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Detection of radical species in haematin-catalysed retinoic acid 5,6-epoxidation by using h.p.l.c.–e.p.r. spectrometry

Hideo IWAHASHI, Akihiko IKEDA, Youichi NEGORO and Ryo KIDO
Department of Biochemistry, Wakayama Medical College, Wakayama 640, Japan

E.p.r. signals were detected in an all-*trans*-retinoic acid/haematin incubation mixture by using an e.p.r. spin-trapping technique. The spin adducts are presumably attributable to some intermediates in haematin-catalysed retinoic acid 5,6-epoxidation, since addition of nitrosobenzene to the reaction mixture dose-dependently inhibited the epoxidation. Analysing the reaction mixture by h.p.l.c.–e.p.r. spectrometry resulted in the detection of three peaks (III-1, III-2, IV) ascribable to the radical species. Two (peaks III-1 and -2) of the three peaks, which appeared 10 min after the reaction had started, seem to be attributable to the radical species directly participating in the epoxidation. The radicals trapped by nitrosobenzene do not appear to be derived from active oxygen, since none of these peaks were detected in a similar h.p.l.c. analysis of O_2^- and OH'-generating systems. They are presumably derived from retinoic acid. This view is also supported by the following results: (*a*) none of these peaks were detected in the h.p.l.c. elution profile of the reaction mixture when retinoic acid was absent; (*b*) peaks III-1 and 2 were detected even under anaerobic conditions, and their peak heights were unchanged under aerobic conditions.

INTRODUCTION

Haemoglobin itself is not considered to be an enzyme; rather it has been classified as an oxygen carrier protein. However, haemoglobin has been implicated as a catalyst in various other reactions [1–8]. Haemoproteins other than haemoglobin also catalyse reactions different from those in which they play a major role, e.g., diphenol oxidase activity by catalase [9] and lipid peroxidation by cytochrome *c* [1]. Recently we reported that haemoglobin catalysed retinoic acid 5,6-epoxidation [10]. In addition, other haemoproteins (myoglobin, catalase and peroxidase) catalysed this epoxidation [11]. Haematin, the prosthetic group of several hydroperoxide-metabolizing enzymes, also catalysed epoxidation, suggesting that the haem moiety participates in the epoxidation [11]. Other haematin-catalysed reactions were reported, e.g., conversion of linoleic acid hydroperoxide to hydroxy, oxo, epoxyhydroxy and trihydroxy fatty acids and epoxidation of 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene [12,13]. Clarification of the mechanism of the reaction catalysed by haematin is important and interesting, since haematin catalyses the oxygenation of several substrates, and the reaction mechanism can be extended to the ones catalysed by other haem-containing proteins.

In previous reports about haematin-catalysed reactions, it was assumed that the haematin-catalysed reactions proceed by a free-radical mechanism, since NADPH, NADH, 2-mercaptoethanol, butylated hydroxyanisole, and butylated hyroxytoluene inhibited the reactions [10–13]. However, direct evidence has not been obtained. The present study has shown that radical species are formed in haematin-catalysed retinoic acid 5,6-epoxidation. Detection of the radical species was done by using an e.p.r. spin-trapping technique involving h.p.l.c.–e.p.r. spectrometry.

MATERIALS AND METHODS

Materials

Haematin, xanthine and all-*trans*-retinoic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Buttermilk xanthine oxidase was obtained from P-L Biochemicals (Milwaukee, WI, U.S.A.). Nitrosobenzene was a product of Nakarai Chemical Co. (Kyoto, Japan). 5,6-Epoxyretinoic acid was synthesized by the method of John *et al.* [14], except for the retinoic acid methylation step, which was done with diazomethane.

Assay conditions

The standard reaction mixture contained, in a total volume of 0.215 ml, 4% Triton X-100, 1.65 mM-retinoic acid (which was added as 10 μl of a solution in dimethyl sulphoxide), 0.1 M-potassium phosphate buffer, pH 7.5, and 5 μl of haematin solution (53 μM-haem). Unless otherwise noted, all reactions were allowed to proceed at 37 °C for 5 min under aerobic conditions and stopped by the addition of 1.6 ml of methanol containing 50 mM-2-mercaptoethanol. The peak height of the product was compared with those of three different concentrations of 5,6-epoxyretinoic acid standards in the range 5–15 pmol to determine the concentration of 5,6-epoxyretinoic acid formed. The concentration of the standard epoxide was determined by using $A^{1\%}_{348} = 1360$ (in chloroform) [14].

H.p.l.c. and e.p.r.

The h.p.l.c. was performed on a Jasco Trirotor V with a variable-wavelength u.v. detector and an e.p.r. spectrometer. The u.v. detector was set at 310 nm. A column (250 mm × 4.9 mm internal diam.) packed with TSK ODS gel (5 μm) was used at flow rate of 1.0 ml/min, with 10 mM-ammonium acetate and aq. 75% (v/v) methanol

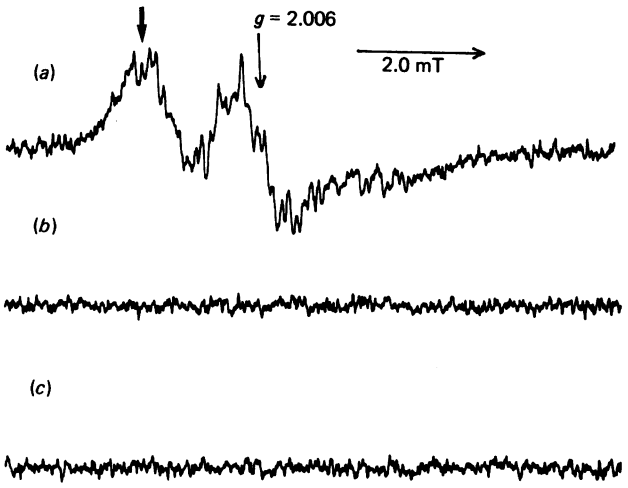


Fig. 1. E.p.r. spectrum of spin adduct of nitrosobenzene in a reaction mixture containing retinoic acid and haematin

The e.p.r. spectrum was obtained after incubation for 10 min at 25 °C. The incubation mixture contained, in a total volume of 0.22 ml, 5 μl of nitrosobenzene (0.1 M in methanol) and standard reaction mixture (described in the text). E.p.r. measurements were made at room temperature by using an aqueous-solution cell. The microwave power was 5 mW, and the modulation frequency was 100 kHz with an amplitude of 63 μT. The sweep time was 25 mT/min, and the receiver gain was 3.2×10^3 with a response time of 0.3 s. The broad arrow (⬇) ($g = 2.017$) indicates the position at which the magnetic field was fixed in the h.p.l.c.–e.p.r. spectrometry experiments. (*a*) Complete; (*b*) nitrosobenzene solubilized in buffer containing 4% Triton X-100 and 0.1 M-potassium phosphate, pH 7.5; (*c*) in the absence of nitrosobenzene.

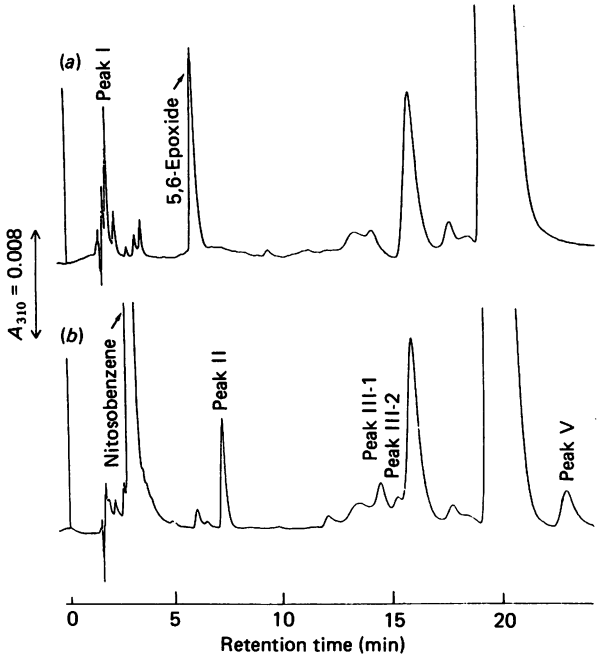


Fig. 2. H.p.l.c. elution profiles of the haematin-catalysed retinoic acid 5,6-epoxidation incubation mixture

H.p.l.c. analyses were done by direct analysis of 10 μl of a reaction mixture where 1.6 ml of 50 mM-2-mercaptoethanol (in methanol) was *not* added to stop the reaction. (*a*) Standard reaction mixture in the absence of nitrosobenzene; (*b*) The standard reaction mixture in the presence of 5 μl of 0.1 M-nitrosobenzene (in methanol). The h.p.l.c. and the other assay conditions are described in the text.

as the mobile phase. The column was kept at 40 °C throughout. An e.p.r. spectrometer (JEOL-FX2XG) was connected to the h.p.l.c. system with Teflon tube, which passed through the e.p.r. cell. The magnetic field of the e.p.r. spectrometer was fixed at the position ($g = 2.017$) indicated by broad arrow in Fig. 1. The e.p.r. spectrometer was operated at room temperature.

Oxygen consumption

Oxygen-consumption studies were performed with a Clark-type electrode (Yellow Spring Instrument Co., Yellow Spring, OH, U.S.A.).

RESULTS

Detection of radical species in haematin-catalysed retinoic acid 5,6-epoxidation by a spin-trapping technique

Haematin-catalysed retinoic acid 5,6-epoxidation was studied using an e.p.r. spin-trapping technique. When the spin-trap reagent nitrosobenzene was added to the standard haematin-catalysed retinoic acid 5,6-epoxidation reaction mixture, e.p.r. signals ($g = 2.006$) were detected (Fig. 1*a*). Signals were not observed when nitrosobenzene was dissolved in the buffer alone or in the absence of nitrosobenzene (Figs 1*b* and 1*c*).

Inhibition of retinoic acid 5,6-epoxidation by nitrosobenzene

To test whether the radical species trapped by nitrosobenzene directly participate in haematin-catalysed

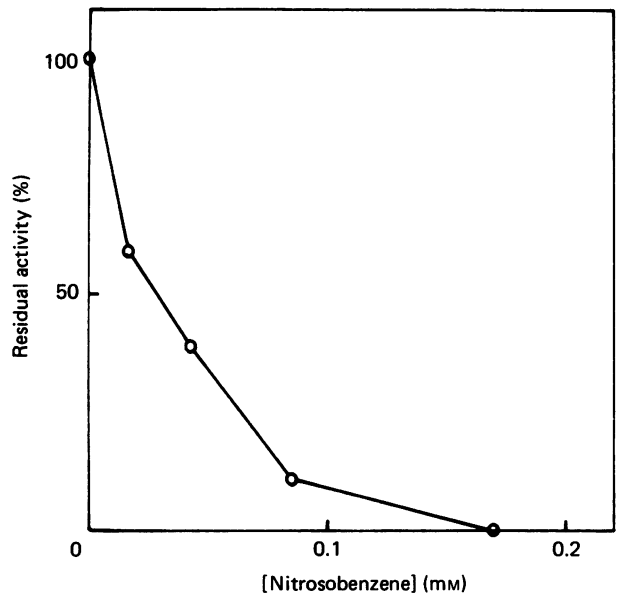


Fig. 3. Effect of nitrosobenzene concentration on the formation of 5,6-epoxyretinoic acid catalysed by haematin

The standard reaction mixture contained various concentrations of nitrosobenzene solubilized in 10 μl of methanol. All the data were collected by subtracting the amount of 5,6-epoxyretinoic acid formed in the reaction mixtures in the absence of haematin. The h.p.l.c. and other assay conditions were as described in the text.

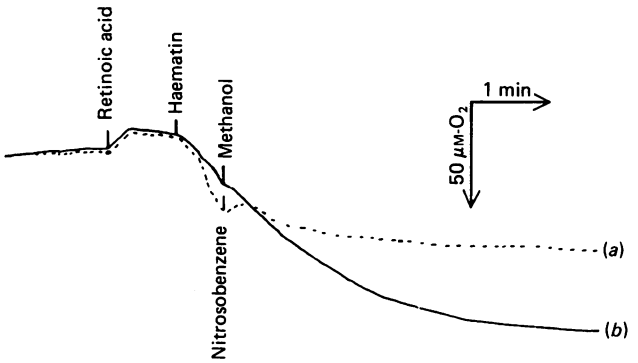


Fig. 4. Oxygen consumption in the haematin-catalysed retinoic acid 5,6-epoxidation reaction mixture

The incubation conditions were as described in the text, except for the total volume (2.2 ml) of the reaction mixture. (*a*) A 50 μl portion of nitrosobenzene (0.1 M in methanol) was added to the reaction mixture; (*b*) instead of nitrosobenzene, 50 μl of methanol was added. The amount of oxygen consumed was estimated by use of the value 217 μM for the saturated oxygen concentration [16].

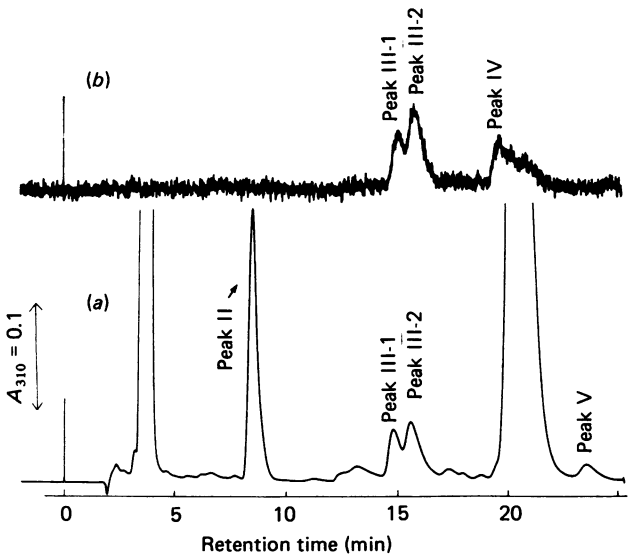


Fig. 5. H.p.l.c. elution profiles of standard reaction mixture in the presence of 5 μl of nitrosobenzene (0.1 M in methanol)

The analyses were done by h.p.l.c. equipped with an e.p.r. spectrometer. The magnetic field of the e.p.r. spectrometer was fixed at the position ($g = 2.017$) shown by broad arrow in Fig. 1. The microwave power was 5 mW and the modulation frequency was 100 kHz with an amplitude of 63 μT. The receiver gain was 3.2×10^3 with a response time of 1.0 s. A 100 μl portion of the 1 h incubation mixture was subjected to h.p.l.c. analysis. Other h.p.l.c. and incubation conditions were as described in Fig. 1. (*a*) H.p.l.c. analysis of the complete reaction mixture detected by u.v. absorbance at 310 nm; (*b*) h.p.l.c. analysis of the complete reaction mixture detected by e.p.r.

retinoic acid 5,6-epoxidation, the formation of 5,6-epoxyretinoic acid was measured by h.p.l.c. on addition of various concentrations of nitrosobenzene to the standard reaction mixture (Figs. 2 and 3). Peak assignment of 5,6-epoxyretinoic acid was done by comparing the h.p.l.c. retention time with that of

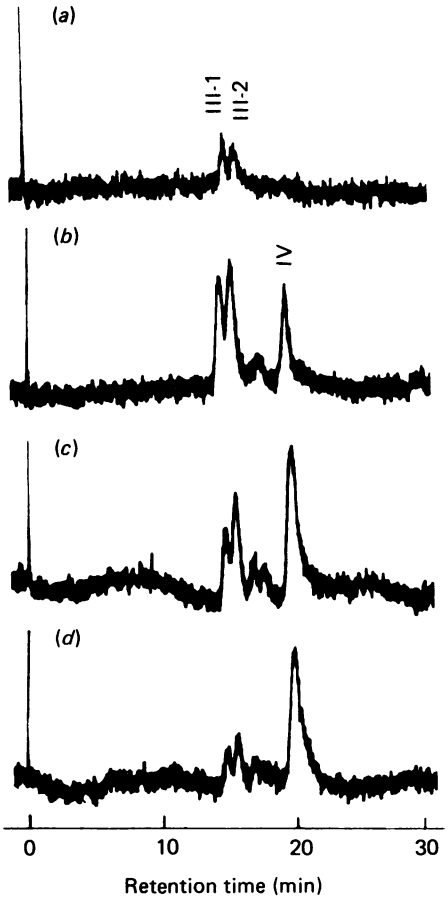


Fig. 6. Time course of the peak heights of radical species detected by h.p.l.c. equipped with an e.p.r. spectrometer

H.p.l.c. and e.p.r. conditions were the same as those in Fig. 5. Incubations conditions were as described in the text. (*a*) 10 min incubation; (*b*) 1 h incubation; (*c*) 4 h incubation; (*d*) 19 h incubation.

synthetic 5,6-epoxyretinoic acid. As shown in Fig. 2, formation of 5,6-epoxyretinoic acid completely stopped on the addition of nitrosobenzene (2.3 mM). A minor peak at the position corresponding to the 5,6-epoxyretinoic acid in Fig. 2(*b*) was also detected in the reaction mixture in the absence of haematin. It could be attributable to 5,6-epoxyretinoic acid contamination of the substrate, retinoic acid. Peak I, which appeared concomitantly with the formation of 5,6-epoxyretinoic acid in the retinoic acid/haematin incubation mixture, decreased on addition of nitrosobenzene. In contrast with the peak-I compound and 5,6-epoxyretinoic acid, the peak-II, peak-III-1 and -2 and peak-V compounds appeared on addition of nitrosobenzene (Fig. 2*b*). The effect of the addition of various concentrations of nitrosobenzene on haematin-catalysed retinoic acid 5,6-epoxidation was examined. Formation of 5,6-epoxyretinoic acid dose-dependently decreased with increase of nitrosobenzene in the reaction mixture (Fig. 3).

Oxygen consumption on addition of nitrosobenzene to the reaction mixture

Oxygen consumption was measured on addition of nitrosobenzene to the standard reaction mixture (Fig. 4).

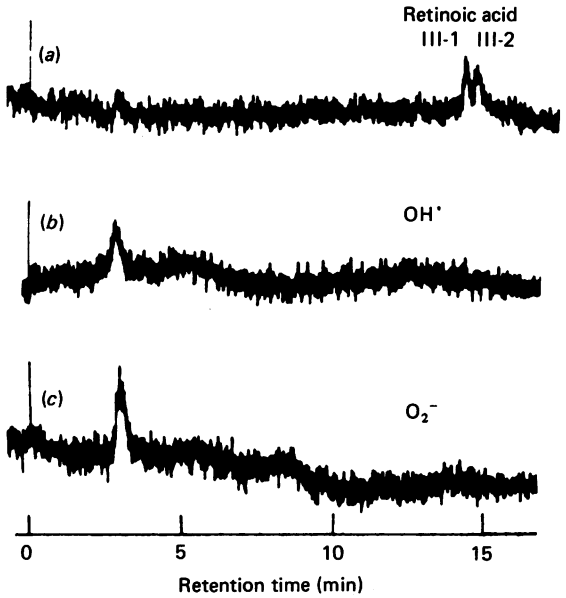


Fig. 7. H.p.l.c. analyses of spin adducts of nitrosobenzene in the reaction mixtures of O_2^-- and OH·-generating systems and retinoic acid 5,6-epoxidation

The O_2^--generating system contained 0.5 ml of 0.1 mM-xanthine solubilized in 4% Triton X-100 and 0.1 M-potassium phosphate buffer, pH 7.5, 50 μl of 0.1 M-nitrosobenzene in methanol and 5 μl of xanthine oxidase (cytochrome *c*-reduction activity 84 pmol/min per μl). The reaction was carried out for 10 min at 25 °C. The OH·-generating system contained 0.2 ml of 4% Triton X-100 and 0.1 M-potassium phosphate buffer, pH 7.5, 50 μl of 0.1 M-nitrosobenzene in methanol, 5 μl of 5 mM-$FeSO_4$ and 10 μl of H_2O_2 (31%, v/v). The reaction was allowed to proceed for 10 min at 25 °C. The reaction conditions for retinoic acid 5,6-epoxidation are described in the text. H.p.l.c. and e.p.r. conditions were the same as those in Fig. 5.

Even in the absence of haematin, a small amount of oxygen was consumed. This slight oxygen consumption is consistent with the observation that retinoic acid is spontaneously oxidized to form 5,6-epoxyretinoic acid [10,11]. When haematin was added to the reaction mixture, molecular oxygen was consumed to a considerable extent. To test whether addition of nitrosobenzene to the reaction mixture inhibits oxygen consumption, 2.3 mM-nitrosobenzene was added in the course of haematin-catalysed retinoic acid 5,6-epoxidation. The oxygen consumption stopped on addition of nitrosobenzene to the reaction mixture (Fig. 4).

Detection of the radical species by h.p.l.c.–e.p.r. spectrometry

By using h.p.l.c.–e.p.r. spectrometry, peaks attributable to radical species were detected in the h.p.l.c. elution profile of the standard haematin-catalysed retinoic acid 5,6-epoxidation reaction mixture in the presence of nitrosobenzene (Fig. 5). The peaks (III-1 and -2) attributable to the radical species appeared at the same retention time as those of peaks III-1 and -2 in Fig. 2 (*b*) in the h.p.l.c. elution profile. The peaks were not detected in the reaction mixture in the absence of retinoic acid.

The time course of formation of the radical species was examined by h.p.l.c.–e.p.r. spectrometry (Fig. 6). The peak heights of III-1 and -2 increased to 1 h and then gradually decreased. In contrast with peaks III-1 and -2, the peak height of IV continued to increase for 19 h.

In order to characterize the spin adducts, the elution profile of the haematin-catalysed retinoic acid 5,6-epoxidation reaction mixture was compared with those of O_2^- and OH·-generating systems (Fig. 7). In the h.p.l.c. elution profile of O_2^-- and OH·-generating systems, the corresponding peaks were not detected. In addition, peaks III-1 and -2 were observed in the h.p.l.c.–e.p.r. elution profile of the standard reaction mixture under anaerobic conditions, and the peak heights were unchanged under aerobic conditions (Fig. 8). In contrast with peaks III-1 and -2, peak IV was small in the elution profile obtained under anaerobic conditions compared with that under aerobic conditions.

DISCUSSION

There have been few reports concerning h.p.l.c. separation of radical species [15]. In particular, this is the first report about the elucidation of enzyme-like reactions by h.p.l.c.–e.p.r. spectrometry. We believe that the separation of radical intermediates by h.p.l.c.–e.p.r. spectrometry represents a new strategy for the elucidation of the reaction mechanisms, including radical intermediates in reaction mixtures, and such a method enables us to isolate the radical intermediates and determine the chemical structure by other analytical methods such as n.m.r., mass, u.v. and i.r. spectroscopies.

In the present investigation, e.p.r. signals were observed in the course of haematin-catalysed retinoic acid 5,6-epoxidation in the presence of the spin-trap reagent nitrosobenzene. The hfs constant (1.5 mT) of the e.p.r. signal appears to originate from interaction of an unpaired electron with a nitrogen in the nitrosobenzene. The broad peaks may be attributable to an interaction between the spin adduct and paramagnetic haematin. The distorted signal could be attributable to the interaction between the spin adduct and a Triton X-100 micelle, since the interaction disturbs isotropic rotation of the spin adduct. The e.p.r. signals seem to be attributable to adducts between nitrosobenzene and short-lifetime radical intermediates formed in the course of the haematin-catalysed retinoic acid 5,6-epoxidation, since no e.p.r. signals were detected in the haematin-catalysed retinoic acid 5,6-epoxidation reaction mixture in the absence of nitrosobenzene or in nitrosobenzene solubilized in 4% Triton X-100/0.1 M-potassium phosphate buffer, pH 7.5. The two results obtained here, namely that addition of nitrosobenzene completely inhibits both oxygen consumption and formation of 5,6-epoxyretinoic acid, support the view that the short-lifetime radical intermediates directly participate in retinoic acid 5,6-epoxidation.

By using h.p.l.c.–e.p.r. spectrometry, three peaks attributable to radical species were detected in the h.p.l.c. profile of the haematin-catalysed retinoic acid 5,6-epoxidation reaction mixture. These peaks seem to be attributable to spin adducts between nitrosobenzene and radicals generated from retinoic acid in the course of the haematin-catalysed retinoic acid 5,6-epoxidation, since the peaks were not detected in the reaction mixture in the absence of retinoic acid. Of the three peaks, peaks III-1

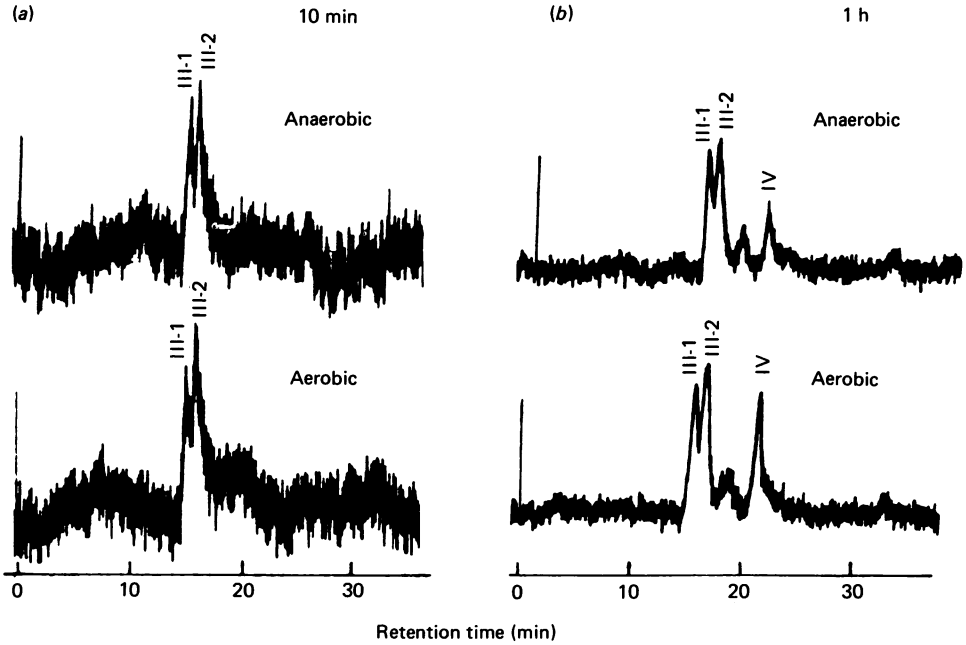
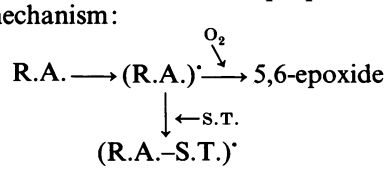


Fig. 8. Formation of spin adducts of nitrosobenzene in the standard reaction mixture under aerobic and anaerobic conditions

(*a*) Incubation was done at 25 °C for 10 min after mixing 200 μl of retinoic acid (1 mg/0.1 ml of dimethyl sulphoxide) and haematin solution under anaerobic conditions. The haematin solution contained, in a total volume of 4.1 ml, 4% Triton X-100, 0.1 M-potassium phosphate buffer, pH 7.5, 100 μl of haematin in 1 mM-NaOH (53 μM-haem), and 100 μl of nitrosobenzene (0.1 M in methanol). Procedures of h.p.l.c. and e.p.r. analyses were the same as those in Fig. 5, except for the e.p.r. receiver gain, which was 10×10^3. (*b*) After mixing the reaction mixture under anaerobic conditions, incubation was performed under aerobic conditions. Other experimental procedures were the same as those in Fig. 5.

and -2 seem to participate directly in the retinoic acid 5,6-epoxidation, because the peaks appeared 10 min after the reaction had started. Of course the spin adducts between nitrosobenzene and $OH^{\cdot}$ or O_2^{-} should be considered candidates for the peaks. However, no corresponding peaks were detected in the h.p.l.c. elution profile of the O_2^{-}- and $OH^{\cdot}$-generating system. In addition to the above results, the h.p.l.c. retention time of the spin adducts enables us to exclude the possibility that peaks III-1 and -2 compounds are spin adducts between nitrosobenzene and $OH^{\cdot}$ or O_2^{-}, since the spin adducts between nitrosobenzene and the active oxygen radicals, more polar than nitrosobenzene, should have been eluted earlier than nitrosobenzene itself (Fig. 5). The radical intermediates seem to be formed before reaction of retinoic acid with oxygen, because the peaks III-1 and -2 were formed under anaerobic conditions.

The above results allow us to propose the following reaction mechanism:



First, retinoic acid, R.A., becomes a radical, (R.A.)$^{\cdot}$, which corresponds to those that react with nitrosobenzene, S.T., to form the spin adduct, (R.A.–S.T.)$^{\cdot}$, observed in this experiment. Although the structure of the retinoic acid radical is unclear at present, radicals formed by hydrogen subtraction from retinoic acid are considered plausible candidates for those detected. At the second step the radicals formed react with oxygen to form 5,6-epoxyretinoic acid ('5,6-epoxide' above). The reaction mechanism described here is not comprehensive, for the details of each step have yet to be obtained. It will be necessary to elucidate the chemical structure of the trapped radical in order to understand the reaction mechanism in detail.

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