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Oscillatory Oxido-Reductive Reaction of Intracellular Hemoglobin in Human Erythrocyte Incubated with *o*-Aminophenol

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Akazawa, M., Takasaki, M. and Tomoda, A. *Oscillatory Oxido-Reductive Reaction of Intracellular Hemoglobin in Human Erythrocyte Incubated with o-Aminophenol.* Tohoku J. Exp. Med., 2000, **192** (4), 301-312 —— When human erythrocytes were incubated with *o*-aminophenol at pH 7.0 at 37°C for 46 hours, intracellular oxyhemoglobin was completely oxidized to methemoglobin during the initial 6 hours, and methemoglobin formed was then reduced to oxyhemoglobin during the following 20 hours. This was demonstrated by the changes in absorption spectra of intracellular hemoglobin. Such oscillatory behavior of intracellular hemoglobin during reaction with *o*-aminophenol was explained by the fact that *o*-aminophenol has the ability to both oxidize oxyhemoglobin and reduce methemoglobin. In order to study the mechanism of oxido-reductive reactions of hemoglobin with aromatic reductants including *o*-aminophenol, the oxidation of ferrous hemoglobin and reduction of methemoglobin with various aromatic reductants such as *o*-aminophenol, 2-amino-4-methyl-phenol, 2-amino-5-methylphenol, and homogentisic acid were investigated under various conditions. It was found that oxyhemoglobin was oxidized by these aromatic compounds, and the oxidation rate was accelerated in the presence of inositol hexaphosphate, but was not affected in the presence of catalase and superoxide dismutase, except for the case with homogentisic acid. The oxidation of ferrous hemoglobin by these compounds did not proceed under anaerobic conditions. Methemoglobin was reduced by these aromatic compounds, and the reduction rate was much accelerated in the presence of inositol hexaphosphate, but was not affected in the presence of catalase and superoxide dismutase, except for the case with homogentisic acid. The reduction of methemoglobin by these compounds proceeded under anaerobic conditions, suggesting that ferric heme of hemoglobin reacts directly with aromatic reductants. On the basis of these results, the mechanism of oxido-reductive reaction of ferrous and ferric hemoglobin with aromatic reductants was proposed. —— oscillatory oxido-reductive reaction; hemoglobin; erythrocytes; *o*-aminophenol

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It has been well known that aminophenols causes methemoglobinemia when they are adsorbed in the human body (Kiese and Rachor 1964). Kiese and Weger (1969) observed that after intravenous injection of *o*-aminophenol and 4-dimethylaminophenol, rapid oxidation of erythrocytic hemoglobin occurred. They also showed that quick reduction of methemoglobin occurred after the oxidation of hemoglobin in the erythrocytes of various animal species (Kiese and Weger 1969). Howerver, there are little descriptions on the ability of *o*-aminophenol and its derivatives to reduce methemoglobin.

Winterbourn et al. (1979) firstly showed that menadione (2-methyl-1, 4-naphthoquinone), an aromatic compound, can oxidize oxyhemoglobin and reduce methemoglobin, but cannot oxidize deoxyhemoglobin. We also observed that the metabolites of tryptophan, an aromatic amino acid, such as 3-hydroxykynurenine and 3-hydroxyanthranilic acid have the ability to oxidize oxyhemoglobin, and to reduce methemoglobin, but not to oxidize deoxyhemoglobin (Tomoda et al. 1986a). These two compounds are characteristic, because they contain amino group and hydroxyl group at the adjacent position as *o*-aminophenol does. Furthermore, *o*-aminophenol is supposed to have stronger oxidative action on hemoglobin than 3-hydroxyanthranilic acid and 3-hydroxykynurenine (unpublished results). In this sense, it is interesting to investigate how the oxido-reductive reaction of erythrocytic hemoglobin may occur, when human erythrocytes are incubated with *o*-aminophenol.

In this study we presented the patterns of the oxidation and reduction of erythrocytic hemoglobin caused by *o*-aminophenol during 46 hours incubation at 37°C in vitro. In order to study the mechanism of human hemoglobin with *o*-aminophenol, we investigated the reactions of purified ferrous and ferric hemoglobin with *o*-aminophenol, 2-amino-4-methylphenol, 2-amino-5-methylphenol, and homogentisic acid under various conditions.

Materials and Methods

Materials

Human erythrocytes were obtained after removal of plasma and buffy coats from outdated ACD (acid/citrate/dextrose) bloods by centrifugation and washed twice with 0.9% NaCl. Then, the erythrocytes were suspended in 0.9% NaCl solution to make the hematocrit value 15%, and were poured in a 100 ml glass beaker. The pH of the suspension was adjusted to 7.0 at 37°C by addition of 0.1 M NaOH containing 0.9% NaCl. *o*-Aminophenol solution neutralized with 0.1 M NaOH was added to the erythrocyte suspension; the final concentration of *o*-aminophenol was 2 mM. Then the erythrocyte suspension was incubated at 37°C for 46 hours in a thermostatic water bath shaker. Glucose was not added in erythrocyte suspension, so as to prevent the action of NADH methemoglobin reductase. Samples were taken out at constant intervals for analysis of hemoglobin.

Measurement of absorption spectra of intracellular hemoglobin

Erythrocyte samples, which were taken out from the erythrocyte suspension at the indicated time points (see Results), were lyzed with 30-vol of distilled water. Then, 0.5 ml of hemolysate was applied on a column (0.3 cm × 10 cm) of Sephadex G-25 (Amersham-Pharmacia, Uppsala, Sweden) previously equilibrated with 10 mM potassium phosphate buffer (pH 7.0). The hemolysates were eluted with 10 mM potassium phosphate buffer (pH 7.0). Cell membranes were eluted firstly, and were discarded. Then all the red colored portions containing hemoglobin were collected in a mess glass tube. After the collection of the red colored portions, 10 mM potassium phosphate buffer (pH 7.0) was added to make the final volume of 5 ml in a mess glass tube. After mixing the eluates well, optical absorption spectra of the hemoglobin solution were measured between 450 nm and 650 nm. By these procedures, it was possible to obtain the absorption spectra of hemoglobin with an clear isosbestic points.

Purification of human oxyhemoglobin and methemoglobin

Human bloods were freshly drawn from a normal adult after obtaining the informed consents. After collecting erythrocytes by centrifugation, erythrocytes were lyzed with distilled water, and hemoglobin was purified according to the method described previously (Tomoda et al. 1986a). Hemoglobin solution was free of catalase and superoxide dismutase. It was passed through a column of Sephadex G-25 previously equilibrated with 100 mM potassium phosphate buffer (pH 7.0) to remove organic phosphates such as 2, 3-bis phosphoglycerate (Tomoda et al. 1986a). A portion of this purified hemoglobin was converted to methemoglobin by potassium ferricyanide and then passed through a column of Sephadex G-25 (fine) previously equilibrated with 100 mM potassium phosphate to remove ferricyanide.

Reactions of human hemoglobin with various aromatic reductants

Oxido-reductive reactions of human hemoglobin were performed as follows. A 2-ml aliquot of oxyhemoglobin (60 μM as heme) in 100 mM potassium phosphate buffer (pH 7.0) or 2-ml of methemoglobin (60 μM as heme) in the same buffer was mixed with 0.01-ml solution of each of various aromatic reductants such as *o*-aminophenol (Tokyo Kasei, Tokyo), 2-amino-4-methylphenol (Tokyo Kasei), 2-amino-5-methylphenol (Tokyo Kasei), and homogentisic acid (Wako Pure Chemical, Tokyo). The reductant solutions were freshly prepared before use. The final concentration of each aromatic reductant was 350 μM. The reactions were performed aerobically at 25°C with or without 200 μM inositol hexa-kis-phosphate (P_6-inositol; Sigma, St.Louis, MO, USA), 1300 units of catalase (Boehringer Mannheim, Mannheim, Germany) or 29 units of superoxide dismutase (Sigma). The oxidation and reduction rates of hemoglobin were

spectrophotometrically measured by following the decrease of absorbance at 578 nm and the decrease of absorbance at 630 nm, respectively. The measurements of the rates of oxidation of ferrous hemoglobin under anaerobic conditions were performed with a Thunberg-type quartz cell after replacing the gas phase with Q gas (helium/isobutane, 99.05 : 0.95). The concentration of hemoglobin (as heme) was estimated using a millimolar extinction coefficient of 3.84 at 630 nm at pH 7.0 for methemoglobin, and that of 15.3 at 578 nm for oxyhemoglobin (van Assendelft and Zijlstra 1975).

Results

We investigated the changes in absorption spectra of the intracellular hemoglobin between 450 nm and 650 nm during the incubation of human erythrocytes with *o*-aminophenol (Fig. 1). During 2-hour incubation, intracelluar hemoglobin changed to methemoglobin quickly (Fig. 1A). This was shown by the increase of absorbance at 630 nm, the decrease of absorbance at 578 nm and the clear isobestic points at 528 nm and 588 nm. As shown in Fig.1B, intracellular hemoglobin was completely oxidized to methemoglobin after 6 hours, judging from the absorption spectra of methemoglobin (van Assendelft and Zijlstrta 1975). Thereafter intracellular methemoglobin began to be reduced, because the absorbance at 630 nm and 578 nm decreased and increased, respectively, with the clear isobestic points at 528 nm and 588 nm (Fig. 1B). After 26 hours, the absorption spectrum obtained was identical with that of oxyhemoglobin, indicating that erythrocytic methemoglobin was completely reduced to ferrous oxyhemoglobin during 26 hours incubation.

The changes in absorbance at 630 nm, which were obtained from the results in Figs. 1A and 1B and those at 46 hour, are depicted in Fig. 1C. The absorbance at 630 nm increased for the initial 6 hours, indicating that intracellular hemoglobin was oxidized by *o*-aminophenol to methemoglobin. The oxidation proceeded biphasically, but in a first order as shown in Fig. 1D. Between 6 hours and 10 hours, no reaction occurred, because no change of absorbance was observed (the optical densitiy at 630 nm during these period was not depicted in the figure.) Then, methemoglobin that had been formed was began to be reduced to oxyhemoglobin by 26 hours. After 26 hours, no oxidation was observed until the end of experiment (46 hours). During the 46-hour incubation of the erythrocytes, little hemolysis was observed. We also observed that production of 2-aminophenoxazine-3-one during the incubation (data not shown) as reported previously (Tomoda et al. 1986b).

Figs. 2A and 2B show the oxidation of purified oxyhemoglobin and reduction of purified methemoglobin by *o*-aminophenol, respectively, in the presence or absence of P_6-inositol, catalase, or superoxide dismutase. The oxidation of oxyhemoglobin by *o*-aminophenol was accelerated in the presence of P_6-inositol but was affected by neither catalase, superoxide dismutase, nor catalase plus

superoxide dismutase (Fig. 2A). The reduction of methemoglobin by *o*-aminophenol was also highly accelerated in the presence of P_6-inositol, but was affected by neither catalase, superoxide dismutase nor catalase plus superoxide dismutase (Fig. 2B). Tomoda et al. (1979a) showed that the rates of oxidation of oxyhemoglobin by ferricyanide, hydrogen peroxide, hydroxylamine and *β*-naphthoquinone-4-sulfonate were accelerated in the presence of P_6-inositol, and were correlated with the conformational changes in oxyhemoglobin by the binding of P_6-inositol to oxyhemoglobin. It was also suggested by Perutz et al. (1974) that the quaternary structure of methemoglobin might be changed by the binding of P_6-inositol to methemoglobin. Thus, the acceleration of the reduction of methemoglobin by *o*-aminophenol in the presence of P_6-inositol may be explained by the conformational change in methemoglobin, which is consistent with our previous report (Tomoda et al. 1976).

Table 1 shows the comparison of oxidation of purified oxyhemoglobin with *o*-aminophenol, 2-amino-4-methylphenol, 2-amino-5-methylphenol, and homogentisic acid. Though the oxidation of oxyhemoglobin by these aromatic compounds did not occur under anaerobic conditions, it occurred under aerobic conditions. The rates of oxidation of oxyhemoglobin were markedly accelerated in the presence of P_6-inositol. However, catalase, superoxide dismutase and catalase plus superoxide dismutase had no effect on the oxidation of oxyhemoglobin by *o*-aminophenol and its derivatives.

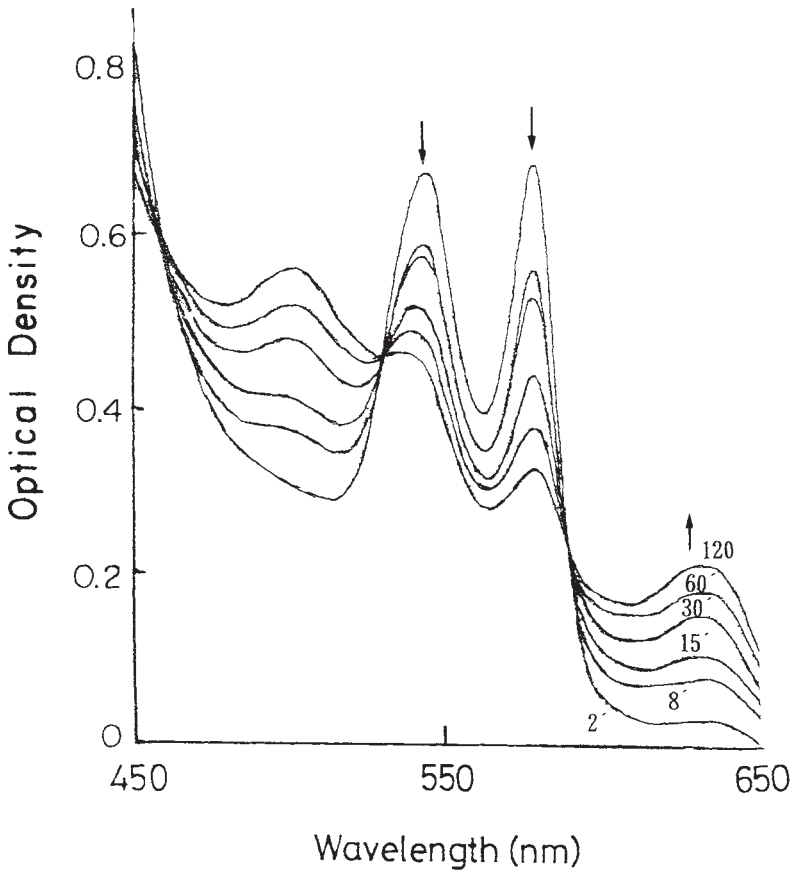
In the case of the oxidation of oxyhemoglobin with homogentisic acid, the oxidation rate was much accelerated in the presence of superoxide dismutase, but was inhibited in the presence of catalase, suggesting that the mechanism of oxidation of ferrous hemoglobin by homogentisic acid is different from those of other aromatic reagents used.

Table 2 shows the reduction of purified methemoglobin with various aromatic reductants. Methemoglobin was reduced by all reagents used under aerobic and anaerobic conditions, and was accelerated in the presence of P_6-inositol under

TABLE 1. *Initial rates of oxidation of oxyhemoglobin and deoxyhemoglobin by o-aminophenol, its derivatives and homogentisic acid (μM heme/minute)*

Aromatic Compound	Anaerobic	Aerobic				
		Control	P_6-inositol (+)	Catalase (+)	SOD (+)	Catalase + SOD
o-aminophenol	0	1.2	2.0	1.2	1.2	1.2
2-amino-4-methylphenol	0	8.2	11.6	8.2	8.2	8.2
2-amino-5-methylphenol	0	0.18	0.23	0.18	0.18	0.18
homogentisic acid	0	1.96	15.3	0.7	8.2	0.9

A


0.8
0.6
0.4
0.2
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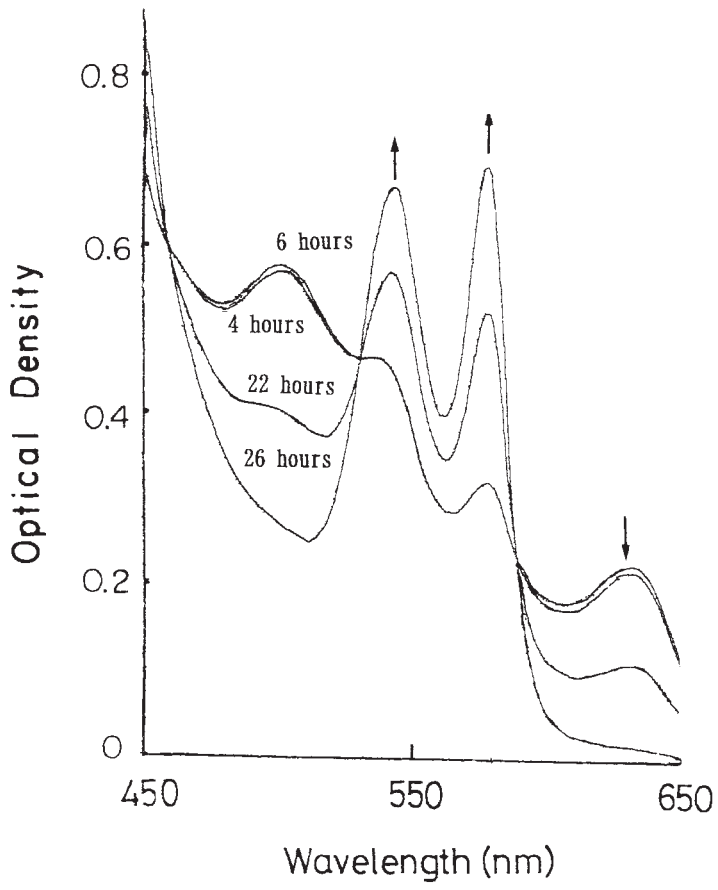
Optical Density
120
60´
30´
15´
8´
2´
450
550
650
Wavelength (nm)

B


0.8
0.6
0.4
0.2
0
Optical Density
6 hours
4 hours
22 hours
26 hours
450
550
650
Wavelength (nm)

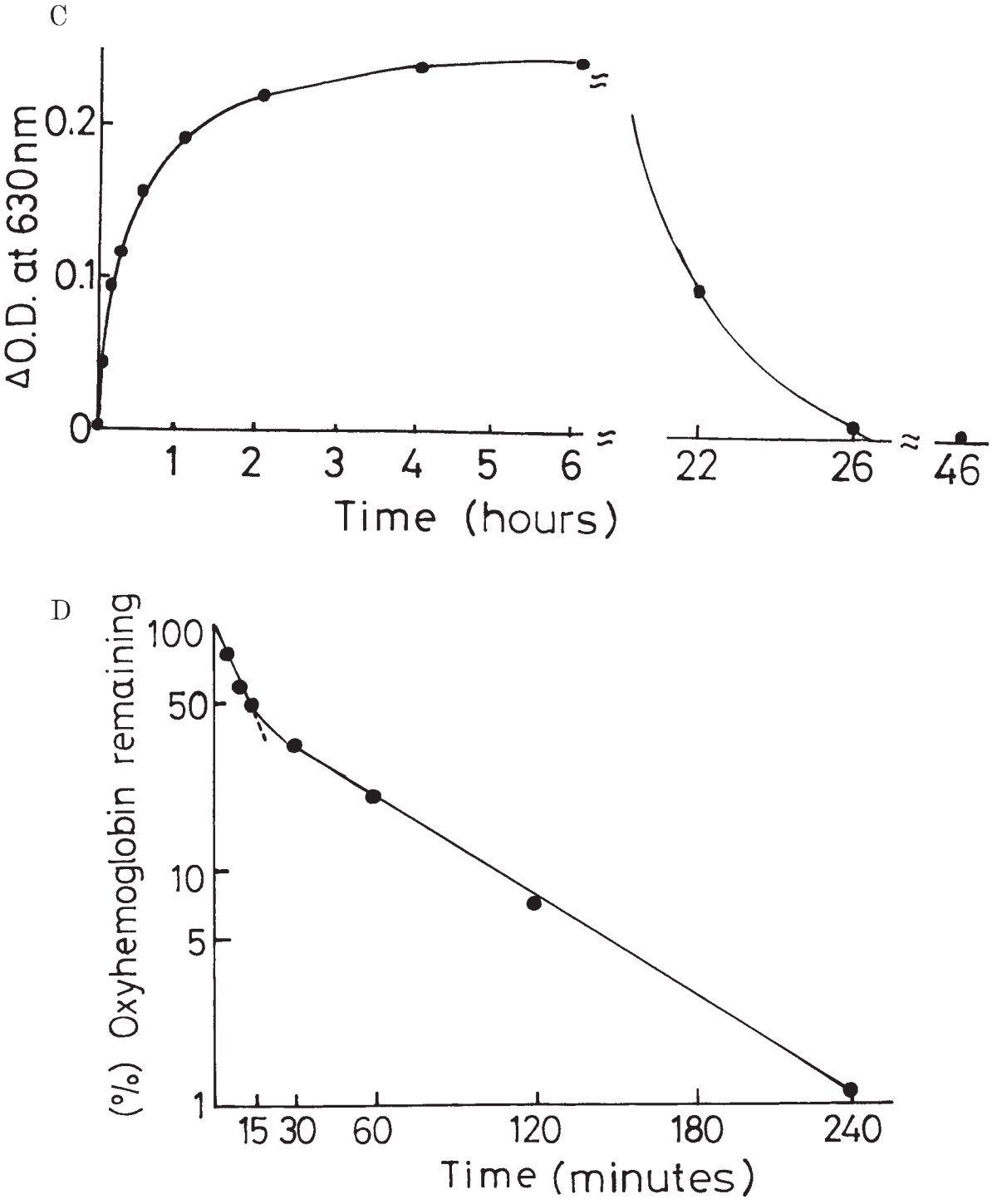


Fig. 1. Spectral changes of intracellular hemoglobin during the incubation of human erythrocytes with *o*-aminophenol at pH 7.0 at 37°C A: Spectral changes between 450 nm and 650 nm of intracellular hemoglobin during initial 120 minutes. B: Spectral changes during the incubation for 4 to 26 hours. C: Changes in absorbance at 630 nm during the incubation of human erythrocytes with o-aminophenol for 46 hours. This figure was depicted from the data shown in Figs. 1A and B. D: Semi-logarithmic plot of the rate of oxidation of erythrocytic oxyhemoglobin by *o*-aminophenol. The results shown in Fig. 1C were depicted by semi-logarithmic plot (% remaining of oxyhemoglobin).

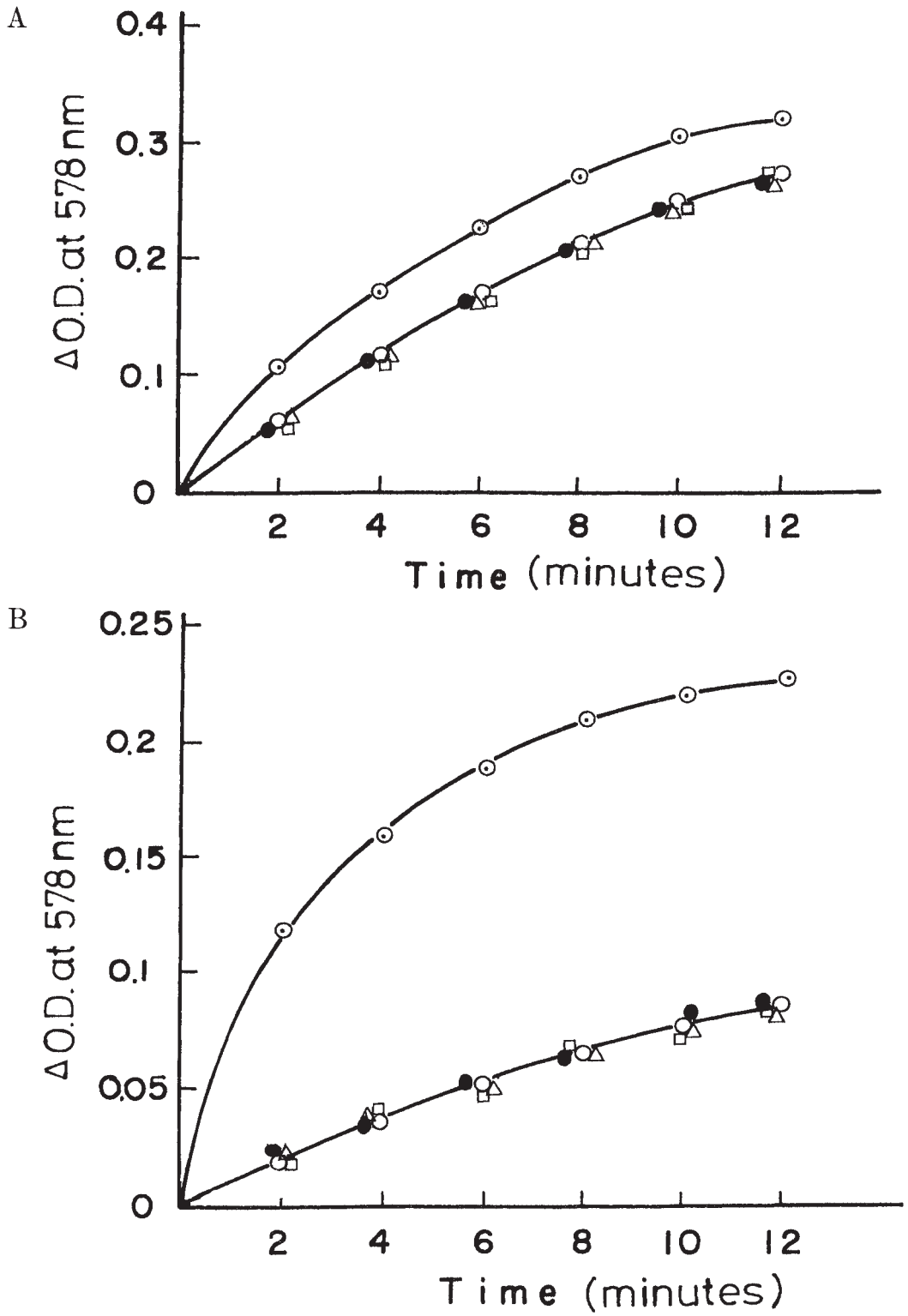


Fig. 2. Oxidation of purified oxyhemoglobin or the reduction of purified methemoglobin by *o*-aminophenol under various conditions. A: the oxidation of oxyhemoglobin. B: reduction of methemoglobin. (○), control; (●), catalase (+); (△), superoxide dismutase (SOD) (+); (□), catalase+SOD; (◉), P_6-inositol (+).

TABLE 2. *Initial rates of reduction of methemoglobin by o-aminophenol, its derivatives and homogentisic acid (μM heme/minute)*

Aromatic Compound	Anaerobic	Aerobic				
		Control	P_6-inositol (+)	Catalase (+)	SOD (+)	Catalase + SOD
o-aminophenol	0.13	1.6	7	1.6	1.6	1.6
2-amino-4-methylphenol	very slow	1.6	3.5	1.6	1.6	1.6
2-amino-5-methylphenol	0.57	1.45	8.2	1.45	1.45	1.45
homogentisic acid	10	2.5	5.4	2.5	0.7	—

aerobic conditions. The reduction rates of methemoglobin by *o*-aminophenol, 2-amino-4-methylphenol, and 2-amino-5-methylphenol were not affected by catalase, superoxide dismutase, or catalase plus superoxide dismutase.

However, the reduction rate of methemoglobin by homogentisic acid under the aerobic condition was not affected by catalase, but was significantly inhibited by superoxide dismutase.

DISCUSSION

It was found that intracellular oxyhemoglobin was oxidized to methemoglobin at an initial rate of 0.8 mM heme/hour in 6 hours, when human erythrocytes (hematocrit value, 15%; total heme concentrations, 3 mM) were incubated with 2 mM *o*-aminophenol (Fig. 1). The reaction proceeded in a first order and biphasically (Fig. 1D), suggesting that erythrocytic oxyhemoglobin reacts directly with *o*-aminophenol. The biphasic reaction of erythrocytic hemoglobin with *o*-aminophenol may be exlained by the differences in the reactivity with *o*-aminophenol between α and α chains in oxyhemoglobin, as suggested for the oxidation of oxyhemoglobin with ferriycanide and nitrite in vitro (Tomoda and Yoneyama 1979b; Tomoda et al. 1981a). The contribution of the autoxidation of hemoglobin in human erythrocytes may be negligible, because the autoxidation rate of hemoglobin in human erythrocytes is approximately 0.025 mM heme/hour (Tomoda et al. 1981b).

After 6-hour incubation of human erythrocytes with *o*-aminophenol at 37°C, methemoglobin in the erythrocytes began to be reduced during the following 20-hour incubation (Fig. 1C). Such an oscillatory reaction, i.e., the oxidation and subsequently occurring reduction of intracellular hemoglobin with aromatic compounds such as *o*-aminophenol has not been previously reported. In the experiments of methemoglobin reduction in the erythrocytes, the incubation of human erythrocytes was performed without glucose; under these conditions NADH methemoglobin reductase (NADH cytochrome b_5 reductase) cannot work.

Thus, the reduction of intracellular methemoglobin after 6 hours is likely due to the direct reaction of methemoglobin with *o*-aminophenol. Kiese and Weger (1969) showed that 4-dimethylphenol, which has chemical properties similar to *o*-aminophenol, induces quick oxidation of oxyhemoglobin and subsequent quick reduction of methemoglobin in the erythrocytes, when this compound was intravenously injected to various animal species. They also observed the quick oxidation of erythrocytic oxyhemoglobin by *o*-aminophenol in humans in 60 minutes, aiming at applying this compound for the treatment of cyanide poisoning, after intravenous injection of the compound. However, they did not mentioned the time course of the oxidation and reduction of erythrocytic hemoglobin after 60 minutes. But, it is possible that methemoglobin formed in the erythrocytes during 60 minutes may be reduced by *o*-aminophenol itself, thereafter, in vivo, as is suggested by our present results.

Furthermore, we found that oxyhemoglobin was oxidized, methemoglobin was reduced, but deoxyhemoglobin was not oxidized by *o*-aminophenol (Figs. 2A and B, Tables 1 and 2). We also observed that during the incubation of human erythrocytes with *o*-aminophenol at pH 7.0 at 37°C, 2-amino-phenoxazine-3-one was progressively produced (data not shown [Tomoda et al. 1986b]). Wallace et al. (1978) suggested that aminophenols (=electron donor [ED]) oxidize oxyhemoglobin after one-electron reduction of hemoglobin bound oxygen, producing methemoglobin and peroxide:

$$Hb(Fe^{2+}\text{-}O_2) + ED \rightarrow Hb(Fe^{2+} - O_2^-) + ED^+$$
$$Hb(Fe^{2+}\text{-}O_2^-) + 2H^+ \rightarrow Hb(Fe^{3+}) + H_2O_2$$

Concerning the reduction of methemoglobin by *o*-aminophenol, it is likely that direct reduction of methemoglobin by *o*-aminophenol proceeds, because the reaction proceeded even under the anaerobic conditions (Table 2). Thus, the reaction of intracellular hemoglobin with *o*-aminophenol may be explained, in

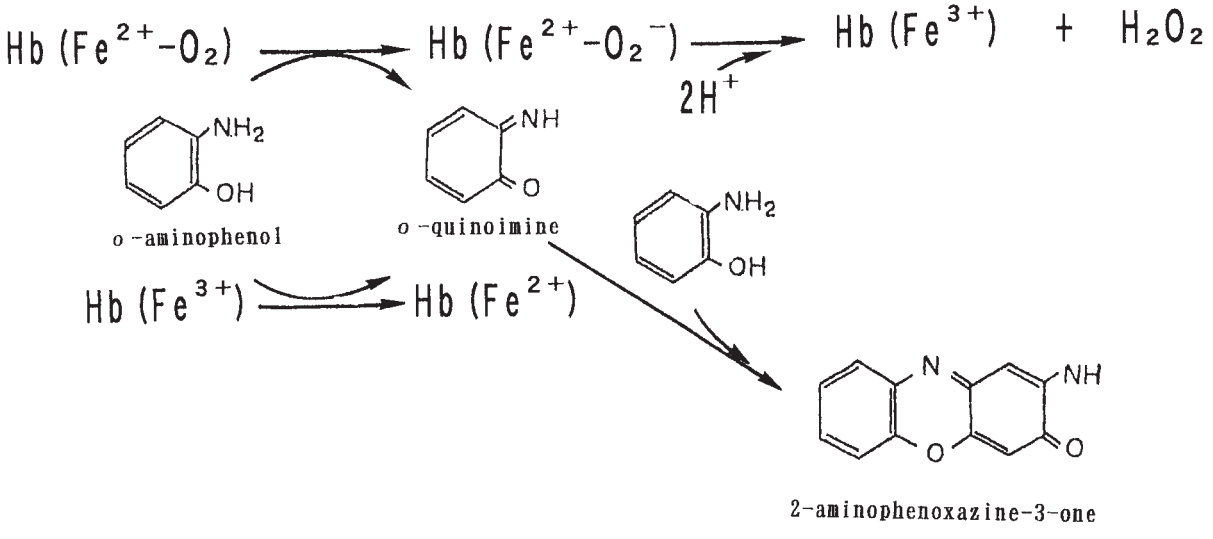


Fig. 3.

part, by the process in Fig. 3, based on the reactions of purified oxyhemoglobin and methemoglobin with *o*-aminophenol.

Hydrogen peroxide, which may be produced according to Fig. 3, will be used for the oxidation of oxyhemoglobin (Eyer et al. 1975). Thus, it would be expected that the oxidation of oxyhemoglobin by *o*-aminophenol might be inhibited by catalase, a scavenger of hydrogen peroxide. However, the reactions were not affected by the addition of catalase and superoxide dismutase (Figs. 2A and B, Tables 1 and 2). Plausible mechanism will be explained by two different reactions: 1. Oxidation of oxyhemoglobin with *o*-aminophenol described in Fig. 3, where the scavenging of hydrogen peroxide by catalase will promote the the oxidation of oxyhemoglobin:

$$Hb(Fe^{2+}-O_2^-)+2H^+ \rightarrow Hb(Fe^{3+})+H_2O_2$$

2. Oxidation of oxyhemoglobin with hydrogen peroxide, where the scavenging of hydrogen peroxide by catalase will inhibit the oxidation of oxyhemoglobin:

$$Hb(Fe^{2+})+H_2O_2 \rightarrow Hb(Fe^{3+})+H_2O+1/2\ O_2$$

As a result of the sum of these reactions, it is likely that the oxidation of oxyhemoglobin by *o*-aminopohenol was not affected by the addition of catalase.

We investigated various aromatic compounds which exert bifunctional (oxidative and reductive) effects on human hemoglobin, and found that *o*-aminophenol, 2-amino-4-methylphenol, 2-amino-5-methylphenol, and homogentisic aicd, had the ability to both oxidize oxyhemoglobin and reduce methemoglobin under aerobic and anaerobic conditions, but did not oxidize deoxyhemoglobin (Tables 1 and 2). We also observed that 3-hydroxykynurenine and 3-hydroxyanthranilic acid have the same ability (Tomoda et al. 1986a). Thus, there may be a group of aromatic compounds which oxidize hemoglobin under aerobic conditions, do not oxidize under anaerobic conditions, and reduce methemoglobin under both aerobic and anaerobic conditions.

The mechanism of oxidation of ferrous hemoglobin and reduction of ferric hemoglobin by homogentisic acid is different from that by *o*-aminophenol and its derivatives, and seems to be similar to that by menadione (Winterbourn et al. 1979), because the superoxide dismutase and catalase exerted similar effects, in both cases with homogentisic acid (Tables 1 and 2) and menadione (Winterbourn et al. 1979).

It is likely that oxygen radicals such as superoxide and hydrogen peroxide and semiquinone radicals might be involved in the reactions of hemoglobin with homogentisic acid and menadione, as suggested by Winterbourn et al. (1979).

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