{ mM}$ . Curve 1 shows how the course of the reaction changes after an increase in  $[H_2O_2]$  to 5.73 mM and in  $[Fe(CN)_6^{4-}]$  to 2.88 mM. The times of  $H_2O_2$  and  $Fe(CN)_6^{4-}$  additions are marked by arrows *a* and *b*, respectively. For curves 2 and 3, the moments of HRP addition are marked by arrows *c*. For curve 2,  $[HRP] = 2.2 \times 10^{-8} \text{ M}$ , and for curve 3,  $[HRP] = 4.4 \times 10^{-9} \text{ M}$ .



**Figure 2.** Dependence of pH on time for the reactionary system  $H_2O_2-SO_3^{2-}-Fe(CN)_6^{4-}$  with and without HRP. At the initial moment of time marked by arrows (at the moment  $H_2O_2$  was added),  $[H_2SO_4]_0 = 0.394 \text{ mM}$ ,  $[Na_2SO_3]_0 = 3.02 \text{ mM}$ , and  $[H_2O_2]_0 = 7.16 \text{ mM}$ . For curve 1,  $[Fe(CN)_6^{4-}]_0 = 0$ , and for curves 2–5,  $[Fe(CN)_6^{4-}]_0 = 1.44 \text{ mM}$ . For curves 1 and 2,  $[HRP] = 0$ . For curve 3,  $[HRP] = 1.1 \times 10^{-8} \text{ M}$ . For curve 4,  $[HRP] = 2.2 \times 10^{-8} \text{ M}$ . For curve 5,  $[HRP] = 10^{-7} \text{ M}$ .

then, on condition that  $[Fe(CN)_6^{4-}] \cong [Fe(CN)_6^{4-}]_0$ , the constant  $k_{12}'$  may be found from the slope of the straight section. The calculation shows that  $k_{12}' \cong (0.8-1.0) \times 10^{10} \text{ M}^{-2} \text{ s}^{-1}$ , which is in good accordance with literature data.<sup>18</sup>

Figure 2 shows typical experiments on the study of the peroxidase effect on the whole  $H_2O_2-SO_3^{2-}-Fe(CN)_6^{4-}$  system, when reactions 1 and 2 take place together. Curve 1 presents solely reaction 1, while curve 2 presents the sum of reactions 1 and 2. An addition of HRP does not affect reaction 1, but when HRP is added to the whole  $H_2O_2-SO_3^{2-}-Fe(CN)_6^{4-}$  system (curves 3–5 of Figure 2), the time of the system's stay at low pH ( $pH < 5$ ) markedly shortens. At rather high  $E_0$  (curve 5) an autocatalytic growth of  $[H^+]$  does not occur at all, and the system remains at high pH ( $pH > 7$ ). From relation 17 we estimated  $(E_0)_{\max} = 10^{-5} \text{ M}$ , above which autocatalysis should be dampened, though it follows from the experiment that autocatalysis is inhibited at much smaller concentrations of  $E_0$ , at  $E_0 \cong 10^{-7} \text{ M}$ . Hence, for the estimation of  $(E_0)_{\max}$  at  $pH > 7$ , we should compare the rate of reaction 6,  $v_6 = k_6[SO_3^{2-}][H_2O_2]$ , with the rate  $v_{2t}$  (expression 16) at neutral pH,  $v_{2t} \cong k_{12}''E_0[Fe(CN)_6^{4-}] + k_2[H_2O_2]$ ,

$$k_6[\text{SO}_3^{2-}][\text{H}_2\text{O}_2] < k_{12}''(E_0)_{\max}[\text{Fe}(\text{CN})_6^{4-}] + k_2[\text{H}_2\text{O}_2] \quad (21)$$

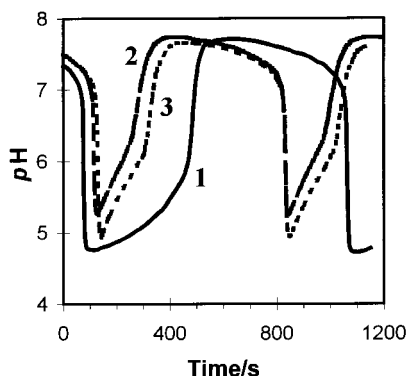
From (21) for  $k_{12}'' \cong 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ,  $k_2 = 1.6 \times 10^{-4} \text{ s}^{-1}$ ,  $k_6 = 0.2 \text{ M}^{-1} \text{ s}^{-1}$ ,  $[\text{H}_2\text{O}_2]_0 = 7 \times 10^{-3} \text{ M}$ ,  $[\text{SO}_3^{2-}]_0 = 3 \times 10^{-3} \text{ M}$ , and  $[\text{Fe}(\text{CN})_6^{4-}]_0 = 1.5 \times 10^{-3} \text{ M}$ , we find that  $(E_0)_{\max} \cong 1.4 \times 10^{-7} \text{ M}$ , which is much closer to the experimentally found value  $(E_0)_{\max} \cong 10^{-7} \text{ M}$ . Probably a more exact account of all stages will give practically the same  $(E_0)_{\max}$  as the one found experimentally.

Figure 2 also shows that for the moderate values of  $E_0$  ( $E_0 \cong 10^{-8} \text{ M}$ ) the rate of pH growth at  $\text{pH} > 5-6$  in the peroxidase-containing system differs only slightly from the rate of pH growth in a system without peroxidase. Hence, the maximum difference in the oscillation pattern after HRP addition to the  $\text{H}_2\text{O}_2\text{--SO}_3^{2-}\text{--Fe}(\text{CN})_6^{4-}$  system should be expected at pH values ranging from 4 to 5-6.

**Oscillations in a CSTR.** The most typical oscillation patterns of the  $\text{H}_2\text{O}_2\text{--SO}_3^{2-}\text{--Fe}(\text{CN})_6^{4-}$  oscillator with and without HRP are presented in Figure 3. An addition of HRP markedly shortens the time of the system's stay at low pH, shortens the oscillation period, and makes the oscillation amplitude smaller at the expense of the lowest pH value growth. The limit cycle built in the  $\text{pH--Fe}(\text{CN})_6^{4-}$  plain also shrinks after HRP addition, but its shape remains unchanged.

When the rate of the reagent supply increases, the oscillatory state, as is known,<sup>12</sup> turns to the stationary state with low pH at some critical value of  $k_0 = (k_0)_{\text{cr}}$ ,  $k_0$  being the reciprocal of the residence time. HRP addition increases the value of  $(k_0)_{\text{cr}}$ . Table 1 presents the maximum and minimum values of pH in the course of pH oscillations, as well as the oscillation period  $T$  at various  $k_0$  and  $[\text{HRP}] = E_0$  for some fixed set of initial reagent concentrations. It can be seen from Table 1 that the stationary state of the system may turn to the oscillatory state after HRP addition (compare rows 2 and 6 or rows 3, 7, and 9). Naturally, it is easy to turn the oscillatory state to the stationary one by adding HRP.

By varying the concentrations of the initial reagents, as well as the concentration of HRP and residence time ( $k_0^{-1}$ ), one can get oscillations of different shapes including rather complex ones, though this topic is not the subject of the present paper. In this work we only wanted to show that we can modify the properties of pH oscillators by adding peroxidase to them. The most interesting consequences of this step, however, may be expected after examining the stationary structures of distributed



**Figure 3.** Effect of HRP addition on pH oscillations in the  $\text{H}_2\text{O}_2\text{--SO}_3^{2-}\text{--Fe}(\text{CN})_6^{4-}$  system under flow conditions. Input concentrations are:  $[\text{H}_2\text{O}_2]_0 = 6.45 \text{ mM}$ ,  $[\text{Na}_2\text{SO}_3]_0 = 2.7 \text{ mM}$ ,  $[\text{Fe}(\text{CN})_6^{4-}]_0 = 2.02 \text{ mM}$ , and  $[\text{H}_2\text{SO}_4]_0 = 0.374 \text{ mM}$ . For curve 1  $[\text{HRP}] = 0$ . For curves 2 and 3  $[\text{HRP}] = 1.38 \times 10^{-9} \text{ M}$ . For curves 1 and 2,  $k_0 = 5.5 \times 10^{-4} \text{ s}^{-1}$ , and for curve 3,  $k_0 = 7 \times 10^{-4} \text{ s}^{-1}$ .

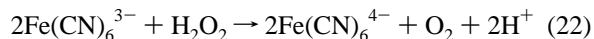
systems in thin unmixed layers. To visualize these structures, it is reasonable to add some pH indicator to a pH oscillator. Therefore, we checked if such pH indicators could inhibit peroxidase functioning. As in our preceding paper,<sup>20</sup> it turned out that the use of bromocresol purple is most preferential. This indicator hardly affects the  $\text{H}_2\text{O}_2\text{--SO}_3^{2-}\text{--Fe}(\text{CN})_6^{4-}$  pH oscillator and does not change peroxidase activity.

## 5. Simple Model

To model the HRP-modified  $\text{H}_2\text{O}_2\text{--SO}_3^{2-}\text{--Fe}(\text{CN})_6^{4-}$  pH oscillator, we used the following simple model:



where  $\text{A} = \text{H}_2\text{O}_2$ ,  $\text{B} = \text{Fe}(\text{CN})_6^{4-}$ ,  $\text{C} = \text{Fe}(\text{CN})_6^{3-}$ ,  $\text{H} = \text{H}^+$ ,  $\text{X} = \text{SO}_3^{2-}$ , and  $\text{HX} = \text{HSO}_3^-$ . Reactions R1, R2, and R3 + R4 correspond to reactions 1, 2, and 4, respectively. Reaction R5 corresponds to reaction 2 catalyzed by HRP. For reactions R1–R5, we used the following reaction rates:  $v_1 = (k_1 + k_1'[\text{H}])[\text{HX}][\text{A}]$ ,  $v_2 = k_2[\text{A}][\text{H}]/(K_2 + [\text{H}])$ ,  $v_3 = k_3[\text{H}][\text{X}]$ ,  $v_4 = k_4[\text{HX}]$ ,  $v_5 = (k_5'' + k_5'[\text{H}])E_0[\text{B}]$ , respectively. In the last expression for  $v_2$  we used the additional (in comparison with expression 9) multiplier  $[\text{H}]/(K_2 + [\text{H}])$  with  $K_2 = 10^{-7} \text{ M}$ , which implies that at  $\text{pH} > 7$  reaction R2 stops because of the ferricyanide reduction.<sup>12</sup>



A set of corresponding equations are as follows:

$$d[\text{H}]/dt = v_1 - 2v_2 - v_3 + v_4 - 2v_5 + k_0([\text{H}]_0 - [\text{H}])$$

$$d[\text{C}]/dt = 2v_2 + 2v_5 - k_0[\text{C}]$$

$$d[\text{HX}]/dt = -v_1 + v_3 - v_4 - k_0[\text{HX}]$$

$$d[\text{X}]/dt = -v_3 + v_4 + k_0([\text{X}]_0 - [\text{X}])$$

$$d[\text{B}]/dt = -2v_2 - 2v_5 + k_0([\text{B}]_0 - [\text{B}])$$

$$d[\text{A}]/dt = -v_1 - v_2 - v_5 + k_0([\text{A}]_0 - [\text{A}])$$

Figure 4 presents the calculated curves for the same concentrations of the reagents as those used in the experiment. As can be seen from the comparison between Figures 4 and 3, model indicated by reactions R1–R5 satisfactorily describes the effect induced by adding HRP to the  $\text{H}_2\text{O}_2\text{--SO}_3^{2-}\text{--Fe}(\text{CN})_6^{4-}$  pH oscillator. Table 2 shows the dependence of the oscillation period on the constant  $k_0$  and on concentration  $E_0$ . From the comparison between Tables 1 and 2 it follows that the experimental and theoretical dependencies are identical. To be more exact, a growth in  $E_0$  causes a decrease in the period  $T$  and an increase in the critical value of  $(k_0)_{\text{cr}}$  at which the oscillatory state turns to the stationary one.

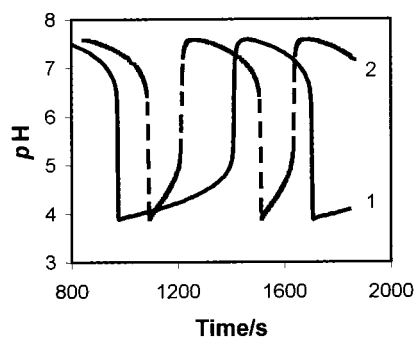
## 6. Conclusion

The  $\text{H}_2\text{O}_2\text{--SO}_3^{2-}\text{--Fe}(\text{CN})_6^{4-}$  pH oscillator modified by adding horseradish peroxidase is suggested and investigated. It

**TABLE 1: Experimentally Obtained Dependence of pH Oscillation Amplitude (Minimum and Maximum) and Oscillation Period  $T$  on  $k_0$  and [HRP]<sup>a</sup>**

$k_0/10^{-4} \text{ s}^{-1}$	[HRP]/nM	pH	$T/\text{min}$
5.5		4.75–7.75	16.5
7		5	—
9		4.6	—
4.2	1.38	5.83–7.75	11.5
5.5	1.38	5.2–7.75	12
7	1.38	4.95–7.7	12
9	1.38	5.8	—
7	3.22	5.6–7.65	9.5
9	3.22	5.2–7.6	9
11	3.22	6.1	—

<sup>a</sup> The data in Table 1 are obtained at  $[\text{H}_2\text{O}_2]_0 = 6.45 \text{ mM}$ ,  $[\text{Na}_2\text{SO}_3]_0 = 2.7 \text{ mM}$ ,  $[\text{Fe}(\text{CN})_6^{4-}]_0 = 2.02 \text{ mM}$ ,  $[\text{H}_2\text{SO}_4]_0 = 0.374 \text{ mM}$ . A single number in the column “pH” and a dash in the column “ $T$ ” characterize stationary state.



**Figure 4.** Calculated curves for eqs R1–R5 model at  $k_1 = 7 \text{ M}^{-1} \text{ s}^{-1}$ ,  $k_1' = 1.48 \times 10^7 \text{ M}^{-2} \text{ s}^{-1}$ ,  $k_2 = 1.6 \times 10^{-4} \text{ s}^{-1}$ ,  $K_2 = 10^{-7}$ ,  $k_3 = 5 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ ,  $k_4 = 3 \times 10^3 \text{ s}^{-1}$ ,  $k_5' = 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ,  $k_5'' = 2.5 \times 10^{10} \text{ M}^{-2} \text{ s}^{-1}$ ,  $[\text{H}]_0 = 0.75 \text{ mM}$ ,  $[\text{X}]_0 = 2.7 \text{ mM}$ ,  $[\text{B}]_0 = 2.02 \text{ mM}$ ,  $[\text{A}]_0 = 6.45 \text{ mM}$ , and  $k_0 = 1.2 \times 10^{-3} \text{ s}^{-1}$ . For curve 1  $E_0 = 0$ . For curve 2  $E_0 = 2 \times 10^{-10} \text{ M}$ .

**TABLE 2: Calculated Dependence of Oscillation Period  $T$  for Eqs R1–R5 Model on  $k_0$  and  $E_0$ <sup>a</sup>**

$k_0/10^{-3} \text{ s}^{-1}$	$E_0/\text{nM}$	$T/\text{min}$
1.3	0	15
1.3	0.1	8.5
1.3	0.2	7.1
1.3	0	15
1.4	0	21.8
1.5	0	—
1.4	0.1	9.1
1.5	0.1	10.5
1.6	0.1	—
1.4	0.2	7.23
1.5	0.2	7.5
1.6	0.2	8.3
1.7	0.2	—

<sup>a</sup> All the reaction rate constants and input concentrations of the variables are the same as in Figure 4.

might be said that it is the first homogeneous pH oscillator with the enzyme. The use of this system in the studies of stationary and wave patterns may prove to be of much importance because of the following reasons: peroxidase is practically immobile in a matrix and can be spatially selectively immobilized according to a given template.

The use of peroxidase in the  $\text{H}_2\text{O}_2$ – $\text{SO}_3^{2-}$ – $\text{Fe}(\text{CN})_6^{4-}$  pH oscillator allowed us to vary the strength of negative feedback and to change its dependence on  $[\text{H}^+]$ . It is noteworthy that

the possibility of varying the value of the negative feedback in the  $\text{H}_2\text{O}_2$ – $\text{SO}_3^{2-}$ –marble pH oscillator<sup>6a</sup> and in the  $\text{H}_2\text{O}_2$ – $\text{HSO}_3^-$ – $\text{HCO}_3^-$ – $\text{Fe}(\text{CN})_6^{4-}$  pH oscillator<sup>6b,c</sup> led to the discovery of the chaotic pH oscillations. It may be possible that the use of HRP in the  $\text{H}_2\text{O}_2$ – $\text{SO}_3^{2-}$ – $\text{Fe}(\text{CN})_6^{4-}$  pH oscillator will also lead to the chaotic pH oscillations, which, unlike the twice heterogeneous  $\text{H}_2\text{O}_2$ – $\text{SO}_3^{2-}$ –marble pH oscillator and the heterogeneous  $\text{H}_2\text{O}_2$ – $\text{HSO}_3^-$ – $\text{HCO}_3^-$ – $\text{Fe}(\text{CN})_6^{4-}$  pH oscillator, may be discovered in a homogeneous pH oscillator.

It should be noted that the rate of  $\text{H}^+$  consumption in the  $\text{H}_2\text{O}_2$ – $\text{SO}_3^{2-}$ – $\text{Fe}(\text{CN})_6^{4-}$  system may be increased not only by the addition of HRP but also by the illumination of the system.<sup>6c,20</sup> The photoconstituent of the reaction 2 rate,  $v_{2,L}$ , is written as follows:<sup>20</sup>

$$v_{2,L} = k_L[\text{Fe}(\text{CN})_6^{3-}]I_0(1 - \exp(-2.3A))/A \quad (23)$$

where  $k_L$  is the constant,  $I_0$  is the incident light intensity, and  $A$  is the total absorbency of the system. The addition of HRP and illumination are the parallel ways of reaction 2 acceleration. All three constituents of the reaction 2 rate,  $v_2$ ,  $v_{2,e}$ , and  $v_{2,L}$ , which are determined by eqs 9, 14, and 23, respectively, are dependent on concentrations of the different chemical components of reaction 2, on  $[\text{H}_2\text{O}_2]$ ,  $[\text{Fe}(\text{CN})_6^{4-}]$ , and  $[\text{Fe}(\text{CN})_6^{3-}]$ , respectively. This ensures various methods of reaction 2 rate control. Either it is possible to create chaotic oscillations by varying the rates  $v_2$ ,  $v_{2,e}$ , and  $v_{2,L}$ , or it is necessary to have for this purpose quite another kind of proton consumption channel connected, for instance, with the heterogeneous reaction of  $\text{CO}_2$  removal, as it was in the work by Rábai et al.<sup>6</sup> This has still remained a question.

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