Haemoglobin-catalysed retinoic acid 5,6-epoxidation

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Examination of the subcellular distribution of retinoic acid 5,6-epoxidase activity in rat liver and human liver homogenates showed that there is a prominent peak of activity in a high-density fraction. A corresponding peak was also detected in rat blood and human blood. Retinoic acid 5,6-epoxidation was catalysed by human blood cells but not by human plasma, and purified human haemoglobin also catalysed the epoxidation of retinoic acid to 5,6-epoxyretinoic acid. These results suggest that retinoic acid 5,6-epoxidase activity in human liver and rat liver homogenates is partially due to the presence of residual blood cells, and particularly haemoglobin, in the homogenates. In the retinoic acid 5,6-epoxidation catalysed by human haemoglobin, molecular O₂ was required and its reaction was stimulated by Triton X-100. Boiling of haemoglobin solution resulted in an 94% decrease in the activity. NADPH (1 mM) and NADH (1 mM) completely [2-mercaptoethanol (5 mM) almost completely] inhibited the 5,6-epoxidation catalysed by haemoglobin, but catalase, superoxide dismutase and mannitol showed no inhibitory effect. CN⁻ ion (100 mM) inhibited the reaction, but N₃⁻ ion (100 mM) did not.

INTRODUCTION

A number of metabolites of retinoic acid have previously been identified, such as 4-hydroxyretinoic acid, 4-oxoretinoic acid (Frolik *et al.*, 1978, 1979; Roberts *et al.*, 1979) and 5,6-epoxyretinoic acid (Fig. 1) (McCormick *et al.*, 1978) *in vivo*. Sietsema & DeLuca (1979, 1982) showed the existence of 5,6-epoxidase activity in rat liver tissue, and mentioned that the epoxidation is probably not necessary for the function of retinoic acid in maintaining growth and differentiation.

Haemoglobin is not itself normally considered to be an enzyme, but rather has been classified as an inert carrier protein. However, haemoglobin has also implicated as a catalyst in various reactions (Tappel, 1953; Yamabe & Lovenverg, 1972; Juchan & Symms, 1972; Dairnan & Christenson, 1973; Symms & Juchan, 1974; Mieyal *et al.*, 1976; Mieyal & Blumer, 1976). In the present paper we report that human haemoglobin also catalyses the epoxidation of retinoic acid, and we discuss the properties of the retinoic acid epoxidation in comparison with the other reactions catalysed by haemoglobin.

MATERIALS AND METHODS

Materials

all-*trans*-Retinoic acid was a gift from Hoffman–La Roche (Nutley, NJ, U.S.A.). The purity of the sample was checked by reversed-phase h.p.l.c. before use. Retinoic acid contained traces of the *cis* isomer and the 5,6-epoxide, but these do not interfere with the assay of retinoic acid 5,6-epoxidase activity. Human haemoglobin (type IV), bovine liver catalase, NADPH, NADH and xanthine 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.). All other chemicals used were commercial products of the highest grade available. Human blood cells were prepared as follows. Whole blood containing heparin was

centrifuged at 200 *g* for 5 min. The supernatant was used as the plasma fraction. Sedimented blood cells were resuspended in 0.9% NaCl (5 ml) and centrifuged at 200 *g* for 15 min. The supernatant was discarded and the sedimented blood cells were used for assay of retinoic acid 5,6-epoxidase activity. Superoxide dismutase was purified from bovine erythrocytes and the activity was assayed by the method of McCord & Fridovich (1969). The specific activity was 35000 units/ml. Oxyhaemoglobin and methaemoglobin were prepared according to the method of Mieyal & Blumer (1976).

Tissue preparation

Male Wistar-strain rats (200–300 g) were obtained from Kiwa Animal laboratory (Wakayama, Japan). Human liver was obtained from autopsy of a man (43 years old) who had died accidentally. Rats were killed by decapitation. The livers were quickly removed and placed in ice-cold 0.9% NaCl.

Preparation of subcellular fractions

All procedures were carried out at 0–4 °C. Rat and human livers were removed, cut into small pieces and homogenized by several excursions in 0.25 M-sucrose in a Teflon/glass homogenizer. The homogenates were centrifuged at 500 *g* for 3 min to sediment nuclei and whole cells. Then 5 ml of the supernatant was layered on to a 45 ml linear sucrose gradient (27–60%, w/w) for rat liver and a 45 ml linear sucrose/Percoll gradient (0.25–0.63 M-sucrose in 80%, w/w, Percoll) for human liver in a RPS25-2 swing-out rotor in a Hitachi 70p-72

Fig. 1. Structure of 5,6-epoxyretinoic acid

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Fig. 2. H.p.l.c. separation of the metabolite of retinoic acid formed during incubation with rat liver homogenate

(a) Incubation mixture contained 5 μ l of homogenate preparation (280 μ g of protein/ml), 0.1 M-potassium phosphate buffer, pH 7.8, 4% Triton X-100 and 1.65 mM retinoic acid [added in 10 μ l of methanol/dimethyl sulphoxide (1:1, v/v)] in a total volume of 0.215 ml. Incubation was at 37 °C for 5 min, and the reaction was stopped by the addition of 1.6 ml of methanol containing 50 mM-2-mercaptoethanol. The stopped reaction mixture was directly analysed by h.p.l.c. under the conditions described in the Materials and methods section. (b) Incubation with boiled rat liver homogenate. The peak (I) of 5,6-epoxyretinoic acid formed is shown by the arrow.

automatic preparative ultracentrifuge. The gradient was centrifuged at 54000 *g* for 2 h.

Determination of haem concentration

Haemoglobin haem concentration was determined by the pyridine haemochromogen method, by using $\epsilon_{557} = 33.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Paul *et al.*, 1953). Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. All spectrophotometric measurements were carried out, in a cuvette with a 10 mm light path, with a Hitachi 100-50 spectrophotometer.

Activity assays

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 μ l of solution in methanol/dimethyl sulphoxide (1:1, v/v)], 0.1 M-potassium phosphate buffer, pH 7.8, and 80 μ l of enzyme solution (280 μ g of protein/ml) or 5 μ l of haemoglobin preparation (24 μ M of haem). Each reaction was carried out in 15 ml centrifuge tubes, and the tubes were shaken at 37 °C for 5 min under aerobic conditions in the dark. The reaction was stopped by addition of 1.6 ml of

methanol containing 50 mM-2-mercaptoethanol. The reaction mixture was subjected to h.p.l.c. on a Jasco Trirotor III apparatus with a variable-wavelength u.v. detector. The u.v. detector was set at 310 nm. A Finepak C₁₈ prepacked column (250 mm $\times$ 4.9 mm internal diam.) was used at a flow rate of 1.0 ml/min of 10 mM-ammonium acetate and 80% (v/v) methanol as a mobile phase. The column was kept at 40 °C throughout. The 5,6-epoxyretinoic acid was detected and its concentration determined by measurement of the absorption ($A_{346}^{1\%} = 1360$ in chloroform; John *et al.*, 1967).

Cytochrome *c* oxidase and esterase activities were assayed as described by Wharton & Tzagoloff (1967) and Beaufay *et al.* (1974) respectively.

Isolation of compound I

In order to obtain peak I compound on a preparative scale, 10 ml of complete mixture was incubated at 37 °C for 30 min, and the reaction was then stopped by adding 10 ml of methanol containing 50 mM-2-mercaptoethanol. After addition of 200 μ l of 1 M-ammonium acetate to the reaction mixture, 1 ml of the reaction mixture was applied to the h.p.l.c. system and fractions corresponding to peak I were collected. Each collected peak I was combined and concentrated to 1 ml under reduced pressure. The concentrated peak I fraction was rechromatographed. The peak I compound was again fractionated and subjected to u.v., m.s. and ¹H-n.m.r. analyses.

M.s. and n.m.r. measurements

A 500 MHz n.m.r. spectrum of the compound was recorded on a JEOL GX-500s n.m.r. spectrometer, in [²H₄]methanol with tetramethylsilane as an internal standard.

A field-desorption mass spectrum was obtained by use of a JEOL JMS-DX 300 mass spectrometer. An electron-impact mass spectrum was recorded on a Shimadzu GCMS-QP 1000 mass spectrometer.

RESULTS

Isolation and identification of 5,6-epoxyretinoic acid

Fig. 2(a) shows a typical h.p.l.c. elution profile obtained after incubation of retinoic acid with rat liver homogenate. Comparison with the h.p.l.c. elution profile obtained after incubations of retinoic acid with boiled rat liver homogenate (Fig. 2b) shows that peak I increased in the h.p.l.c. chromatogram of the complete system. The results indicate that formation of peak I compound is catalysed by heat-labile agent(s) in the rat liver homogenate, because peak I was not detectable in the homogenate itself. Some peaks appeared at retention time 2.2 min in the h.p.l.c. elution profiles of the complete system, and hence more-polar compounds other than peak I also seemed to be formed. However, in the present investigation we devoted our attention to peak I. The compound had a λ_{max} at 346 nm in chloroform. The value agreed with that of 5,6-epoxyretinoic acid already reported by John *et al.* (1967). An electron-impact mass spectrum had typical prominent ions of 5,6-epoxyretinoic acid (Reid *et al.*, 1973) at m/z 316 (M^+), 301 ($M^+ - 15$) and 271 ($M^+ - 45$) (Fig. 3a). A field-desorption mass spectrum showed a peak at m/z 316 (M^+), which corresponds to the molecular ion of 5,6-epoxyretinoic acid (Fig. 3b).

Fig. 3. Electron-impact (a) and field-desorption (b) mass spectra of the peak I compound

Fig. 4. ^1H -n.m.r. spectrum of peak I compound in $[\text{D}_4]\text{methanol}$

The peaks marked ** are due to an impurity, and those marked * to a spinning side band.

Fig. 5. Subcellular distribution of epoxidase activity in rat liver

Preparation of the particulate fraction and centrifugation in the sucrose density gradient are described in the text. (a) Homogenate; (b) homogenate perfused by 0.9% NaCl; (c) rat blood. Density (----) and epoxidase activity (●) are plotted. Cytochrome *c* oxidase and esterase activities were assayed as described by Wharton & Tzagoloff (1967) and Beaufay *et al.* (1974) respectively. The peak of activity of cytochrome *c* oxidase appeared at fraction no. 26 (b) (○) and those of esterase appeared at fraction nos. 26 and 42 (b) (▽).

A ^1H -n.m.r. spectrum (Fig. 4) of the compound in $[\text{D}_4]\text{H}_2\text{O}$ supported the assumed chemical structure, 5,6-epoxyretinoic acid. Peaks of the methyl group were obtained at 0.923 p.p.m. (5-CH_3), 1.144 p.p.m. and 1.122 p.p.m. (1-CH_3), and 2.162 p.p.m. (13-CH_3). The splitting of 1-CH_3 proton resonances is ascribed to the formation of 5,6-epoxide. In the lower field (5.8–6.8 p.p.m.) all the six protons on the double bonds were available, i.e. 5.888 p.p.m. (s), 5.972 p.p.m. (d), 6.154 p.p.m. (d), 6.248 p.p.m. (d), 6.328 p.p.m. (d) and 6.766 p.p.m. (t). Morgan & Thompson (1966) reported a similar ^1H -n.m.r. spectrum of synthetic methyl 5,6-epoxyretinoate. The u.v., m.s. and ^1H -n.m.r. spectra indicate that the peak I substance is 5,6-epoxyretinoic acid. U.v. spectroscopy, mass spectrometry and h.p.l.c. on a sample of 5,6-epoxyretinoic acid synthesized by the method of John *et al.* (1967) provided final confirmation that compound I was indeed 5,6-epoxyretinoic acid.

Fig. 6. Subcellular distribution of epoxidase activity in human liver

Preparation of the particulate fraction and concentration in the sucrose/Percoll gradient are described in the text. (a) Human liver homogenate; (b) human blood. Density (----), A_{415} (○) and epoxidase activity (●) are plotted.

Subcellular distribution of retinoic acid 5,6-epoxidase activity of human liver and rat liver

Cell fractionation was performed by the sucrose-density-gradient method (Figs. 5 and 6). Peaks of activity were detected at several density values in rat liver homogenate (Fig. 5). A prominent peak was observed in a high-density fraction (1.25 g/ml, fraction no. 16). A corresponding activity peak was also observed in the sucrose-density-gradient profile of rat blood (Fig. 5c). On the other hand the prominent peak disappeared after perfusion of rat liver, although the retinoic acid 5,6-epoxidase activities in microsomal (fraction no. 40) and mitochondrial (fraction no. 23) fractions still remained (Fig. 5b). These results suggest that the epoxidase activity that appeared in the high-density fraction of the sucrose-density-gradient profile of rat liver homogenate probably arises from residual blood cells in the homogenate. An activity peak that appeared in a low-density fraction of rat blood seems to come from lysed erythrocytes, probably haemoglobin.

Similar results were also obtained from subcellular fractionation of human liver homogenate and blood (Fig. 6). The absorbance of each fraction at 415 nm was also plotted here. The peaks of absorbance and the epoxidase activity coincided, suggesting that the activity was due to haemoglobin.

Table 1. Factors required for retinoic acid 5,6-epoxidation by rat liver homogenate and human haemoglobin

For assays with rat liver homogenate activities relative to complete systems are presented in parentheses (percentage of control). For assays with human haemoglobin in the absence of O₂, methaemoglobin was used as catalytic agent and percentage of control (in parentheses) is relative to activity (1200 pmol/min) of the complete system with methaemoglobin. The other activities with the human haemoglobin system were measured by using oxyhaemoglobin as the catalytic agent.

System	Activity (pmol/min)	
	Rat liver homogenate (22.4 µg of protein)	Human haemoglobin (120 pmol of haem)
Complete	170 (100%)	1400 (100%)
– O ₂	36 (21%)	35 (3%)
– Enzyme	28 (16%)	43 (3%)
– Triton X-100	58 (34%)	170 (12%)
Boiled (5 min)	81 (48%)	86 (6%)

Table 2. Effect of some agents on the retinoic acid 5,6-epoxidase activity of oxyhaemoglobin

The results are given as percentages of the activity of the complete system with oxyhaemoglobin as catalytic agent.

Agent	Concn. of agent	Epoxidase activity (% of control)
NADPH	1 mM	4
	5 mM	0
NADH	1 mM	0
	5 mM	0
2-Mercaptoethanol	1 mM	62
	5 mM	21
CN [–]	1 mM	76
	10 mM	56
	100 mM	13
N ₃ [–]	1 mM	113
	10 mM	98
	100 mM	64
Mannitol	100 mM	94
	200 mM	94
Catalase	0.5 µg/ml	71
	100 µg/ml	95
Superoxide dismutase	1.8 units/ml	110
	1800 units/ml	117

Retinoic acid 5,6-epoxidase activity of human haemoglobin

Human blood was separated into blood cells and plasma. The blood cells and plasma were suspended in 0.25 M-sucrose, and both preparations were tested for retinoic acid 5,6-epoxidase activity. The epoxidase activity was detected in the blood cells, at a specific activity of 2.9 mol/min per mol of haem, but no epoxidase activity was detected in the plasma.

To test whether haemoglobin catalyses the epoxidation, the epoxidase activity of purified oxyhaemoglobin and methaemoglobin was measured. Both oxyhaemoglobin

Fig. 7. Inhibition by NADPH of the retinoic acid 5,6-epoxidase activity of haemoglobin

NADPH (5 mM) was added to the incubation mixture at 5 min (●) and 10 min (□) after the reaction had been started. The incubation mixture contained 0.56 µM-oxyhaemoglobin, 0.1 M-potassium phosphate buffer, pH 7.8, 4% Triton X-100 and 1.65 mM-retinoic acid [added in 50 µl of methanol/dimethyl sulphoxide (1:1, v/v)] in total volume of 1.075 ml. The reaction was stopped by mixing 50 µl of incubation mixture with 400 µl of methanol containing 50 mM-2-mercaptoethanol. The result obtained with no addition of NADPH is also shown (○).

and methaemoglobin catalysed retinoic acid 5,6-epoxidation, at specific activities of 11 and 10 mol/min per mol of haem respectively. This suggests that the epoxidase activities of rat and human liver homogenates are partially due to residual blood cells, and particularly to haemoglobin in erythrocytes, in the homogenate.

Factors required for retinoic acid 5,6-epoxidation by rat liver homogenate and human haemoglobin

The retinoic acid 5,6-epoxidation by rat liver homogenate and human haemoglobin was measured under a variety of conditions (Table 1). It was clear that rat liver homogenate was required for the reaction with molecular O₂. Removal of Triton X-100 from the complete system decreased the activity to 34% of that of complete system. A 5 min or larger boiling of the rat liver homogenate decreased the formation of 5,6-epoxyretinoic acid to 48% of that of complete system.

Similar results were also obtained for the epoxidation catalysed by human haemoglobin.

Effects of some agents on oxyhaemoglobin-catalysed epoxidase activity

NADPH and NADH completely (2-mercaptoethanol almost completely) inhibited the haemoglobin-catalysed retinoic acid 5,6-epoxidase activity (Table 2). NADP⁺ and NAD⁺ did not show any inhibitory effect (results not shown). CN[–] ion (100 mM) inhibited the reaction, but N₃[–] (100 mM) did not. Catalase and superoxide dismutase did not exhibit any inhibitory effect.

Fig. 8. Effect of H_2O_2 concentration on the retinoic acid 5,6-epoxidase activity of haemoglobin

(a) Assay mixture contained $0.56 \mu\text{M}$ -oxyhaemoglobin, 100 mM-potassium phosphate buffer, pH 7.8, 4% Triton X-100, various concentration of H_2O_2 , as shown, and 1.65 mM-retinoic acid [added in $10 \mu\text{l}$ of methanol/dimethyl sulphoxide (1:1, v/v)] in a total volume of 0.215 ml. The mixture was incubated at 37°C for 5 min (●). The result obtained without oxyhaemoglobin is also shown (○). (b) Assay mixture contained 100 mM-potassium phosphate buffer, pH 7.8, 4% Triton X-100, various concentrations of H_2O_2 , as shown, and $1.7 \mu\text{M}$ -5,6-epoxyretinoic acid [added in $10 \mu\text{l}$ of methanol/dimethyl sulphoxide (1:1, v/v)] in a total volume of 0.215 ml. The mixture was incubated at 37°C for 5 min (□).

Fig. 9. Effect of $\text{O}_2^{\cdot -}$ concentration on the retinoic acid 5,6-epoxidase activity of haemoglobin

$\text{O}_2^{\cdot -}$ anion was produced by use of the xanthine/xanthine oxidase system. (a) Assay mixture contained $0.56 \mu\text{M}$ -oxyhaemoglobin, 100 mM-potassium phosphate buffer, pH 7.8, 4% Triton X-100, 0.1 mM-xanthine, various concentrations of xanthine oxidase and 1.65 mM-retinoic acid [added in $10 \mu\text{l}$ of methanol/dimethyl sulphoxide (1:1, v/v)] in a total volume of 0.215 ml. The mixture was incubated at 37°C for 5 min (●). The result obtained without oxyhaemoglobin is also shown (○). (b) Assay mixture contained 100 mM-potassium phosphate buffer, pH 7.8, 4% Triton X-100, 0.1 mM-xanthine, various concentrations of xanthine oxidase and $1.7 \mu\text{M}$ 5,6-epoxyretinoic acid [added in $10 \mu\text{l}$ of methanol/dimethyl sulphoxide (1:1, v/v)] in a total volume of 0.215 ml. The mixture was incubated at 37°C for 5 min (□).

Effect of NADPH on epoxide formation

As just mentioned, NADPH, NADH and 2-mercaptoethanol inhibited the haemoglobin-catalysed epoxidase activity. These findings suggest two possible mechanisms, namely that the compounds inhibit the reaction itself or degrade the epoxide formed. Addition of NADPH (5 mM) during an incubation inhibited production of the epoxide completely but did not decrease the amount of the epoxide formed (Fig. 7). This indicates that NADPH inhibits formation of the epoxide. Addition of 50 mM-2-mercaptoethanol alone (i.e. with no methanol or dimethyl sulphoxide) completely inhibits formation of the epoxide.

Inhibition of epoxidation by H_2O_2 and $\text{O}_2^{\cdot-}$

Addition of a low concentration (100 μM) of H_2O_2 to the complete system with oxyhaemoglobin as catalytic agent had no effect on the epoxidase activity (Fig. 8a). With higher concentrations of H_2O_2 the epoxidase activity was decreased. Since H_2O_2 did not degrade the epoxide (Fig. 8b), the decrease in activity is due to inhibition of epoxide formation by H_2O_2 .

Addition of the $\text{O}_2^{\cdot-}$ -generating system xanthine/xanthine oxidase (Muraoka *et al.*, 1967) to the complete system (with oxyhaemoglobin as catalytic agent) did not stimulate the epoxidase activity (Fig. 9a). As with the addition of H_2O_2 , the activity was decreased in the presence of high concentrations of the $\text{O}_2^{\cdot-}$ generating system. The fact that H_2O_2 and $\text{O}_2^{\cdot-}$ did not stimulate the epoxidase activity is consistent with the lack of inhibitory effect of catalase and superoxide dismutase (Table 2).

DISCUSSION

In the present investigation we have shown that haemoglobin catalyses the epoxidation of retinoic acid to 5,6-epoxyretinoic acid. The nature of the reaction is different from that reported by Sietsema & DeLuca (1979, 1982), since the haemoglobin-catalysed retinoic acid 5,6-epoxidation is inhibited by NADPH completely, whereas NADPH is essential to the epoxidase activity reported by Sietsema & DeLuca (1979, 1982). The difference indicates that two retinoic acid 5,6-epoxidase activities, haemoglobin and some other enzyme, exist in rat liver. In elucidation of retinoic acid 5,6-epoxidation it is necessary to consider both these activities.

The retinoic acid 5,6-epoxidation catalysed by haemoglobin may not be physiologically important, because epoxidation is probably not necessary for the function of retinoic acid in maintaining growth and differentiation (Sietsema & DeLuca, 1982) and the K_m value of the epoxidation catalysed by oxyhaemoglobin is in any case too high (approx. 2 mM) (A. Ikeda, H. Iwahashi & R. Kido, unpublished work), much greater than the concentration *in vivo* (McCormick & Napoli, 1982). Nevertheless account must be taken of the retinoic acid 5,6-epoxidation catalysed by haemoglobin in any study of the mechanism of the other reactions catalysed by haemoglobin.

Haemoglobin is a readily available major protein in the human body and it has the well-known function of the transport of O_2 from the lungs to peripheral tissues and of CO_2 from the peripheral tissues back to the lungs. Haemoglobin, however, has been implicated as a catalyst in various other reactions, such as lipid peroxidation

(Tappel, 1953), decarboxylation of dopa (3,4-dihydroxyphenylalanine) (Yamabe & Lovenverg, 1972; Dairnan & Christenson, 1973) and hydroxylation of aniline (Juchan & Symms, 1972; Symms & Juchan, 1974; Mieyal *et al.*, 1976; Mieyal & Blumer, 1976). Of these haemoglobin-catalysed reactions the hydroxylation of aniline can be compared with the 5,6-epoxidation of retinoic acid, since both are of the mono-oxygenase type. These two reactions are, however, quite different from one another. NADPH and NADPH-dependent cytochrome *P*-450 reductase are essential to the hydroxylation of aniline, whereas NADPH inhibited the epoxidation of retinoic acid completely. Thus haemoglobin-catalysed retinoic acid 5,6-epoxidation is a new type of haemoglobin-catalysed reaction.

Removal of Triton X-100 from the complete system decreased the retinoic acid 5,6-epoxidase activity of both rat liver homogenate and human haemoglobin (Table 1). Solubility of retinoic acid is low in water, and retinoic acid is scarcely solubilized when Triton X-100 is absent from the assay system. Hence an increase in the solubility of retinoic acid may explain the stimulation of the activity by Triton X-100.

Boiling of the crude rat liver homogenate decreased the activity to 48% of the initial value, and complete loss of activity was not observed (Table 1). The epoxidase is thus somewhat resistant to heat treatment at up to 100 °C. A similar result was also obtained with haemoglobin as the catalytic agent (Table 1). It is possible that denatured haemoglobin may retain some of its epoxidase activity.

The inhibitory effect of NADPH, NADH and 2-mercaptoethanol on the epoxidase activity suggests that radical species participate in the reaction. The absence of an inhibitory effect of active-oxygen scavengers, such as mannitol, superoxide dismutase and catalase, does not preclude the possibility that active oxygen participates in the epoxidation, since these active-oxygen scavengers may not be able to function in this experimental system. It will therefore be necessary to elucidate the relation between radical species, including active oxygen, and the retinoic acid 5,6-epoxidation catalysed by haemoglobin.

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