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Transformation of Hemoglobin by Various Peroxides *in Vitro*

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Hydrogen peroxide converted HbO_2 and DeoxyHb into MetHb, and MetHb into the complex [MetHbOOH], which readily regenerated MetHb. *tert*-Butyl hydroperoxide converted HbO_2 and DeoxyHb into MetHb, and MetHb into a complex which was rather stable. The formation of MetHb was mainly due to the direct action of each of these peroxides, and was influenced by the allosteric effector, IHP. Ascorbic acid affected the hydrogen peroxide-induced MetHb formation, probably due to hydroxyl radical produced during the interaction of ascorbic acid with hydrogen peroxide. HbO_2 and DeoxyHb treated with linoleic acid hydroperoxide at pH 7—8 produced MetHb in the presence of albumin or KCN. Complex formation between MetHb and linoleic acid hydroperoxide occurred at pH 8, and it was prevented by albumin and KCN. The reaction of MetHb with the hydroperoxide at pH 7 produced precipitates, and that at pH 7.4 yielded the complex and then precipitates. It was suggested that the interaction of MetHb with hydroperoxide first produced the complex, which regenerated MetHb with the production of some active species for cross-linking of hemoglobin tetramer upon treatment at a lower pH value or in the presence of albumin.

Keywords——hemoglobin; oxyhemoglobin; methemoglobin; deoxyhemoglobin; myoglobin; hydrogen peroxide; *tert*-butyl hydroperoxide; linoleic acid hydroperoxide; inositol hexaphosphate; ascorbic acid

Under physiological conditions, oxygen is taken up into red cells for binding to hemoglobin, and the transport of oxygen through the red cell membrane may cause peroxidation of unsaturated fatty acid residues of the membrane lipid under certain conditions to produce lipid hydroperoxides. Hydrogen peroxide at up to 0.1 μM concentration may also be present in the cells as a consequence of the intracellular metabolism of the red cells. These physiologically important peroxides may damage intracellular and membrane constituents.[1,2] It is interesting to note that hydrogen peroxide is converted into some active species of oxygen by interaction with hemoglobins *in vitro*,[2] and is able to oxidize organic amines such as benzidine and phenolic compounds contained in gum guaiac to form the characteristic color;[3,4] the reactions have been applied to the detection of blood in the fields of clinical chemistry and forensic science. These *in vitro* reactions suggest that the interaction of peroxides with hemoglobins may produce other molecules with oxidative activity *in vivo*. However, the mechanisms of the reactions of peroxides and hemoglobins still remain obscure with regard to the conversion of hemoglobins and to the generation of other species with oxidative activity.

Some earlier studies demonstrated that hydrogen peroxide and ethyl hydroperoxide convert oxyhemoglobin into methemoglobin, convert methemoglobin into complexes or destroy the heme nucleus.[5-7] We investigated the reactions of hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (BHPO) and linoleic acid hydroperoxide (LAHPO) with hemoglobins under mild conditions, and we report here the transformation of hemoglobins by these peroxides.

Experimental

Commercial reagent-grade hydrogen peroxide (31% H_2O_2) was estimated to be 9.73 M by iodometric titration. *tert*-Butyl hydroperoxide (BHPO), 70%, 7.6 M (Nakarai Chemicals, Ltd.), was adjusted for use as follows: 0.1 ml of stock solution was mixed with 5.6 ml of dimethylsulfoxide and made up to 20 ml with

water. Myoglobin (type I, equine skeletal muscle), inositol hexaphosphate (IHP) (phytic acid from corn, type IV, sodium salt), catalase (bovine liver, 2000 U/mg solid) and superoxide dismutase (bovine blood, type 1, 2900 U/mg solid) were obtained from Sigma Chemical Company. Bovine serum albumin was a product of Tokyo Kasei Kogyo Company, Ltd.

Human normal oxyhemoglobin (HbO_2) was obtained from the hemolyzate of washed red cells from normal blood and partially purified by passage through a Sephadex G-25 column. Methemoglobin (MetHb) was prepared by treatment with an approximately five-fold excess of potassium ferricyanide followed by passage through a Sephadex G-25 column. Oxymyoglobin (MbO_2) was prepared from a commercial preparation: 70 mg of myoglobin in 2.0 ml of water was treated with 10 mM ascorbic acid under anaerobic conditions at room temperature for 2 h and it was rapidly passed through a column of Sephadex G-25. Metmyoglobin (MetMb) was prepared by treatment of commercial myoglobin with an approximately 10-fold excess of potassium ferricyanide. Concentrations were determined by use of the following extinction coefficients: $\epsilon_{576}=16500$ for HbO_2, $\epsilon_{630}=4010$ (pH 7.0) for MetHb,[8] and $\epsilon_{410}=1.4\times10^5$ for MbO_2 and $\epsilon_{408}=1.8\times10^5$ for MetMb[9] on a heme basis.

Linoleic acid hydroperoxide (LAHPO) was prepared according to the method of Gardner.[10] Linoleic acid (P-L Biochemicals, Inc.), 1.0 g, was placed in a round-bottomed flask (50 ml) which was rotated under aerobic conditions in a rotary evaporator at 25—30°C for 60—70 h. About 0.5 g of the oxidized linoleic acid was purified through a column (3×14 cm) of silicic acid (Mallinkrodt) by the use of a developing solvent composed of *n*-hexane-ethyl ether. Each fraction was checked by thin-layer chromatography on silica gel plates with ethyl ether-petroleum ether-glacial acetic acid (8:7:0.5). The fractions giving a single spot, which showed ultraviolet (UV) absorption (233 nm), a positive reaction to 5% KI-10% glacial acetic acid spray, and a negative reaction to saturated 2,4-dinitrophenylhydrazine·HCl-10% sulfuric acid spray, were collected and dried. The yield of LAHPO was 0.1—0.18 g (9—17%). The peroxide value for the preparation was 3800—4900 meq/kg compared with the theoretical value of 6401 meq/kg. The extinction coefficient at 233 nm was 15000—18000 (lit.[10] 25250).

HbO_2, deoxyhemoglobin (DeoxyHb) and MetHb, at concentrations of 40—50 μM, were incubated in 0.1 M phosphate buffer (pH 7.0—8.0) in quartz cuvettes under the indicated conditions, and the absorption spectra were recorded with a Shimadzu UV-200S double beam spectrophotometer equipped with a thermostatic control system. DeoxyHb was prepared by deoxygenation of HbO_2 with nitrogen gas, and treated under anaerobic conditions by the use of cuvettes attached to Thunberg tubes.

Results

Transformation of Oxyhemoglobin by Hydrogen Peroxide and *tert*-Butyl Hydroperoxide

Oxyhemoglobin (HbO_2) was treated with hydrogen peroxide (H_2O_2) and *tert*-butyl hydroperoxide (BHPO) at pH 7.0 under various conditions, and the changes of absorption spectrum were followed.

When 40 μM HbO_2 was mixed with a large excess of H_2O_2 (2 mM) at 37°C, spontaneous evolution of gas was observed and the absorption spectrum of the mixture gradually altered:

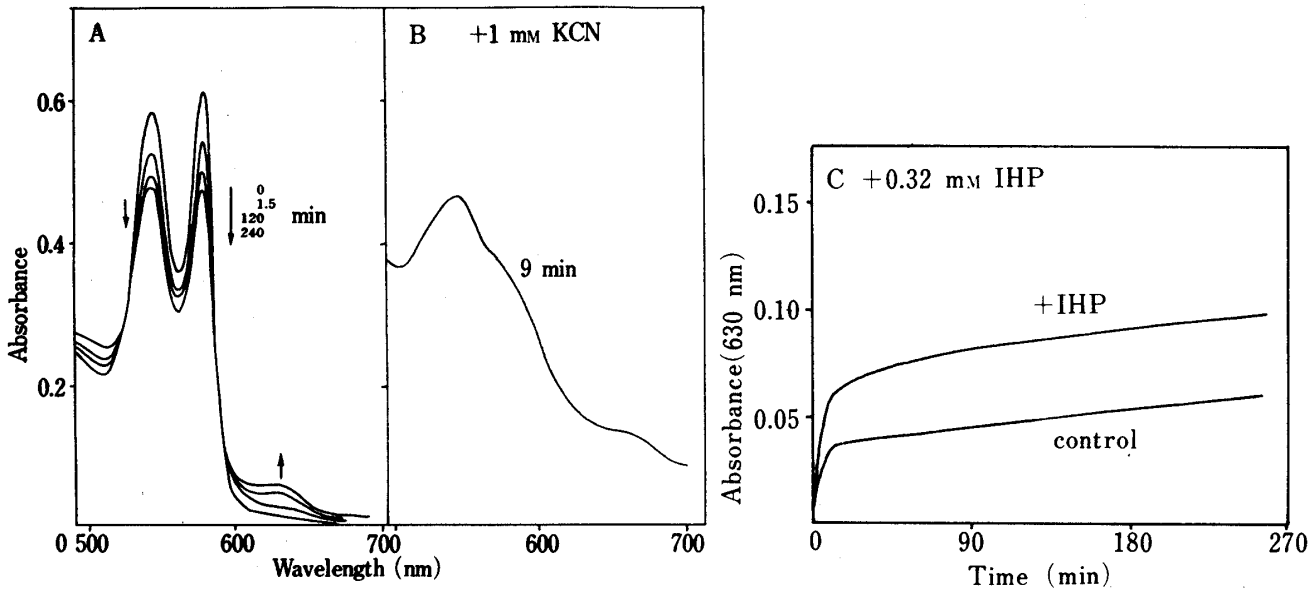

Fig. 1. Transformation of 40 μM HbO_2 by 2 mM H_2O_2 at pH 7.0 and 37°C

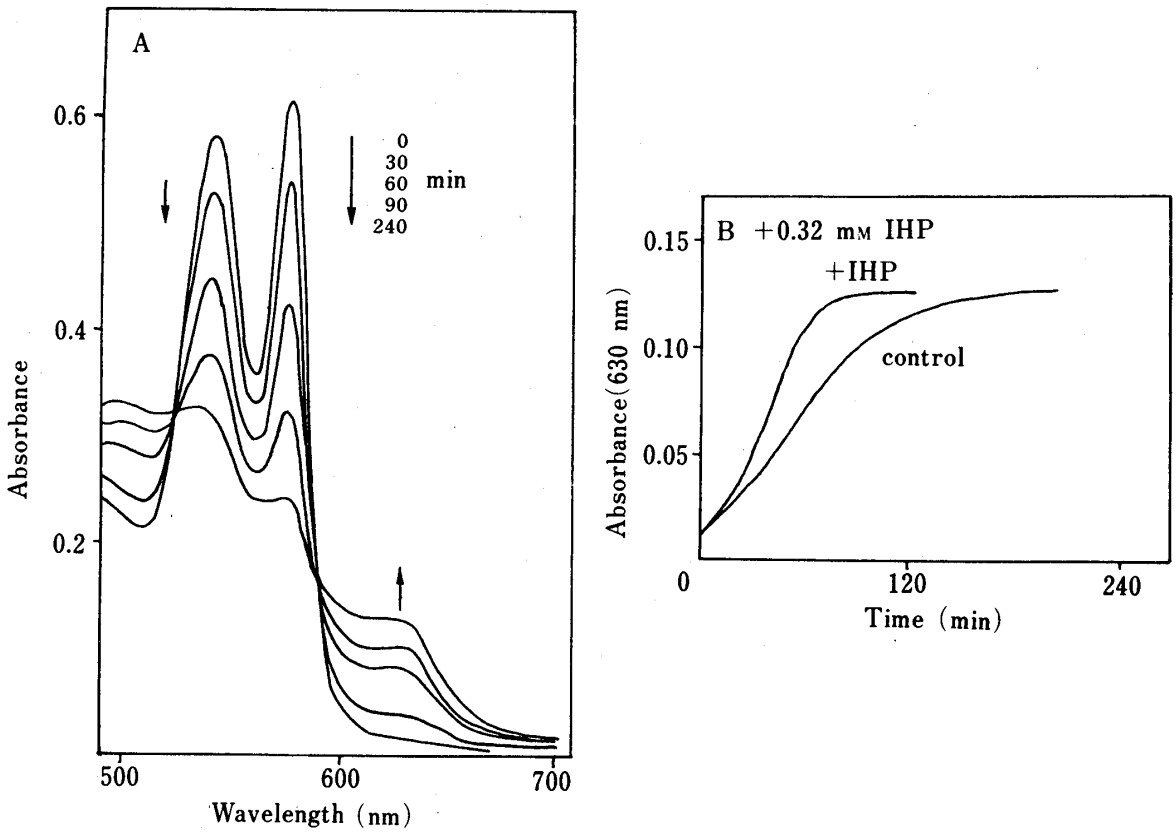

Fig. 2. Transformation of 40 μM HbO_2 by 0.1 mM BHPO at pH 7.0 and 30°C

the absorbance at 540 nm and 576 nm due to HbO_2 decreased, and the absorbance at 630 nm due to methemoglobin (MetHb) increased (Fig. 1A). The formation of MetHb was confirmed by the cyano-trapping technique.[11] The absorption spectrum of HbO_2 treated with H_2O_2 in the presence of 1 mM KCN showed that HbO_2 was rapidly converted into cyanomethemoglobin having the absorption maximum at 540 nm (Fig. 1B). Hence, the primary product of the reaction must be MetHb. The MetHb formation was greatly accelerated by addition of 0.32 mM inositol hexaphosphate (IHP), which converts the R state of HbO_2 into the T state,[12] indicating that the H_2O_2-induced MetHb formation occurred preferentially in the T state rather than in the R state (Fig. 1C).

When 40 μM HbO_2 was treated with 0.1 mM BHPO at 30°C, the time-dependent spectral changes indicated that HbO_2 was transformed into MetHb with clear isosbestic points at 523 and 591 nm (Fig. 2A). The formation of MetHb by BHPO was also stimulated by IHP (Fig. 2B).

It is well known that H_2O_2 produces superoxide anion (O_2^-), hydroxyl radical (·OH) and singlet oxygen ($^1O_2$) under various conditions.[13] The formation of MetHb by H_2O_2 was not prevented by addition of 180 U of superoxide dismutase,[14] 1 mM D-mannitol or 0.6% ethanol, scavengers of hydroxyl radicals,[15] or 1 mM L-methionine, a trapper of singlet oxygen.[16] Naturally, the formation of MetHb was largely prevented by 1200 U of catalase. These results indicated that the formation of MetHb was caused by the direct action of H_2O_2 and was hardly accelerated by superoxide anion,[17,18] singlet oxygen or hydroxyl radical, which might be produced during the interaction of HbO_2 and H_2O_2.[2] The MetHb formation by BHPO could not be prevented by catalase, superoxide dismutase, D-mannitol, ethanol or L-methionine, indicating that the reaction may be due to the direct action of BHPO on HbO_2.

The effect of ascorbic acid on H_2O_2- and BHPO-induced MetHb formation was investigated. When 0.2 mM ascorbic acid was added to the reaction mixture of HbO_2 and H_2O_2, MetHb

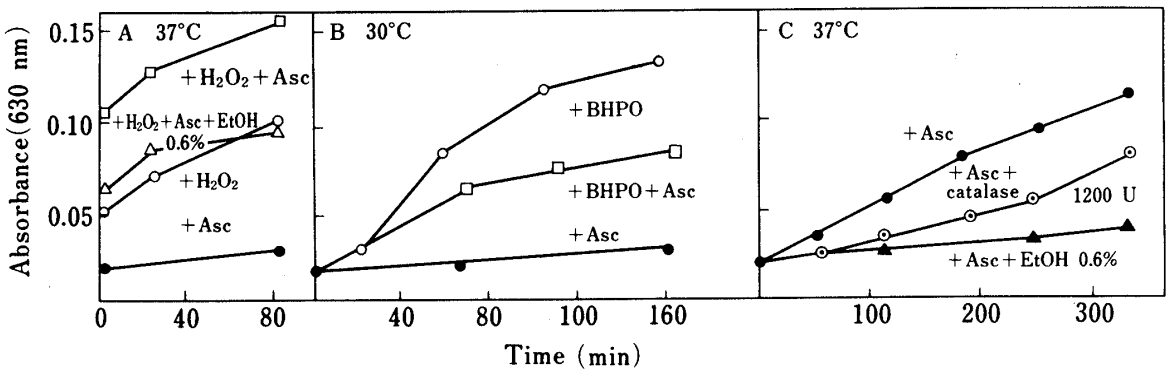

Fig. 3. Effect of 0.2 mM Ascorbic Acid on 2 mM H_2O_2-induced and 0.1 mM BHPO-induced MetHb Formation from 40 μM HbO_2 at pH 7.0

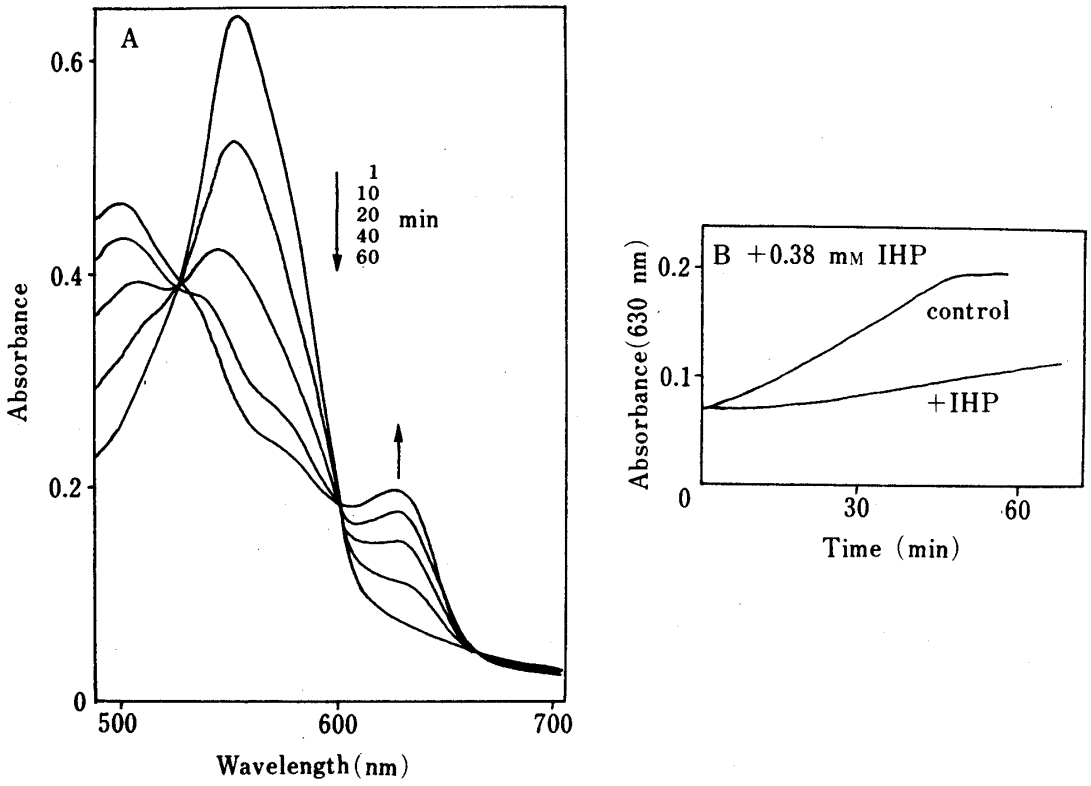

Fig. 4. Transformation of 48 μM DeoxyHb by 0.12 mM BHPO at pH 7.0 and 30°C

formation was greatly enhanced, and the enhancement was diminished by the addition of 0.6% ethanol (Fig. 3A). The production of hydroxyl radical from the reaction of ascorbic acid and H_2O_2[19] might affect MetHb formation. In contrast, MetHb formation by BHPO was retarded by ascorbic acid, probably because the activity of BHPO was decreased by ascorbic acid (Fig. 3B). Ascorbic acid is known to convert HbO_2 into MetHb,[20] and the MetHb formation was prevented by catalase and ethanol (Fig. 3C). Transformation of HbO_2 by ascorbic acid involved the formation of H_2O_2 and hydroxyl radical, which enhanced the transformation.

Transformation of Deoxyhemoglobin by Hydrogen Peroxide and *tert*-Butyl Hydroperoxide

Deoxyhemoglobin (DeoxyHb) was treated with H_2O_2 and BHPO at pH 7.0 under anaerobic conditions. Treatment of 48 μM DeoxyHb with 96 μM H_2O_2 at 37°C caused no apparent spectral changes, but treatment with 2.4 mM H_2O_2 caused gas evolution and spectral changes due to the formation of both HbO_2 and MetHb. When 48 μM DeoxyHb was treated with 0.12

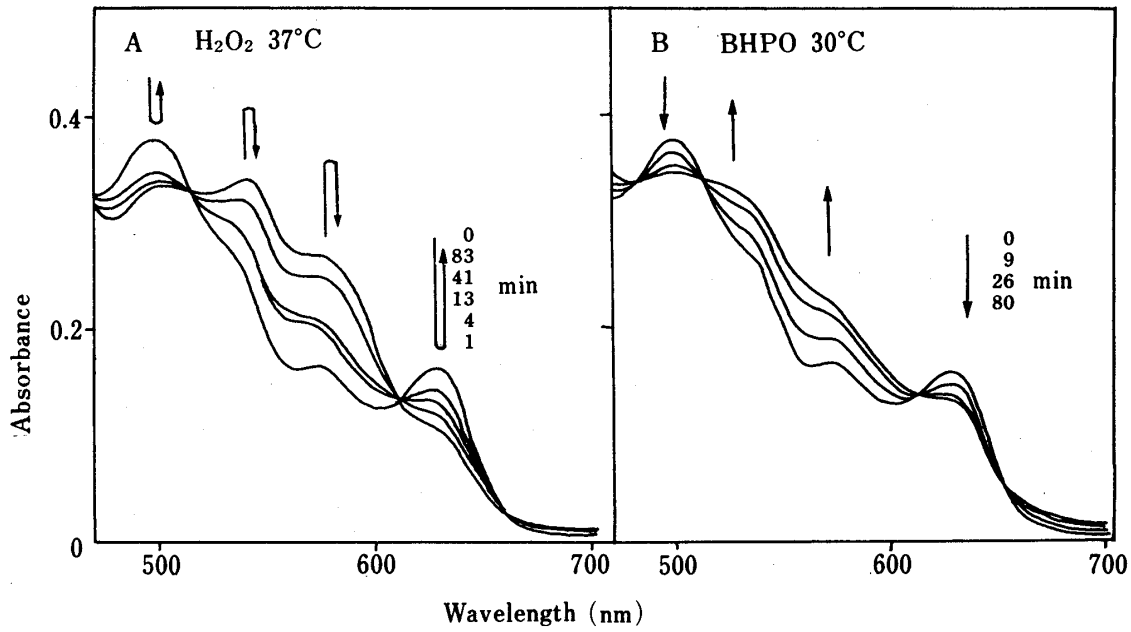

Fig. 5. Transformation of 40 μM MetHb by 2 mM H_2O_2 or 0.1 mM BHPO at pH 7.0

mM BHPO at 30°C, the absorbance at 555 nm due to DeoxyHb gradually decreased and that at 630 nm due to MetHb increased with clear isosbestic points at 524 and 600 nm (Fig. 4A). IHP prevented the production of MetHb (Fig. 4B), indicating that the R state of DeoxyHb favored the transformation rather than the T state. The MetHb formation by BHPO was not affected by catalase, superoxide dismutase or scavengers of hydroxyl radical and singlet oxygen.

Transformation of Methemoglobin by Hydrogen Peroxide and *tert*-Butyl Hydroperoxide

MetHb was treated with H_2O_2 and BHPO at pH 7.0 under various conditions. When 40 μM MetHb was treated with 2 mM H_2O_2 at 37°C, the solution rapidly turned from brown to red and the absorbances at 500 and 630 nm rapidly decreased then gradually increased. Rapid increase and subsequent decrease of the absorbances at 541 and 574 nm with clear isosbestic points at 520 and 611 nm were observed (Fig. 5A). It is obvious that intermediary unstable hemoglobin was rapidly produced (within one min) and gradually transformed. The spectrum of the unstable hemoglobin showed two absorption maxima at around 541 and 574 nm. Keilin and Hartree reported that MetHb treated with H_2O_2 produced a complex[5] [MetHbOOH] having two diffuse absorption maxima at 545 and 585 nm, and the complex was readily converted into HbO_2. The spectral changes within one minute in Fig. 5A could be due to the formation of this type of complex. The gradual changes of the spectrum may be due to the regeneration of MetHb and not to the conversion into HbO_2. Regeneration of MetHb was confirmed by the absorption spectrum of cyanomethemoglobin after addition of 1 mM KCN to the mixture of MetHb and H_2O_2 treated for 140 min. The spectral changes of MetHb treated with H_2O_2 were not affected by 90 μM bovine serum albumin.

When 40 μM MetHb was treated with 0.1 mM BHPO at pH 7.0 and 30°C, the absorbance at 630 nm gradually decreased and that at 535 and 574 nm gradually increased (Fig. 5B), producing a spectrum similar to that observed for the unstable hemoglobin produced by MetHb and H_2O_2 (Fig. 5A). The results suggest that MetHb was converted by BHPO to a complex [MetHbOO*tert*-Bu] which may be rather stable. The reaction was not affected by bovine serum albumin.

Transformation of Oxyhemoglobin by Linoleic Acid Hydroperoxide

HbO_2 was treated with linoleic acid hydroperoxide (LAHPO) at pH 7.0 or 8.0 under various conditions. When 40 μM HbO_2 was treated with 0.35 mM LAHPO at pH 7.0 and at

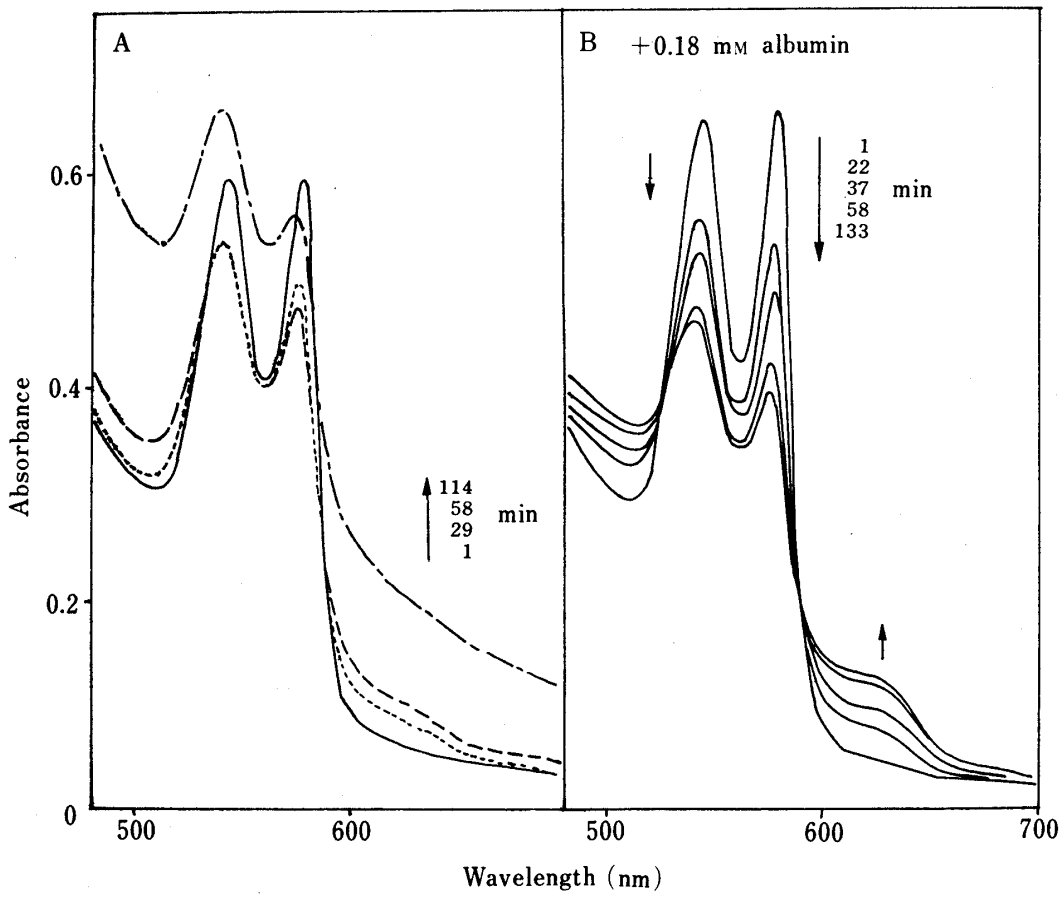

Fig. 6. Transformation of 40 μM HbO_2 by 0.35 mM LAHPO at 7.0 and 37°C

37°C, precipitates were formed instantaneously and the spectral changes were complex (Fig. 6A). When the reaction was performed in the presence of 0.18 mM bovine serum albumin, the mixture produced no precipitates and the spectrum of the mixture changed into that of MetHb with clear isosbestic points at 523 and 591 nm (Fig. 6B). Preincubation of LAHPO with albumin for up to 70 min did not affect the MetHb formation. When the reaction was performed in the presence of 1 mM KCN, the mixture produced no precipitates and showed the absorption spectrum of a mixture of HbO_2 and cyanomethemoglobin. These results indicate that LAHPO attacks the heme moiety of HbO_2 to produce MetHb, whose derivatization could be prevented by albumin or cyanide.

When 40 μM HbO_2 was treated with 0.8 mM LAHPO at pH 8.0 and at 37°C, the mixture produced no precipitates and the spectral changes indicated the formation of MetHb, which was confirmed by addition of KCN.

Transformation of MetHb by Linoleic Acid Hydroperoxide

MetHb was treated with LAHPO at pH 7.0 or 8.0 under various conditions. When MetHb was treated with 0.35 mM LAHPO at pH 7.0 and 37°C, precipitates were formed instantaneously. Addition of 0.18 mM albumin to the reaction mixture prevented the formation of precipitates and the spectral changes.

When 40 μM MetHb was treated with 0.35 mM LAHPO at pH 8.0 and 37°C, no precipitates were formed and the mixture showed the characteristic spectral changes (Fig. 7A) accompanied by the color change from brown to red. The characteristic absorption maximum appeared at 534 nm with a shoulder at around 560 nm, and the spectrum was not altered by addition of KCN. This spectral change may be due to the formation of a complex similar to those produced in the reaction of MetHb with H_2O_2 (Fig. 5A) or BHPO (Fig. 5B). The formation of the complex was markedly decreased by 30 or 60 μM bovine serum albumin (Fig.

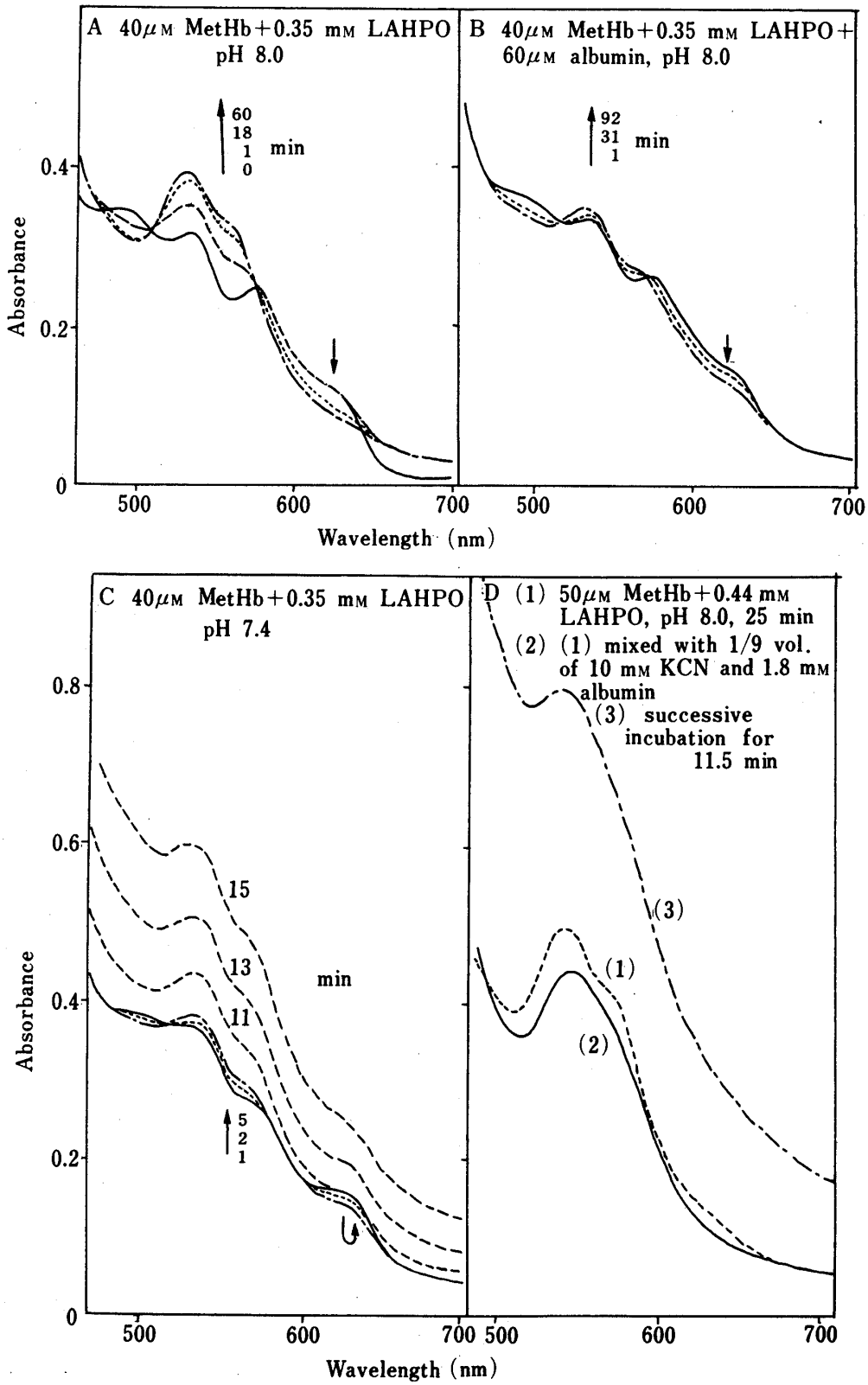

Fig. 7. Transformation of MetHb by LAHPO at 37°C

7B). When 40 μM MetHb was treated with 0.35 mM LAHPO at pH 7.4 and 37°C (Fig. 7C), complex formation was observed during the period of 1—5 min, but after 11 min gradual formation of precipitates occurred. The addition of albumin prevented the formation of both the complex and the precipitates. All these results indicate that LAHPO converted MetHb into the complex and then into precipitates, and albumin prevented the formation of the

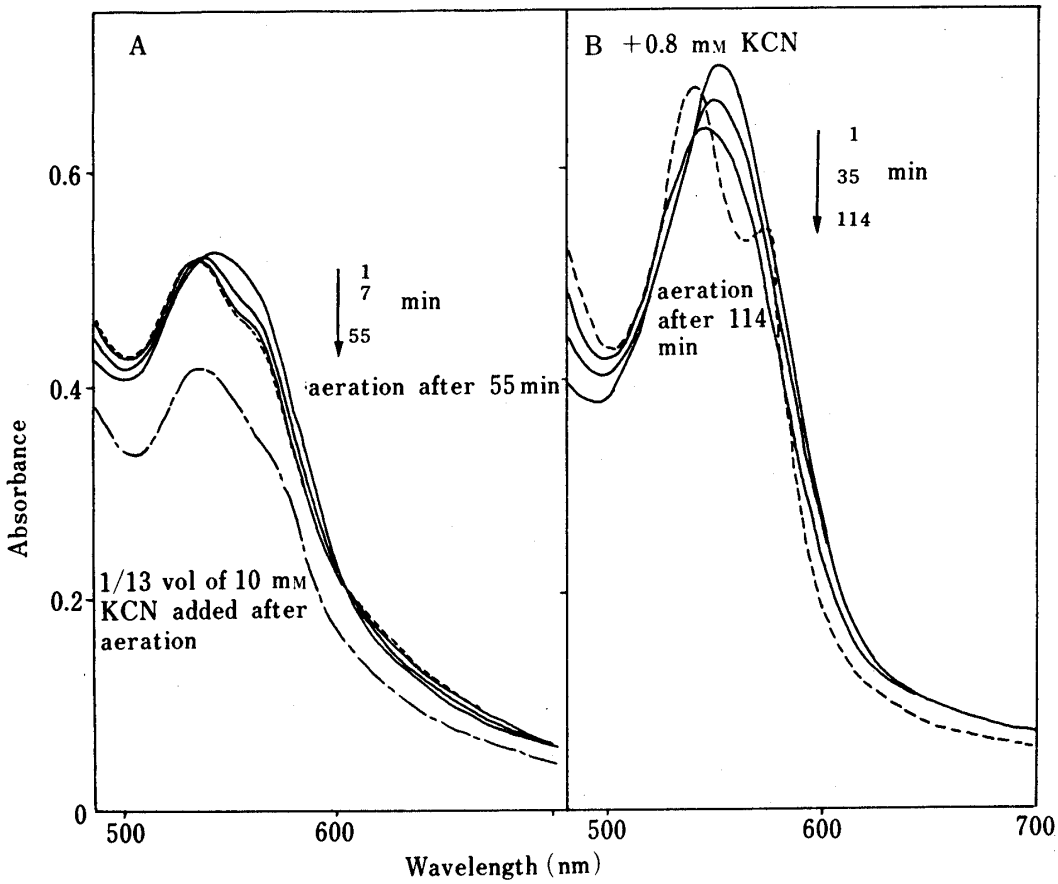

Fig. 8. Transformation of 48 μM DeoxyHb by 0.65 mM LAHPO at pH 8.0 and 37°C

complex and thus the precipitates.

It is interesting that once the complex was formed at pH 8.0, it was repidly changed into precipitates by addition of a large amount of albumin, but when albumin and KCN were added to the complex, the mixture showed a spectral change from the maximum at 534 nm due to the complex to that at 538 nm due to cyanomethemoglobin (Fig. 7D), which indicated that albumin regenerated MetHb. Prolonged incubation of the mixture gradually produced precipitates, which means that apoprotein denaturating factors may be generated by the conversion of the complex into MetHb.

Transformation of Deoxyhemoglobin by Linoleic Acid Hydroperoxide

DeoxyHb was treated with LAHPO under anaerobic conditions. When 48 μM DeoxyHb was treated with 0.4 mM LAHPO at pH 7.0 and 37°C, precipitates were formed, but the product was transformed into MetHb in the presence of 0.14 mM albumin. The reaction of DeoxyHb with LAHPO at pH 7.0 might progress through the formation of MetHb and then precipitates, and albumin inhibited the transformation of MetHb.

When 48 μM DeoxyHb was treated with 0.65 mM LAHPO at pH 8.0 and 37°C, the spectrum after 55 min showed the maximum at 532 nm, which did not change on aeration or the addition of KCN (Fig. 8A). The spectrum was very similar to that obtained by reaction of MetHb with LAHPO (Fig. 7A). When DeoxyHb was treated with LAHPO in the presence of KCN, the spectrum gradually changed into that of cyanomethemoglobin, and after aeration the spectrum was that of a mixture of HbO_2 and cyanomethemoglobin (Fig. 8B). These results may indicate the transformation of DeoxyHb at pH 8.0 to afford the complex *via* MetHb.

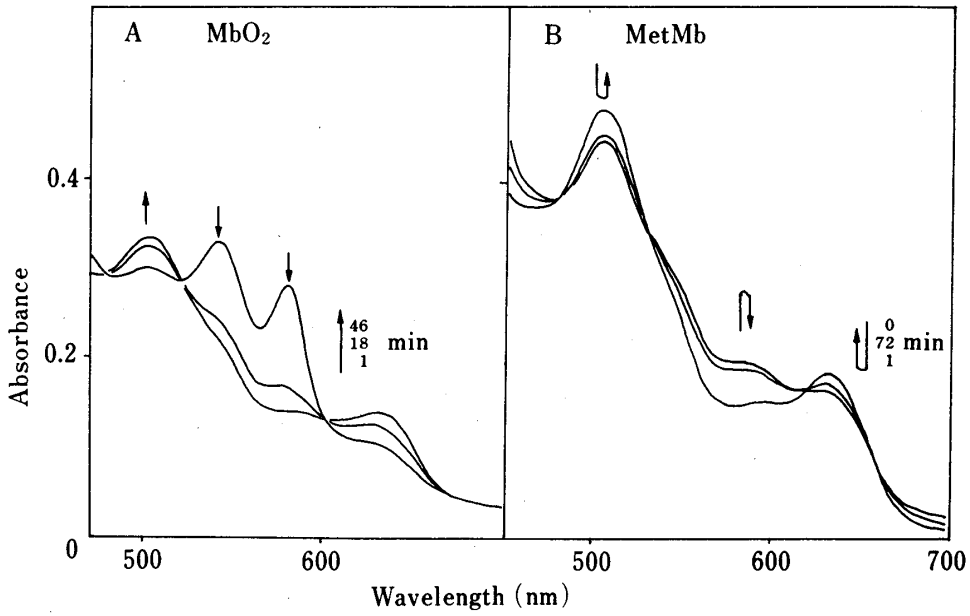

Fig. 9. Transformation of 30 μM MbO_2 and 44 μM MetMb by 0.35 mM LAHPO at pH 7.0 and 37°C

Transformation of Myoglobin by LAHPO

Transformation of oxymyoglobin (MbO_2) with LAHPO at pH 7.0 and 37°C produced metmyoglobin (MetMb), with isosbestic points at 521 and 600 nm (Fig. 9A). Treatment of MetMb with LAHPO at pH 7.0 and 37°C produced a spectrum corresponding to that of the complex which regenerated MetMb (Fig. 9B). No precipitates were formed, in contrast to the case on treatment of hemoglobin.

Discussion

In order to elucidate the transformation of hemoglobins by several kinds of peroxides, systematic investigations were performed. These transformations may be relevant not only to the production of the species with oxidative activity towards organic amines and phenolics,[3,4] but also to the possible *in vivo* interactions of hemoglobin and peroxides in red cells when peroxides are produced.

The transformation of hemoglobins with H_2O_2 and BHPO at pH 7.0 may be postulated to occur as shown in Chart 1. H_2O_2 converted HbO_2 and DeoxyHb into MetHb, and MetHb into a complex which readily regenerated MetHb. The action of BHPO was somewhat different; it converted HbO_2 and DeoxyHb into MetHb, and MetHb into a complex which was rather stable. The MetHb formation occurred irrespective of the presence or absence of oxygen liganded to the ferrous state of hemoglobin.

Ascorbic acid affected H_2O_2-induced MetHb formation from HbO_2. The acid enhanced the MetHb formation, which was greatly suppressed by a hydroxyl radical scavenger, ethanol. Ascorbic acid by itself produces MetHb from HbO_2,[20] and the MetHb formation induced by the acid was prevented by catalase and ethanol. Since it has been demonstrated that the

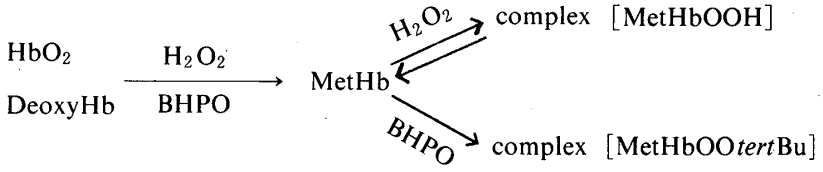

Chart 1

reaction of H_2O_2 with ascorbic acid produces hydroxyl radical,[19] the MetHb formation from HbO_2 and H_2O_2–ascorbic acid may be depicted as follows:

$$HbO_2 \xrightarrow{\text{ascorbic acid}} \text{Met Hb} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{ascorbic acid}} {}^{\bullet}OH$$

$$HbO_2 + H_2O_2 \longrightarrow \text{Met Hb}$$

$$HbO_2 + {}^{\bullet}OH \longrightarrow \text{Met Hb}$$

This scheme can account well for MetHb formation by ascorbic acid. MetHb formation by H_2O_2 was caused mainly by the direct action of the peroxide, and was scarcely inhibited by scavengers of superoxide anion, hydroxyl redical and singlet oxygen,[14-16] indicating that these active oxygens are not involved in the hemoglobin conversion even if they are produced.

While MetHb formation from HbO_2 by H_2O_2 and BHPO was stimulated in the presence of IHP, MetHb formation from DeoxyHb by BHPO was retarded in the presence of IHP. The conformational changes of the hemoglobin subunits affected the MetHb formation. There have been several reports on the effect of the phosphate on the redox reaction of hemoglobin.[21-24] The enhancing effect of IHP on H_2O_2- and BHPO-induced MetHb formation from HbO_2 was identical to those in ferricyanide-,[21] hydroxylamine-,[22] and sesamol-[23,24] induced MetHb formation, but different from that in the nitrite-induced reaction.[21] The inhibitory effect of IHP on MetHb formation from DeoxyHb was similar to that in the reactions of hydroxylamine[22] and sesamol.[23]

The reaction of MetHb with H_2O_2 resulted in the formation of the complex [MetHbOOH] reported by earlier researchers,[5-7] and the complex regenerated MetHb. BHPO seemed to give a similar complex [MetHbOO*tert*-Bu] which did not regenerate MetHb.

LAHPO is a product of peroxidation of linoleic acid and a representative hydroperoxide which may be generated by peroxidation of lipids *in vivo*. The reactions of hemoglobin with LAHPO were not identical to those with H_2O_2 and BHPO, and may be postulated to be as shown in Chart 2. HbO_2 and DeoxyHb treated with LAHPO at pH 7—8 produced MetHb in the presence of albumin or KCN. The formation of precipitates at pH 7.0 in the absence of both albumin and KCN may be caused by further derivatization of MetHb. Cyanomethemoglobin seemed to be stable, and albumin seemed to block the further derivatization of MetHb. The complex formation from MetHb by LAHPO was deduced from spectral measurements; the formation was prevented by albumin, but once the complex was produced, albumin regenerated MetHb and formed precipitates. It is likely that the interaction of MetHb with LAHPO first produces the complex, which regenerates MetHb and produces some active species for apoprotein denaturation upon treatment at lower pH or with albumin. In contrast to the reaction of hemoglobin, myoglobin interacted with LAHPO without

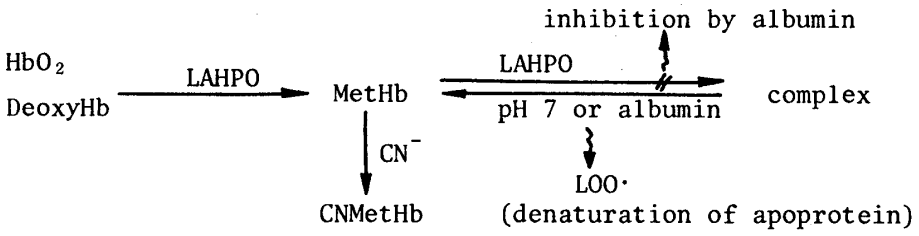

Chart 2

formation of precipitates. The formation of precipitates is characteristic of tetrameric hemoglobin.

Solubility studies[25-28] of oxidized lipid–protein reaction products have shown that they appear to be protein–protein cross-linked polymers produced by free-radical chain polymerization.[27] Studies by O'Brein[29] and Hawco *et al.*[30] suggested that the reaction of MetHb with LAHPO produces a complex whose degradation is accompanied by the formation of peroxy free radical. This active radical species may insolubilize the apoprotein of hemoglobin,

$$\text{LOOH} \xrightarrow{\text{Met Hb}} \text{LOO}^{\bullet}$$

$$2\text{LOO}^{\bullet} \longrightarrow \text{LOOOOL}$$

$$\text{LOOOOL} \longrightarrow \text{LOH, LO, } {}^1O_2$$

probably due to the cross-linking of hemoglobin tetramer. The active species formed in the reaction of MetHb and LAHPO will be discussed in detail in the following paper.

It is interesting to note that albumin affected the formation and degradation of the complex of MetHb with LAHPO, while it was inert as regards the complexes of MetHb with H_2O_2 and BHPO. The MetHb-forming activity of LAHPO was not destroyed or increased by incubation with albumin, and the interaction of LAHPO with albumin did not produce any active species for denaturation of the apoprotein of hemoglobin. The reasons for inhibition by albumin of the conversion of MetHb by LAHPO into the complex are obscure, but it is not surprising that albumin has a stabilizing effect on LAHPO or MetHb, since albumin is well known to interact with fatty acids and some proteins.

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