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Interaction of Hemoglobin A with Sesamol and Polyphenolics

KIYOMI KIKUGAWA,* SHIGEKO ARAI, and TSUTAO KURECHI

Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan

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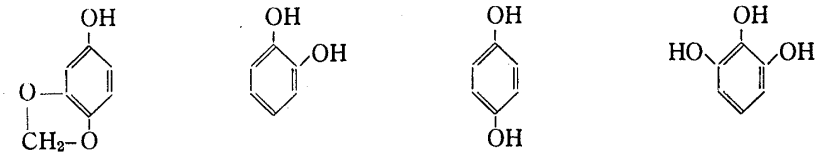
The interactions of sesamol (I), a monophenolic antioxidant in sesame oil, pyrocatechol (II), *p*-hydroquinone (III) and pyrogallol (IV) with hemoglobin A were investigated. A large excess of the phenolic (I, II and III) induced methemoglobin (MetHb) formation from oxyhemoglobin (HbO_2) at pH 7 and 25°, the rates being in the order I>II>III. The diphenolics (II and III) also reduced MetHb to deoxyhemoglobin (DeoxyHb). Pyrogallol (IV) showed a more powerful ability to produce and reduce MetHb. Sesamol (I) readily produced MetHb from HbO_2 and even from DeoxyHb, but did not reduce MetHb. MetHb formation from HbO_2 by I prevailed in the acidic range. The amount of MetHb formed by I was characteristically proportional to the concentration of HbO_2, and more than 10 equivalents of MetHb was produced at high concentrations of HbO_2. MetHb formation by I, II and III from HbO_2 was enhanced by inositol hexaphosphate, whereas MetHb formation from DeoxyHb was inhibited. The mechanisms of action of the di- and triphenolics may involve electron transfer to HbO_2 and MetHb, but that of I could not be explained analogously.

Keywords——hemoglobin A; oxyhemoglobin; methemoglobin; deoxyhemoglobin; sesamol; pyrocatechol; *p*-hydroquinone; pyrogallol; inositol hexaphosphate

Reversible oxygenation of hemoglobin of physiological relevance occurs with hemoglobin [Fe(II)] and not with methemoglobin [Fe(III)].[1] As early as 1947, Jung and Witt[2] studied the actions of some polyphenolics on cat hemoglobin *in vivo*. After the intraperitoneal injection of fairly large doses, polyphenolics such as resorcinol, pyrogallol, *p*-hydroquinone and pyrocatechol oxidized hemoglobin into methemoglobin. It has been shown that *p*-hydroquinone converts oxyhemoglobin into methemoglobin relatively rapidly *in vitro*,[3,4] and the transformation is favored under mild alkaline conditions. We reported earlier[5] that sesamol (3,4-methylenedioxyphenol), a natural antioxidant present in sesame oil, readily converts hemoglobin into methemoglobin *in vitro*. The present study was undertaken to investigate the actions of sesamol (I) on hemoglobin and to compare them with those of polyphenolics such as pyrocatechol (II), *p*-hydroquinone (III) and pyrogallol (IV), and with those of other methemoglobininducing agents such as hydroxylamine and nitrite.



sesamol (I) pyrocatechol (II) *p*-hydroquinone (III) pyrogallol (IV)

Chart 1

Experimental

Materials and Methods——Sesamol (I), obtained from Aldrich Chemical Company, Inc., was recrystallized from chloroform–petroleum ether according to the method of Haslam and Haworth.[6] Pyrocatechol (II), *p*-hydroquinone (III) and pyrogallol (IV) were of reagent grade. IHP (inositol hexaphosphoric acid sodium salt, corn type V), catalase (bovine liver, 2000 units/mg solid) and superoxide dismutase (bovine blood type I, 2900 units/mg solid) were obtained from Sigma Chemical Company.

Hemoglobin was obtained from packed red cells of normal human ACD blood as a crude oxyhemoglobin (crude HbO_2) in saline, and as a purified oxyhemoglobin (purified HbO_2) solution in Tris-HCl buffer (pH 7.0)

by passage through a Sephadex G-25 column, according to the methods reported previously.[5,7] Methemoglobin (MetHb) was prepared by treatment of purified HbO_2 with an approximate five-fold excess of potassium ferricyanide and subsequent passage through a Sephadex G-25 column in order to remove the inorganic salt. Deoxyhemoglobin (DeoxyHb) was prepared from the purified HbO_2 by bubbling nitrogen gas through a solution in a cuvette linked to a Thunberg tube, and the reaction was performed under an atmosphere of nitrogen.

Transformation of hemoglobin was monitored at selected periods by means of a Shimadzu UV-200S double beam spectrophotometer equipped with a thermostatic control apparatus. Concentrations of heme of HbO_2 and MetHb were determined from the extinction coefficients at 576 nm (16500) and at 630 nm (4010), respectively.[8]

Treatment of HbO_2, DeoxyHb and MetHb with Phenolics and Other Reagents——For the treatment of HbO_2 at pH 7.0 (Figs. 1 and 6), 4.0 ml of the purified HbO_2 solution in 0.05 M Tris-HCl buffer (pH 7.0) (absorbance at 576 nm: 1.0—1.5) was mixed with 1.0 ml of 0.1 M potassium sodium phosphate buffer (pH 7.0), 1.0 ml of water, 2.5 mM IHP solution in water or water containing 120 units of superoxide dismutase and/or 800 units of catalase, and 1.0 ml of a test compound in water, in that order, and the solution was kept at the indicated temperature for spectrophotometric assay.

For the treatment of HbO_2 at various pH values (Figs. 2 and 3), 0.10 ml of the purified HbO_2 solution (absorbance at 576 nm: about 30) was mixed with 3.0 ml of 0.1 M phosphate buffer (pH 6.0, 7.0 or 8.0) and 0.50 ml of 100 mM phenolic in water, and the solution was treated in the same way. In some cases (Fig. 3), treatment was performed under an atmosphere of nitrogen gas.

For the treatment of DeoxyHb under anaerobic conditions (Fig. 4), the purified HbO_2 solution (absorbance at 576 nm: 1.5) was mixed with 1/4 volume of the phosphate buffer (pH 7.0), and 1.50 ml of the mixture was mixed with 0.50 ml of water or 2.5 mM IHP solution. The whole was bubbled through with nitrogen gas, and then 0.50 ml of an oxygen-free 100 mM solution of a phenolic in water was added.

For the treatment of MetHb (Fig. 5), a MetHb solution in Tris-HCl buffer was diluted with phosphate buffer (pH 7.0) to make the absorbance at 630 nm equal to 0.2. To 2.0 ml of this solution, 0.50 ml of a 100 mM solution of a phenolic in water was added.

For estimation of the effect of HbO_2 concentration (Fig. 7), 0.30 ml of crude HbO_2 preparation in saline of various concentrations (absorbance at 576 nm: maximum 45) was mixed with 0.10 ml of phosphate buffer (pH 7.0) and 0.10 ml of a solution of a test compound in water. The mixtures were treated under the indicated conditions, then appropriately diluted with water for spectrophotometric assay.

Treatment of Red Cells with Phenolics——To 2.0 ml of diluted packed red cells in 1/2 volume of saline was added 0.50 ml of a solution of 10, 30 or 50 mM phenolic. The mixture (6.0 mM heme) was allowed to stand at 20° for 2 hr. Aliquots of 1.0 ml were removed and washed three times with 2 volumes of saline by centrifugation at 2000 rpm for 10 min. The sedimented red cells were hemolyzed and made up to 5.0 ml with water. Portions of 0.4 ml were mixed with 5.0 ml of water and the mixtures were centrifuged. The spectrum of the supernatant was recorded (Table I).

Infusion of Sesamol (I) and Pyrogallol (IV) into Rats——A solution of 100 mM sesamol (I) or pyrogallol (IV) in saline in an ampoule was deoxygenated by bubbling through nitrogen gas and sterilized in an autoclave at 120° for 20 min. One milliliter of the sterile solution of I or IV was infused into a male Wistar rat (about 200 g) *via* the cardiac vein. Blood (0.9 ml) was collected into 0.1 ml of 3.8% sodium citrate from the vein after 1 hr of infusion. The red cells were washed and collected in the usual manner and hemolyzed by addition of 50 ml of water. The spectrum of the supernatant of the hemolyzate was recorded.

Results

Oxyhemoglobin (HbO_2) solution (52 μM) was treated with a large excess of a phenolic (I, II, III or IV) at pH 7 and 25° under aerobic conditions. The progress of the reaction was followed spectrophotometrically. The reactions with sesamol (I), pyrocatechol (II) and *p*-hydroquinone (III) progressed similarly, with decreases in absorbance at 540 nm and 575 nm and increases in absorbance at 630 nm due to methemoglobin (MetHb). Excellent isosbestic points at 523 and 587 nm were observed in the treatments with I and III, whereas there was no clear isosbestic point at the shorter wavelength in the treatment with II, probably owing to the coloration of the phenolic. The profiles of the spectrophotometric changes after 30 min are illustrated in Fig. 1A. The time course of the increase in absorbance at 630 nm during the period up to 30 min (Fig. 1C) indicated that the rate of MetHb formation by I was greater than those by II and III. Prolonged treatments for up to several hours with these phenolics caused precipitates which disturbed the spectrophotometric estimation. The formation of MetHb was 70% (I), 48% (II) or 42% (III) after treatment at 25° for 30 min. The formations of

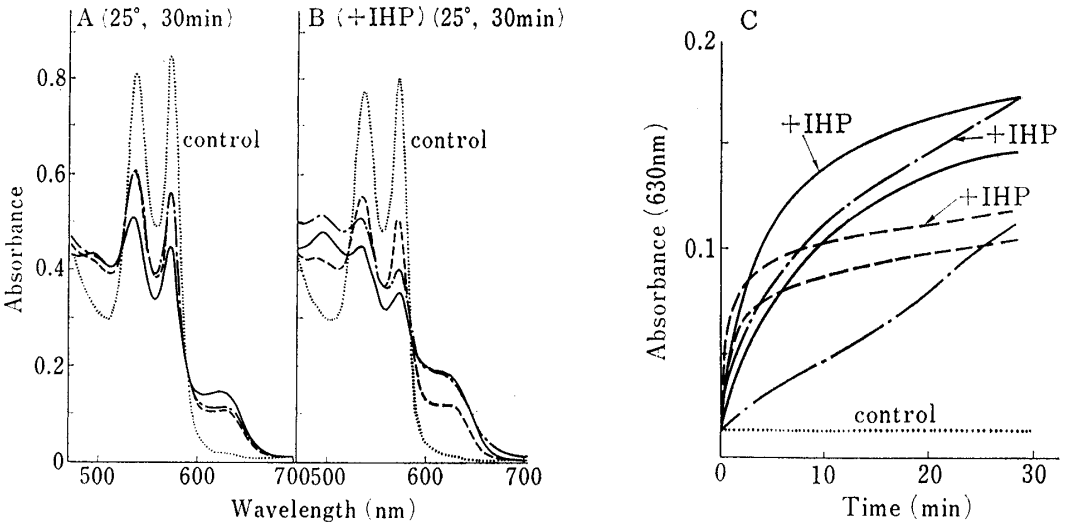


Fig. 1. Transformation of HbO_2 into MetHb by Sesamol (I) (——), Pyrocatechol (II) (— · —) and *p*-Hydroquinone (III) (----)

HbO_2 (52 μM) was treated with 14.3 mM phenolic in the presence or absence of 360 μM IHP at pH 7.0 and 25°.

MetHb by I, II and III were enhanced by inositol hexaphosphate (IHP), an allosteric effector of HbO_2[9,10] (Fig. 1A, B and C). The formation of MetHb by I was little influenced by catalase and/or superoxide dismutase under the conditions used. In the reaction of HbO_2 with pyrogallol (IV), not shown in the figure, the decreases in absorbances at 540 and 575 nm and the increase in absorbance at 630 nm progressed similarly, but the profiles of the spectral changes did not show isosbestic points.

HbO_2 was treated with I, II or III at different pH values (pH 6 and 8) (Fig. 2). HbO_2 did not undergo autoxidation[7] under the test conditions (control experiments in the figure). Sesamol (I) transformed HbO_2 into MetHb at both pH values with the same spectral changes, the rate being much faster at pH 6 than at pH 8. Pyrocatechol (II) and *p*-hydroquinone (III) also transformed HbO_2 into MetHb at these pH values but in contrast to I, the rate of conversion was faster at pH 8 than at pH 6. Thus, the pH-dependence of transformation of HbO_2 into MetHb by I was characteristic and opposite to those of the diphenolics (II and III).

Since the profiles of the spectral changes of HbO_2 treated with IV were not identical with those with other phenolics, HbO_2 was treated with IV at three different pH values under an atmosphere of nitrogen (Fig. 3). The spectral changes at pH 7 indicated that the major

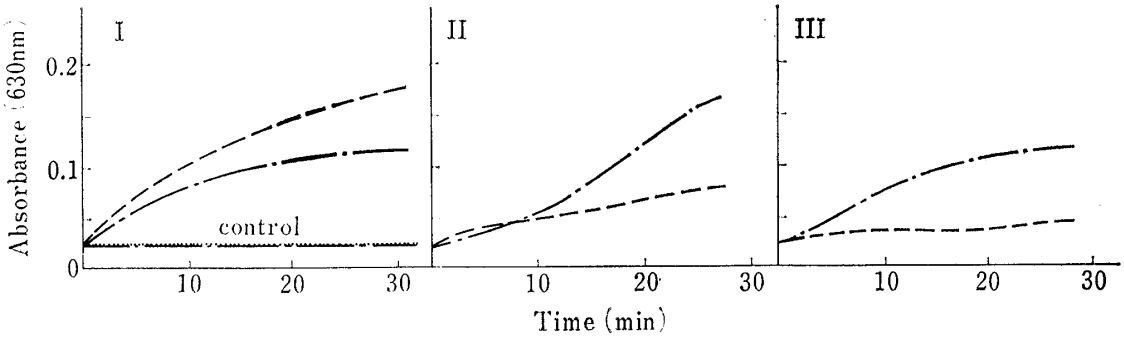


Fig. 2. Time Course of the Increase of Absorbance at 630 nm in the Reaction of HbO_2 with Sesamol (I), Pyrocatechol (II) or *p*-Hydroquinone (III) under Different pH Conditions

HbO_2 (45 μM) was treated with 13.9 mM phenolic at 22° and at pH 6.0 (----) or pH 8.0 (— · —).

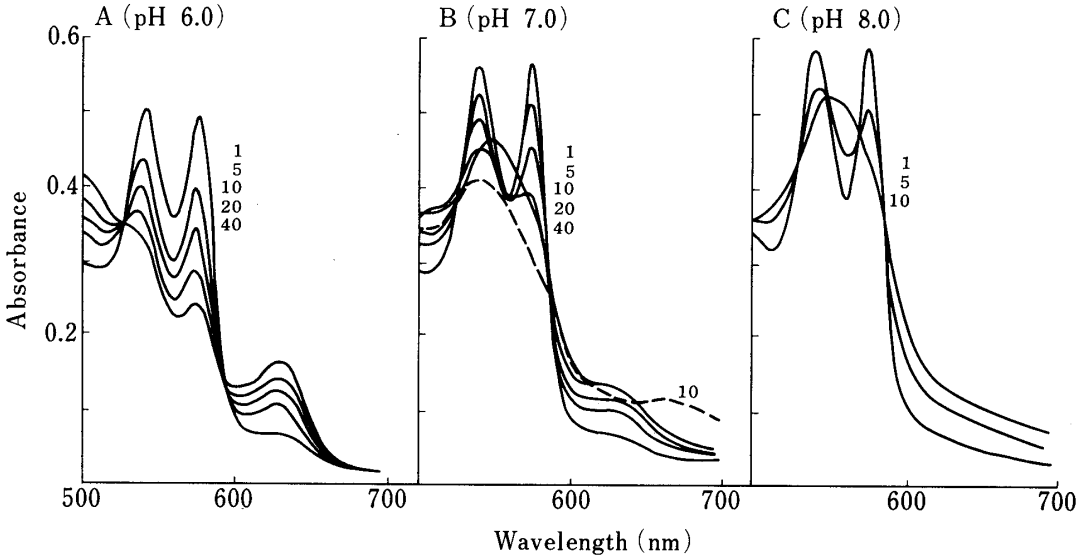


Fig. 3. Transformation of HbO_2 by Pyrogallol (IV) at pH 6, 7 and 8 under an Atmosphere of Nitrogen

HbO_2 (35 μM) was treated with 13.9 mM IV at 20°. The numerals in the figure indicate the treatment time (min.) The dotted line indicates treatment in the presence of 1.4 mM KCN.

reaction was deoxygenation (Fig. 3B). Thus, the spectrum of HbO_2 showed decreases in the absorbances at 540 and 575 nm and increases in the absorbances at around 560 nm and 630 nm, and reached the spectrum of deoxyhemoglobin (DeoxyHb), exhibiting a maximum at 555 nm, after 40 min with concomitant formation of MetHb. The deoxygenation of HbO_2 by IV was more apparent when HbO_2 was treated with IV at pH 8 (Fig. 3C), and complete conversion was accomplished within 10 min. On the other hand, HbO_2 was rapidly converted into MetHb at pH 6 with excellent isosbestic points (Fig. 3A). Thus, in the reaction with IV, deoxygenation prevailed in the alkaline range, while MetHb formation prevailed in the acidic range. Treatment of HbO_2 with IV at pH 7 in the presence of potassium cyanide (cyanide trapping method)[1] rapidly yielded an absorption maximum at 540 nm, characteristic of cyanomethemoglobin (Fig. 3B), which indicated that MetHb was rapidly produced before the formation of DeoxyHb. Treatment of HbO_2 with IV under aerobic conditions resulted in complicated profiles of spectral changes, probably owing to MetHb formation and subsequent reduction and oxygenation by atmospheric oxygen.

DeoxyHb, prepared by deoxygenation of HbO_2 with nitrogen gas, was treated with a large excess of the phenolics (I, II, III and IV) at pH 7 and 25° under anaerobic conditions (Fig. 4). The rate of transformation of DeoxyHb into MetHb was greatest with I and much less with II or III; in all cases there was a decrease in abs orbance at 555 nm and an increase in absorbance at 630 nm with excellent isosbestic points at 523 and 595 nm. Pyrogallol (IV) was found to be quite inert as regards the transformation of DeoxyHb under these conditions. Transformation by I was markedly

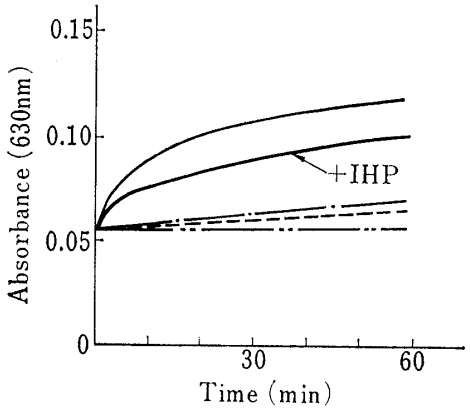


Fig. 4. Transformation of DeoxyHb into MetHb by Sesamol (I) (——), Pyrocatechol (II) (— — —), *p*-Hydroquinone (III) (----) and Pyrogallol (IV) (— · — ·)

DeoxyHb (45 μM) was treated under anaerobic conditions with 20 mM phenolic in the presence or absence of 500 μM IHP at pH 7.0 and 25°.

inhibited by IHP; this effect of IHP was opposite to that observed in the treatment of HbO_2 with I.

MetHb was treated with a large excess of the phenolics (I, II, III and IV) at pH 7 and 25° under aerobic conditions (Fig. 5). While treatment with I for 30 min caused little spectral change, treatments with II, III and IV resulted in marked changes. Treatment of MetHb with *p*-hydroquinone (III) rapidly produced HbO_2 with excellent isosbestic points, the absorbance at 630 nm decreasing and the absorbance at 575 nm increasing with time (Fig. 5B). Pyrocatechol (II) and pyrogallol (IV) also produced HbO_2 but there were no isosbestic points in these cases, probably because the oxidation products of both reductants produced color which influenced the spectral estimation (Fig. 5A). The monophenolic antioxidant, sesamol (I), was found to be almost inert as regards the reduction of MetHb.

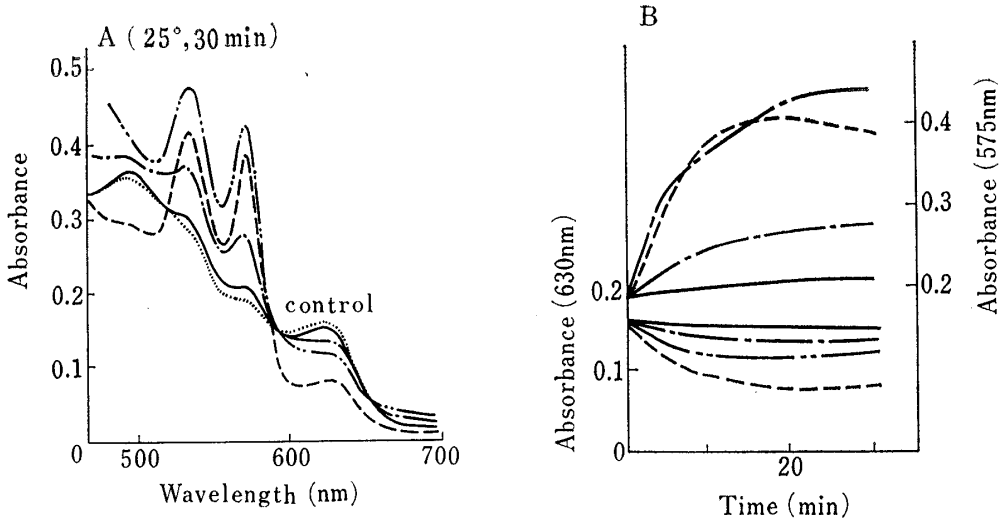


Fig. 5. Transformation of MetHb by Sesamol (I) (——), Pyrocatechol (II) (— — —), *p*-Hydroquinone (III) (----) and Pyrogallol (IV) (— — — —)

MetHb (40 μM) was treated with 20 mM phenolic at pH 7.0 and 25°.

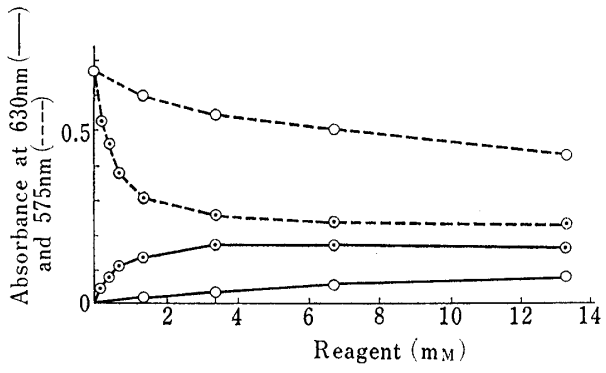


Fig. 6. Dependence of MetHb Formation on the Concentrations of Sesamol (I) and Hydroxylamine

HbO_2 (40 μM) was treated with I (○) or hydroxylamine (⊙) at pH 7.0 and 25° for 10 min.

Profiles of the spectral change of HbO_2 induced by I were similar to those by nitrite[11] and hydroxylamine,[12] but the rate of MetHb formation was slower than that with hydroxylamine. The initial velocities of MetHb formation and HbO_2 decrease caused by I were compared with those caused by hydroxylamine at several reagent concentrations (Fig. 6). Complete conversion of HbO_2 into MetHb was observed when HbO_2 was treated with a 100-fold molar excess of hydroxylamine for 10 min, whereas MetHb formation did not reach maximum even when HbO_2 was treated with a 300-fold molar excess of I. The addition of a large excess of I caused spontaneous precipitation.

Various amounts of HbO_2 were treated at pH 7 with 0.10 mM phenolic (I, II or III) for 5.5 hours (Fig. 7A). The concentration of MetHb formed by I was proportional to the concentration of HbO_2, and I converted 85% of HbO_2 (up to 1.6 mM) into MetHb, indicating that I

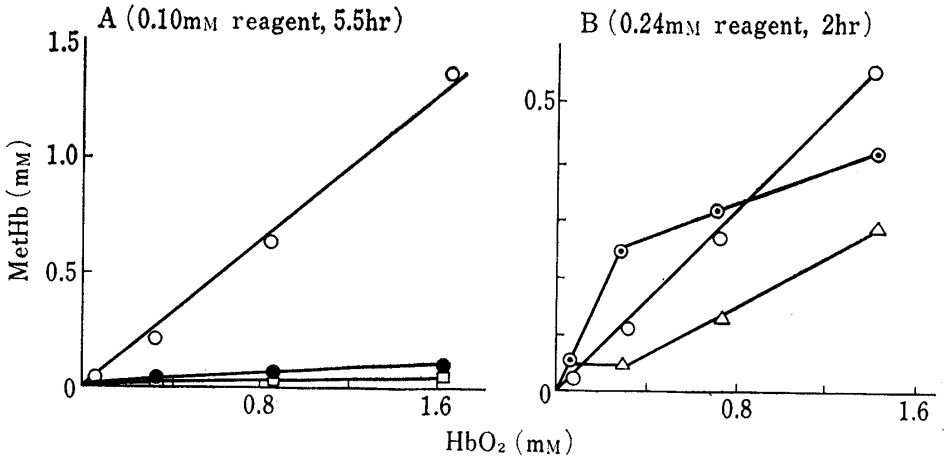


Fig. 7. Dependence of HbO_2 Concentration on MetHb Formation induced by Sesamol (I) (○), Pyrocatechol (II) (●), *p*-Hydroquinone (III) (□), Hydroxylamine (⊙) and Sodium Nitrite (△).

HbO_2 was treated with each reagent at pH 7.0 and 25°.

produces more than ten molar equivalents of MetHb. On the other hand, MetHb produced by II and III did not exceed the concentrations of the reagents under the conditions used. When HbO_2 (up to 1.8 mM) was treated with 0.24 mM I, hydroxylamine or nitrite for 2 hours (Fig. 7B), I showed a constant conversion of 30% of HbO_2 into MetHb over the wohle concentration range of HbO_2, while the MetHb formation by hydroxylamine and nitrite was not proportional to the concentration of HbO_2 but appeared to be higher at low concentrations of HbO_2. The results showed that the MetHb-forming activity of I was proportional to the concentration of HbO_2, and it was found that a large amount of MetHb was formed when HbO_2 was present at a high concentration.

Packed red cells were treated with 2, 6 and 10 mM phenolic (I, II, III or IV) in saline. Transformation of HbO_2 in red cells were estimated by measuring the ratio of the absorbance at 630 nm due to MetHb to that at 587 nm, an isosbestic wavelength of the transformation, after lysis of the cells by water (Table I). Sesamol (I) was the most potent among the phenolics for MetHb formation. Although IV produced little MetHb from HbO_2 at pH 7, it produced MetHb at a relatively high rate when red cells were treated with it. The phenolics such as II and III also induced MetHb formation, but much less effectively.

TABLE I. Formation of MetHb during the Treatment of Red Cells (6 mM Heme) with Phenolics at 20° for 2 Hours

Concentration of phenolic		$A_{630\ nm}/A^{a)}_{587\ nm}$		
		2 mM	6 mM	10 mM
Phenolic	I	0.68	0.83	0.86
	II	0	0.09	0.16
	III	0	0	0.06
	IV	0.38	0.56	0.55

a) An isosbestic point in the conversion of HbO_2 into MetHb.

Sesamol (I) and pyrogallol (IV) (0.1 mmol) were infused into a rat *via* the cardiac vein and the blood was collected affer 1 hour of infusion. Hemoglobin from the red cells was found to be little altered after the red cells had been washed and lyzed. Thus, I and IV did not effectively convert HbO_2 into MetHb *in vivo*.

Discussion

The interactions of sesamol (I), a monophenolic antioxidant present in sesame oil,[13,14] diphenolics (II and III) and a triphenolic (IV) with hemoglobin A were investigated. At pH 7, the diphenolics (II and III) effectively produced MetHb from HbO_2 and reduced MetHb into DeoxyHb, which was subsequently oxygenated under aerobic conditions. Pyrogallol (IV) produced MetHb rapidly as an intermediate, which was in turn rapidly reduced into DeoxyHb at pH 7. The action mechanisms of these di- and triphenolics on hemoglobin can be explained by the following general sequences as noted by Wallace and Caughey[3] and Castro *et al.*[4] HbO_2 might exist in its transition state as a superoxoferrihemoglobin complex.[15] One-electron transfer from each of these di- and triphenolics to the bound superoxide ion would produce MetHb, hydrogen peroxide and phenolic radical (equation 1). MetHb thus formed may subsequently be attacked by one electron of the phenolic or the radical to be reduced into DeoxyHb (equation 2) which is then reoxygenated (equation 3). Pyrogallol (IV) appeared to be the most powerful reductant in this sequence of reactions. The inverse hydrogen ion concentration dependence observed with each of II, III and IV indicates that the active phenolic species may be the phenolate ions; this view is consistent with the observation that the phenolics undergo air oxidation more readily in the alkaline range. IHP, an allosteric effector of HbO_2, which converts the R state of conformation into the T state of conformation,[9,10] accelerated the MetHb formation induced by the diphenolic reductants, indicating that the reactions occurred preferentially in the T state rather than in the R state. This enhancement is identical to those in the ferricyanide- and hydroxylamine-induced MetHb formations[16,17] and opposite to that in the nitrite-induced one.[16]

$$\{[Fe(II)\cdot O_2] \rightleftharpoons [Fe(III)\cdot O_2^-]\} + RH_2 \longrightarrow [Fe(III)] + OOH^- + \cdot RH \quad (1)$$

$$[Fe(III)] + RH_2 \text{ (or } \cdot RH) \longrightarrow [Fe(II)] + \cdot RH \text{ (or R)} \quad (2)$$

$$[Fe(II)] + O_2] \longrightarrow [Fe(II)\cdot O_2] \quad (3)$$

Chart 2

The monophenolic antioxidant, sesamol (I), is a weaker reductant than the di- and triphenolics. The action profiles of I on hemoglobin were different from those of the di- and triphenolics. It more readily produced MetHb from HbO_2 or even from DeoxyHb, but did not reduce MetHb at pH 7. MetHb formation from HbO_2 by I was more prominent in the acidic range than in the alkaline range, in contrast to the case of the di- and triphenolics. MetHb formation from HbO_2 by I was characteristically proportional to the concentration of HbO_2 and thus I produced more than ten equivalents of MetHb at high concentrations of HbO_2. These observations could not be explained by the general reaction sequences illustrated above for the stronger reductants.

In the interaction of I with DeoxyHb, I acted as if it were an oxidant. The action could not be explained without considering the globin moieties of hemoglobin, since the monophenolic with hydrogen- or electron-donating nature could not directly oxidize [Fe(II)] into [Fe(III)] in the absence of oxygen. Interaction of I with the globin moieties might induce intramolecular electron transfer in the DeoxyHb molecule to form MetHb.

The catalytic ability of I to produce a large amount of MetHb from HbO_2 depending upon the amount of HbO_2 might be explained by the above sequence of reactions (equation 1), in which hydrogen peroxide is generated. Hydrogen peroxide is able to transform HbO_2 into MetHb,[18] and thus a larger amount of MetHb may be produced. However, the addition of catalase and/or superoxide dismutase did not reduce the rate of formation of MetHb induced by I. Furthermore, the effects of I were enhanced by increase of the hydrogen concentration, which eliminated the possibility that the electron donating abilities of I contributed to the transformation, and suggested that the promoting effects of hydrogen ion concentration

involved the globin moieties of hemoglobin. Thus, it is difficult to assume the catalytic action of I on HbO_2 to be analogous to those of the di- and triphenolic reductants, and its action mechanisms remains to be clarified.

The effects of IHP on the transformation of HbO_2 and DeoxyHb by I were quite different. Thus, MetHb formation from HbO_2 was enhanced and that from DeoxyHb was inhibited by IHP. This may be because the action mechanism of I on HbO_2 is different from that on DeoxyHb or because this allosteric effector has different effects on the conformations of HbO_2 and DeoxyHb.

It is of interest with regard to the action mechanisms of I to determine how the monophenolic antioxidant (I) is derivatized in the interaction with hemoglobin. A preliminary experiment revealed that a fluorescent dimeric product, 2,2′-dihydroxy-4,5 and 4′,5′-bis-(methylenedioxy)biphenyl, was produced during the interaction. The details will be presented in a separate paper.

The MetHb-forming potencies of the phenolics were in the order I>IV>II>III when red cells were treated *in vitro*. *In vivo* studies with rats showed no apparent MetHb formation when I and IV were infused at a single dose of 50—60 mg/kg.

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