Oxene Transfer, Electron Abstraction, and Cooxidation in the Epoxidation of Stilbene and 7,8-Dihydroxy-7,8-dihydrobenzo[*a*]pyrene by Hemoglobin†

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ABSTRACT: Hemoglobin plus H_2O_2 oxidizes *trans*-stilbene to *trans*-stilbene oxide, *cis*-stilbene to *cis*- and *trans*-stilbene oxide, and *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene to *anti-trans*-7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. Oxidation of *cis*- and *trans*-stilbene to the corresponding *cis*- and *trans*-epoxides proceeds exclusively with incorporation of oxygen from the peroxide. Oxidation of *cis*-stilbene to the *trans*-epoxide, however, proceeds without detectable incorporation of oxygen from the peroxide and partial incorporation of oxygen from O_2. The epoxidations in which stereochemistry is conserved thus appear to involve ferryl oxygen transfer, whereas the epoxidations in which stereochemistry is inverted are proposed to involve protein-mediated cooxidation [Ortiz de Montellano, P. R., & Catalano, C. E. (1985) *J. Biol. Chem. 260*, 9265–9271] and possibly electron abstraction–water addition. The epoxidation of *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene incorporates oxygen from H_2O_2 and H_2O but not O_2. The oxidation of this substrate is thus consistent with ferryl oxygen transfer and electron abstraction but not protein-mediated cooxidation.

The carcinogenicity and toxicity of many organic substrates depends on their metabolic activation by redox enzymes. The activation of benzo[*a*]pyrene into a carcinogen, a case in point, requires epoxidation of the 7,8-double bond, hydrolysis of the epoxide to the *trans*-7,8-dihydrodiol and, finally, oxidation of the diol to the reactive 7,8-diol 9,10-epoxide (Thakker et al., 1985). Catalysis of the two oxidations in this sequence by cytochrome P-450 is well established, but other enzymes are known to also oxidize 7,8-dihydroxy-7,8-dihydrobenzo[*a*]-pyrene to the carcinogenic diol epoxide (Marnett, 1981). The possibility that hemoglobin is one of these enzymes is supported by indirect evidence. A linear correlation exists between the extent of covalent binding of radiolabeled benzo[*a*]pyrene to skin DNA and hemoglobin when the hydrocarbon is painted on the skin of mice (Shugart, 1985). Acidic hydrolysis of the labeled hemoglobin yields the 7,8,9,10-benzo[*a*]pyrenetetrols expected if the protein is alkylated by the *anti*-diol epoxide (Shugart et al., 1984; Marnett et al., 1979; Shugart & Matsunami, 1985). *syn*-tetrols were not detected among the hydrolysis products, a result which suggests that the *syn*-diol epoxide was not involved in the alkylation reaction. *syn*- as well as *anti*-diol epoxides are generally formed if the oxidation is catalyzed by cytochrome P-450 but not if the oxidation is catalyzed by prostaglandin synthase or other peroxidative mechanisms (Marnett, 1981; Sivarajah et al., 1979; Shugart, 1985). Finally, peroxide-dependent covalent attachment of radiolabeled benzo[*a*]pyrene to the hemoprotein has been demonstrated to occur in incubations with hemoglobin (Rice et al., 1983). The specific role of hemoglobin remains ambiguous, however, because (a) the in vivo labeling of hemoglobin could result exclusively from reaction of the hemoprotein with the diol epoxide produced by cytochrome P-450 and (b) the structure of the hemoglobin adduct(s) formed in vitro is not known. The latter ambiguity is important because the fact that peroxygenation of benzo[*a*]pyrene (as distinguished from its 7,8-dihydroxy-7,8-dihydro derivative) yields phenolic and quinonoid products suggests that the adducts formed in vitro may involve quinone rather than diol epoxide metabolites (Marnett, 1981).

Hemoglobin oxidizes arylamines, phenols, and other organic substrates with relatively low oxidation potentials (Mieyal, 1985; Golly & Hlavica, 1983; Lenk & Sterzl, 1984; Cambou et al., 1984; Yamaguchi et al., 1985). These reactions have been investigated primarily with intact erythrocytes or complex systems composed of methemoglobin, a reductive component (usually cytochrome P-450 reductase plus NADPH), and, in some instances, an electron-transfer agent such as methylene blue. The role of the reducing system and the mechanisms of these reactions are not yet clear. In the case of reactions supported by riboflavin and NADPH, the reducing system functions simply as a source of H_2O_2 (Cambou et al., 1984). However, Mieyal et al. (1976) and Hlavica and Golly (1983) argue that H_2O_2 is not the oxidant in the reactions supported by cytochrome P-450 reductase and NADPH even though the reactions are inhibited by catalase. Mieyal et al. based their conclusion on the fact that they could not independently detect H_2O_2 and had to use higher concentrations of hemoglobin when they replaced the reducing system with H_2O_2. Hlavica and Golly derive their conclusion from the observation that their reactions are inhibited by carbon monoxide, a finding that implicates the ferrous state of the hemoprotein in the catalytic process. Inhibition by carbon monoxide does not preclude the involvement of H_2O_2, however, because ferrous hemoglobin in the presence of oxygen and an electron donor may catalyze the production of H_2O_2 in a manner akin to that observed when oxyhemoglobin reacts with electron donors (Wallace & Caughey, 1975; Kawanishi & Caughey, 1985). In sum, it is clear that H_2O_2 is the oxidant in some of the reduction-dependent systems, but the contradiction between the inhibition by catalase and the evidence against involvement of H_2O_2 in the cytochrome P-450 reductase/NADPH system leaves open the possibility that an oxidant other than free H_2O_2

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is involved in some of the reduction-dependent systems.

The mechanistic complications introduced by the presence of reducing agents are avoided if the reducing agents are simply replaced by H_2O_2, although the catalytic properties of the reduction- and H_2O_2-dependent systems are likely to differ even in those instances where H_2O_2 is the active oxidant in the reduction-dependent system. The relative simplicity of the hemoprotein/H_2O_2 sytems makes them more amenable to detailed mechanistic analysis.[1] We recently confirmed that the epoxidation of styrene by erythrocytes, a transformation first reported by Belvedere and Tursi (1981), is catalyzed by methemoglobin plus hydrogen peroxide (Ortiz de Montellano & Catalano, 1985). Studies of the stereochemistry and mechanism of the epoxidation reaction led us to conclude that the oxidation is mediated, at least in part, by a mechanism distinct from that envisioned for cytochrome P-450. In order to rationalize the finding that a large fraction of the epoxide oxygen derives from molecular oxygen, even though the oxidant is H_2O_2, and the fact that the stereochemistry of the olefin is partially lost during the epoxidation reaction, we proposed that the epoxidation is mediated by the peroxy radical formed when molecular oxygen binds to a catalytically generated protein radical. The feasibility of this cooxidation mechanism has been confirmed by model studies with horseradish peroxidase as the oxidant and cresol as a substitute for the protein tyrosine residue postulated to be the seat of the protein radical (Ortiz de Montellano & Grab, 1987). Protein-mediated cooxidation could occur inside or outside the heme crevice because the tyrosine that is probably involved is in contact with both the heme[2] and the exterior of the protein. The mechanistic analysis of the epoxidation of styrene is complicated, however, by the fact that labeled oxygen is incorporated into the epoxide from both $H_2{}^{18}O_2$ and ${}^{18}O_2$ and by the fact that the olefin stereochemistry is only partially lost. In order to more clearly define the mechanisms of hemoglobin- and myoglobin-catalyzed epoxidation reactions, we have investigated the H_2O_2-dependent oxidation of *trans*-stilbene, *cis*-stilbene, and 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene.[3]

Materials and Methods

Methemoglobin (bovine, type I), metmyoglobin (horse muscle, type I), H_2O_2 (30%), and Tween 20 were purchased from Sigma. Styrene, *cis*- and *trans*-stilbene, and *trans*-stilbene oxide were obtained from Aldrich. ${}^{18}O$-Labeled oxygen (98% ${}^{18}O$) was purchased from MSD Isotopes or Cambridge Isotope Labs and was used to synthesize $H_2{}^{18}O_2$ as previously described (Ortiz de Montellano & Catalano, 1985). The ${}^{18}O$ content of the peroxide (76.7 atom %) was determined by mass spectrometric analysis of menadione epoxide produced with the labeled peroxide (Ortiz de Montellano & Catalano, 1985). Racemic *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene and the four 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene isomers were obtained from the National Cancer Institute. Distilled, deionized water was used to prepare the buffers. The buffers were passed through a 2.5 × 30 cm Chelex (Bio-Rad) column to remove trace metal impurities. Electronic spectra were recorded on a Hewlett-Packard Model 8450A diode array spectrophotometer. Electron impact mass spectra were recorded at 70 eV on a Kratos MS-25 instrument coupled to a Varian 3700 gas chromatograph. NMR spectra were obtained in deuteriochloroform on a Varian FT-80 spectrometer. Chemical shift values are reported in parts per million relative to internal tetramethylsilane.

Purification and Chemical Epoxidation of cis-Stilbene. The commercially available *cis*-stilbene was contaminated with 2–3% *trans*-stilbene. The *cis*-stilbene was therefore purified by low-pressure chromatography on a Lichroprep Si-60 size B silica gel column (Merck) eluted with 10% diethyl ether: hexane at a flow rate of 6 mL/min (retention times: cis, 18.1 min; trans, 20.4 min). Fractions (5 mL) were collected and were examined by isothermal (180 °C) gas–liquid chromatography on a Hewlett-Packard 5890A instrument equipped with a 30-m DB-5-coated column. The retention times of the cis and trans isomers under these conditions were, respectively, 9.3 and 15.3 min. Fractions with a low content of the trans isomer were combined and concentrated on a rotary evaporator. The *cis*-stilbene thus obtained was contaminated with no more than 0.2% of the trans isomer. Authentic *cis*-stilbene oxide was prepared by vigorously stirring *cis*-stilbene (178 μL, 1 mmol) and *m*-chloroperbenzoic acid (378 mg, 2 mmol) in 10 mL of CH_2Cl_2 overnight. The organic layer was then washed with 1 N NaOH, water, and saturated NaCl solution and was dried over K_2CO_3. The residue obtained on solvent removal gave a peak with a retention time of 3.4 min on analysis by gas–liquid chromatography on a 6-ft column packed with 3% OV 225 on 100/120 mesh Supelcoport that was programmed to rise from 100 to 200 °C at 12 °C/min: ${}^1H$ NMR (80 MHz) 7.2 (m, 6 H, Ph–) and 4.3 ppm (s, 1 H, –CHO–); EIMS *m/z* 197 ($M^+ + 1$).

Oxidation of cis- and trans-Stilbene. Duplicate 10-mL incubations containing either bovine hemoglobin or equine myoglobin (40 μM heme), *cis*- or *trans*-stilbene (50 μM), and 100 μM Tween 20 in 0.2 M phosphate buffer (pH 7.4) were cooled to 0 °C, and cold aqueous H_2O_2 (600 μM final concentration) was added. The incubations were carried out at 0 °C to minimize decomposition of the hemoprotein by the peroxide. The solutions were kept at 0 °C for 90 min and were then extracted with diethyl ether (2 × 5 mL). The combined extracts were washed with brine, centrifuged to remove precipitated protein, dried over K_2CO_3, and concentrated under vacuum for analysis by isothermal (180 °C) gas–liquid chromatography on a Hewlett-Packard Model 5890 instrument equipped with a flame ionization detector and a 0.5 mm × 30 m DB-5 capillary column (retention times: *cis*-stilbene oxide, 11.4 min; *trans*-stilbene oxide, 15.7 min). The same procedure was employed in studies with ${}^{18}O$-labeled H_2O_2 except that the incubation volumes were raised to 25 mL and the product analyses were carried out by combined gas chromatography–mass spectrometry on a Kratos MS-25 instrument. The incubation volume in studies of the incorporation ${}^{18}O_2$ into the stilbene oxides was 100 mL. The incubation mixture was degassed and placed under an atmosphere of ${}^{18}O_2$ as described below.

Incubation times were shortened to 30 min, and diphenylmethane (1 μg) was added to the incubation mixtures prior

[1] The relationship between the mechanism proposed here for the oxidation by methemoglobin and H_2O_2 of styrene, *cis*- and *trans*-stilbene, and benzo[*a*]pyrene and that involved in the oxidation of aromatic amines by hemoglobin plus a reducing system and O_2 remains to be clarified. Exploratory experiments, however, show that aniline and *N*-methylaniline are oxidized by methemoglobin and H_2O_2 to, respectively, *p*-hydroxyaniline and aniline. The oxidation of aniline to *p*-hydroxyaniline under analogous conditions has been independently reported (Cambou et al., 1984).

[2] Abbreviations: heme, iron protoporphyrin IX regardless of the oxidation state; SCE, standard calomel electrode.

[3] A preliminary account of this work was presented at the Joint Meeting of the Division of Biochemistry, American Chemical Society, and the American Society of Biological Chemists, Washington, DC, 1986 (Catalano & Ortiz de Montellano, 1986).

Table I: Source of the Epoxide Oxygen

	incorporation of $^{18}O^a$ (%)				relative product yield^b (%)			
	bovine hemoglobin		horse myoglobin		bovine hemoglobin		horse myoglobin	
substrate	O_2	H_2O_2	O_2	H_2O_2	O_2	Ar	O_2	Ar
styrene^c	38	46	78	16	100	60	—	—
trans-stilbene	—	100	—	100	—	—	—	—
cis-stilbene								
cis oxide	1	100	<1^d	100	—	—	—	—
trans oxide	32	<7^d	—	—	—	—	—	
BAP-7,8-diol^e	<5^d	66	<1^d	50	100	150	100	88

^{a} Based on an ^{18}O content of 77% in the H_2O_2 (i.e., 100% incorporation indicates 76% ^{18}O in the product). A dash indicates the experiment was not done. ^{b} The product yield in incubations under an argon atmosphere is given relative to that under a normal O_2 atmosphere. The latter yields (see Table II) are assigned a value of 100%. A dash indicates the value has not been determined. ^{c} The values for incorporation of oxygen from H_2O_2 into styrene oxide are lower than those given in Ortiz de Montellano and Catalano (1985). The present experiments were carried out with Chelex-treated buffers, whereas the former experiments were not. ^{d} The actual incorporation of ^{18}O may be zero but in any case does not exceed the indicated value. ^{e} BAP-7,8-diol is *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene.

to workup when the intent was to quantitate product formation. A standard curve was constructed for these quantitative experiments by substituting known amounts (0.1, 0.5, 1.0, 5.0 μg) of the *cis*- or *trans*-stilbene oxides for the stilbenes in a series of otherwise normal incubations.

Oxidation of trans-7,8-Dihydroxy-7,8-dihydrobenzo[a]pyrene. A solution of racemic *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene (35 μM), the appropriate hemoglobin or myoglobin (10 μM in heme), and Tween 20 (100 μM) in 0.2 M Chelex-treated phosphate buffer (pH 7.4) was preincubated at 0 °C for 15 min before H_2O_2 (600 μM final concentration) was added. The volume of the incubation mixture was 10 mL for analytical experiments and 200 or 400 mL, respectively, for preparative incubations with hemoglobin and myoglobin. When required, the incubations were run in tubes that were held in ice while they were evacuated and then filled with nitrogen several times prior to introducing the desired reaction atmosphere. After incubation for 30 min, the solution was extracted with 2 volumes of ethyl acetate, and the combined extracts were washed with 1 volume of brine. The cis isomer of 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene was added as an internal standard to the incubation mixture prior to the extraction when product quantitation was desired. The extract was then dried over anhydrous $MgSO_4$ and was taken to dryness under a stream of nitrogen. The residue was taken up in 50 μL of methanol, filtered (5-μm filter needle), and analyzed by high-pressure liquid chromatography (HPLC) (see below). For further analysis, the HPLC fraction containing the purified tetrols was taken to dryness and the residue was taken up in 1 mL of 1:1 (v/v) pyridine–acetic anhydride. The solution was heated at 65 °C for 120 min. The solvent was then removed under a stream of nitrogen, and the residue was taken up in methanol, filtered, and subjected to high-pressure liquid chromatography (see below). The electron impact spectra of the purified tetrol tetraacetates were obtained by direct probe insertion on a Kratos MS-25 instrument.

High-pressure liquid chromatographic analyses of the benzo[*a*]pyrenetetrols were carried out on an Altech 10-μm C-18 reverse-phase analytical column eluted with 60% methanol–water at a flow rate of 0.7 mL/min. The eluent was monitored at 246 nm with a Hewlett-Packard 1040A diode array detector. The 200–450-nm absorption spectrum of each peak was recorded as it eluted from the column. The tetrol tetraacetates were chromatographed on the same column with 9:1 (v/v) methanol–water at 0.7 mL/min.

Results

trans-Stilbene. Incubation of *trans*-stilbene with bovine methemoglobin and H_2O_2, followed by gas–liquid chromatographic analysis of extracts of the incubation mixture, indicates that a single major product is formed with a retention time of 15.3 min (Figure 1). This product is identified as *trans*-stilbene oxide by cochromatography with authentic material and by the identity of its mass spectrum with that of a reference sample. A minor product with a retention time of 12.0 min is also detected by gas chromatography, but its identity is not known (Figure 1). The formation of both *trans*-stilbene oxide and the unidentified product is strictly dependent on the presence of hemoglobin and H_2O_2 (not shown). Incubation of *trans*-stilbene with methemoglobin and $H_2{}^{18}O_2$ (77 atom %) yields *trans*-stilbene oxide in which the oxygen derives

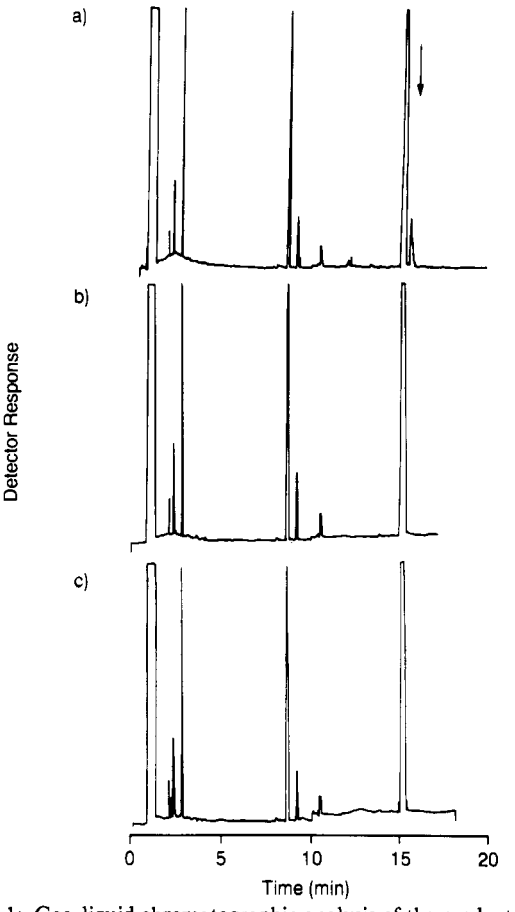


FIGURE 1: Gas–liquid chromatographic analysis of the products from oxidation of *trans*-stilbene by the hemoglobin/H_2O_2 system. The incubation and chromatographic conditions are given under Materials and Methods: (a) complete incubation system (the arrow marks the position of the major metabolite); (b) incubation with hemoglobin omitted; (c) incubation with H_2O_2 omitted.

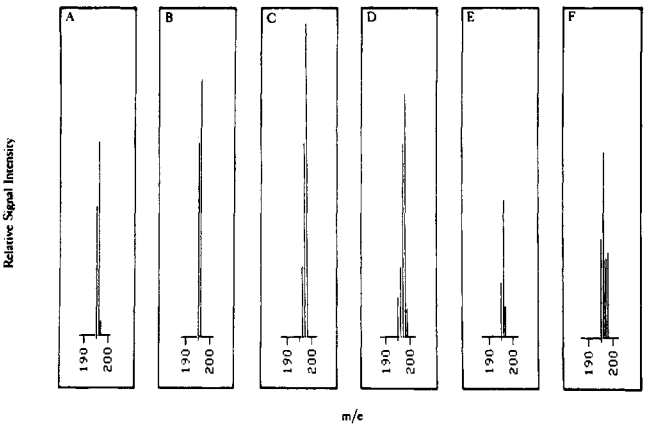


FIGURE 2: Molecular ion region of the mass spectra of (A) unlabeled *trans*-stilbene oxide, (B) unlabeled *cis*-stilbene oxide, (C) *trans*-stilbene oxide produced from *trans*-stilbene by methemoglobin and $H_2{}^{18}O_2$, (D) *cis*-stilbene oxide produced from *cis*-stilbene by methemoglobin and $H_2{}^{18}O_2$, (E) *trans*-stilbene oxide produced from *cis*-stilbene by methemoglobin and $H_2{}^{18}O_2$, and (F) *trans*-stilbene oxide produced from *cis*-stilbene in the presence of $^{18}O_2$.

Table II: Product Yields in the H_2O_2-Dependent Reactions Catalyzed by Hemoglobin and Myoglobin

substrate	product	yield (nmol/mL) Hb	Mb[a]
styrene	styrene oxide	35	12
trans-stilbene	*trans*-stilbene oxide	1.0	0.1
cis-stilbene	*cis*-stilbene oxide	0.7	0.3
	trans-stilbene oxide	0.2	0.05
BAP-7,8-diol[b]	BAP-7,8,9,10-tetrol[b]	0.05–0.1	0.01–0.03

[a] The myoglobin values are estimated from the yields of products obtained relative to those obtained with hemoglobin in 30-min incubations. [b] BAP-7,8-diol is *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; BAP-7,8,9,10-tetrol is *anti-trans*-7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene.

quantitatively from the peroxide (Figure 2; Table I). Horse myoglobin also oxidizes *trans*-styrene to *trans*-styrene oxide with quantitative incorporation of oxygen from the peroxide into the product (Table I).

cis-Stilbene. Two major products are found by gas–liquid chromatography in incubations of *cis*-stilbene with hemoglobin and H_2O_2 (Figure 3, peaks 1 and 3). The two products, identified by retention time and mass spectrometric comparisons with authentic standards, are *cis*- and *trans*-stilbene oxide. The *cis*- and *trans*-stilbene oxides are formed in approximately a 5:1 ratio (Figure 3; Table II), but neither product is formed in the absence of hemoglobin or H_2O_2 (Figure 3). As with *trans*-stilbene, a minor unidentified product (retention time 12.1 min) is also formed. The purified *cis*-stilbene employed in these experiments contains a trace (<0.2%) of *trans*-stilbene, but the proportion of the trans isomer is not measurably increased in the stilbene recovered from incubations with hemoglobin and H_2O_2. Horse myoglobin also oxidizes *cis*-stilbene to a mixture of the *cis*- and *trans*-stilbene oxides, but the yields of the two isomers are much lower than those provided by hemoglobin (Figure 3; Table II).

The *cis*- and *trans*-stilbene oxides obtained in incubations of *cis*-stilbene with hemoglobin and $H_2{}^{18}O_2$ (77 atom %) were analyzed by gas chromatography–mass spectrometry (Figure 2). The ^{18}O content of the *cis*-stilbene oxide (77%) is essentially identical with that of the H_2O_2 employed in the incubations. The oxygen in the cis epoxide thus derives exclusively from the peroxide (Table I). The oxygen in the *cis*-stilbene oxide isolated from incubations of *cis*-stilbene with horse myoglobin and ^{18}O-labeled H_2O_2 also derives quantitatively

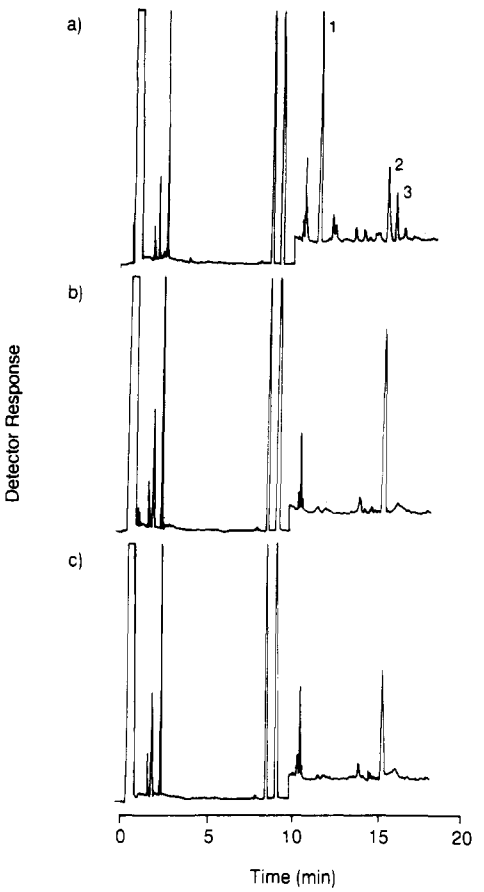


FIGURE 3: Gas–liquid chromatographic analysis of the products from oxidation of *cis*-stilbene by the hemoglobin/H_2O_2 system. The incubation and chromatographic conditions are given under Materials and Methods: (a) complete incubation system (peaks 1, 2, and 3, respectively, are *cis*-stilbene oxide, *trans*-stilbene, and *trans*-stilbene oxide); (b) incubation with hemoglobin omitted; (c) incubation with H_2O_2 omitted. The attenuation of the detector was decreased at approximately 10 min.

from the peroxide (Table I). In contrast, no ^{18}O is detected in the *trans*-stilbene oxide produced from *cis*-stilbene by hemoglobin, so the oxygen in this isomer derives from a source other than the peroxide. When the incubation of *cis*-stilbene is carried out under $^{18}O_2$, approximately 32% of the *trans*-stilbene oxide product is labeled (Figure 2). It has not been possible to carry out the incubation in $H_2{}^{18}O$ to determine if oxygen from water is incorporated into the epoxide because the large incubation volume makes the cost of the experiment prohibitive. It is clear, however, that the *trans*-stilbene oxide derived from *cis*-stilbene incorporates oxygen from the atmosphere and probably from water but not from the peroxide.

trans-7,8-Dihydroxy-7,8-dihydrobenzo[a]pyrene. Incubation of *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene with hemoglobin and hydrogen peroxide gives rise to low yields of two products (Figure 4). These incubations, like the stilbene incubations, were carried out at 0 °C because earlier studies showed that hemoglobin is more resistant at 0 °C than at 37 °C to degradation by H_2O_2 (Ortiz de Montellano & Kerr, 1985). Neither metabolite is formed if hemoglobin or hydrogen peroxide is omitted from the incubation mixture (Figure 4). Comparison of the retention time of the first metabolite with the retention times of the authentic 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene isomers (Figure 4) suggests that the metabolite is the anti-trans isomer of this tetrol (Figure 5). This identification is confirmed by the fact that the absorption spectrum of the free tetrol and the absorption and mass spectra of the tetraacetate

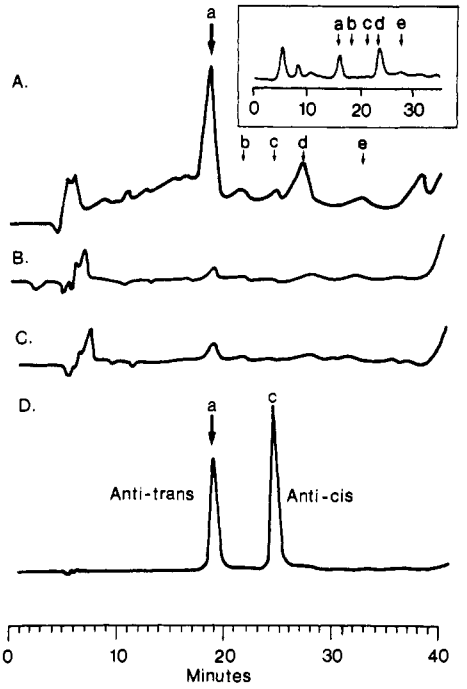


FIGURE 4: High-pressure liquid chromatographic analysis of the products of the oxidation of racemic *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene by bovine methemoglobin and H_2O_2. The chromatographic and incubation conditions are given under Materials and Methods: (A) complete incubation; (B) incubation with hemoglobin omitted; (C) incubation with H_2O_2 omitted; (D) 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene standards. The retention times of the anti-trans, syn-trans, anti-cis, and syn-cis isomers are labeled respectively as a, b, c, and e. Peak d is the methoxy derivative of the anti-trans isomer (see Figure 5). The inset shows the analysis of a second incubation. Comparison of the inset with trace A shows that the ratio of the methoxy ether to tetrol products (a:d) is variable.

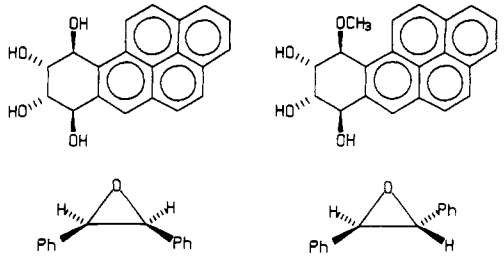


FIGURE 5: Structures of the products isolated from the oxidation of *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene and the stilbenes.

derivative are identical with those of authentic samples (not shown). Traces of the other three possible isomers of 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene are detected, but equal amounts of these isomers are found in the control incubations without the peroxide. The second product, which elutes between the anti-cis and syn-cis isomers, has the same retention time and absorption spectrum as the *anti-trans*-10-methoxy ether (Figure 5) obtained by adding methanol to 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (Dix et al., 1985). This same product is formed in incubations of 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene with hematin and lipid peroxides (Dix et al., 1985). The methyl ether presumably arises by reaction of the unhydrolyzed diol epoxide metabolite with the methanol into which it is taken up for analysis by high-pressure liquid chromatography.

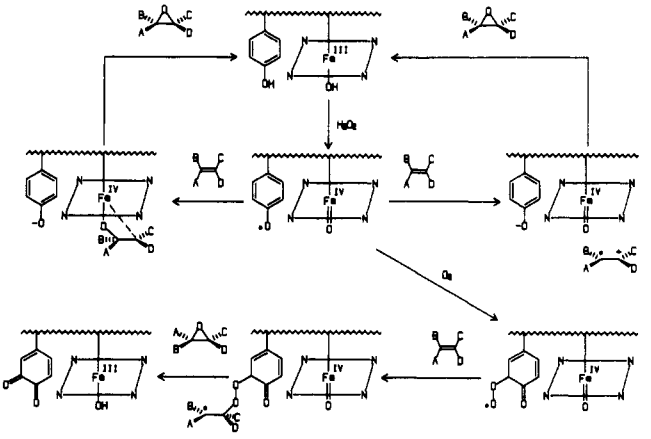
The origin of the oxygen in the *anti-trans*-7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene isolated from incubations with $^{18}O_2$ or $H_2{}^{18}O_2$ was determined by acetylation and mass spectrometry (Table I). Very little, if any, labeled oxygen (<5%) was incorporated into the tetrol obtained from incubations of the diol with methemoglobin and H_2O_2 under an atmosphere of $^{18}O_2$. In contrast, approximately 66% of the tetrol was ^{18}O-labeled when the reaction was carried out with $H_2{}^{18}O_2$ under a normal atmosphere. Confirmation that molecular oxygen is not involved in these reactions is provided by the observation that the yield of tetrol is essentially unchanged if the incubations are carried out under argon (Table I). These results contrast with our earlier demonstration that a large fraction of the styrene oxide is ^{18}O-labeled when styrene is oxidized by hemoglobin in the presence of $^{18}O_2$ or $H_2{}^{18}O_2$. The results also differ from those on the epoxidation of *cis* and *trans*-stilbene in that oxygen from the atmosphere is not incorporated into the product (Table I) (Ortiz de Montellano & Catalano, 1985). The oxidation of *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene by horse myoglobin has therefore also been examined because the fractional incorporation of oxygen from O_2 into styrene oxide is higher with horse metmyoglobin than with methemoglobin (e.g., 78% vs 38%) (Ortiz de Montellano & Catalano, 1985). However, the benzo[*a*]pyrenetetrol generated by horse metmyoglobin, like that produced by methemoglobin, incorporates little or no oxygen from O_2 (Table I), and its yield is not detectably decreased when the reaction is run under argon. The oxygen incorporated into the benzo[*a*]pyrene tetrol thus derives largely, but not exclusively, from the peroxide. The 40% or so of the oxygen that does not derive from the peroxide also does not derive from O_2 and therefore must be provided by the medium.

Discussion

The crystal structures of hemoglobin and myoglobin place the heme in a relatively inaccessible pocket (Ladner et al., 1977; Kendrew et al., 1960), but solution studies show that the heme can be reached by relatively large substrates. The existence of conformational states of the protein that accommodate organic substrates of some size within the heme crevice is demonstrated, inter alia, by the fact that substituted arylhydrazines react with hemoglobin and myoglobin to give complexes in which the aryl moiety is directly bound to the iron atom (Kunze & Ortiz de Montellano, 1983; Ringe et al., 1984; Ortiz de Montellano & Kerr, 1985). Comparison of the crystal structure of the myoglobin phenyl–iron complex with that of native myoglobin shows that the lateral displacement of several amino acid residues makes room for the aryl moiety (Ringe et al., 1984). NMR studies of the aryl–iron complexes, however, indicate that rotation about the carbon–iron bond is restricted by the protein (Ortiz de Montellano & Kerr, 1985). The heme crevice thus opens to accommodate relatively large organic substrates but then closes upon them and constrains their motion.

The similarities in the size and shape of styrene and phenylhydrazine suggest that the heme crevice is sufficiently large to bind styrene. The oxidation of styrene by methemoglobin and metmyoglobin, however, incorporates oxygen from both H_2O_2 and O_2 into the epoxide and proceeds with partial loss of the olefin stereochemistry. Since up to 78% of the oxygen incorporated into the epoxide derives from O_2, it appears that a major fraction of the epoxidation is accomplished by a process other than ferryl oxygen transfer. We have proposed an alternative mechanism for this reaction involving cooxidation of styrene by a protein-bound peroxy radical (Ortiz de Montellano & Catalano, 1985). The actual ratio of ferryl oxygen transfer to cooxidation, however, could not be determined in the earlier study because it was not possible to determine if incorporation of oxygen from the peroxide is linked

Scheme I: Hypothetical Mechanisms for the Hemoglobin-Catalyzed Epoxidation of *cis*-Stilbene, *trans*-Stilbene, and *trans*-7,8-Dihydroxy-7,8-dihydrobenzo[*a*]pyrene[a]



[a] The wavy line represents the protein surface. A tyrosine is shown as the amino acid that carries the unpaired electron, although this has not been unambiguously demonstrated. The oxygen transfer, protein cooxidation, and radical cation alternatives are shown.

to retention of the olefin stereochemistry. Furthermore, the catalatic generation of O_2 from H_2O_2 by hemoglobin and myoglobin left open the possibility that the labeled peroxide was cooxidatively incorporated into the product after its catalatic conversion to molecular oxygen. These ambiguities have been resolved by the present experiments with *cis*-stilbene, *trans*-stilbene, and *trans*-7,8-dihydroxy-7,8-dihydrobenzo-[*a*]pyrene.

trans-Stilbene is oxidized almost exclusively to *trans*-stilbene oxide (Figure 1). This reaction proceeds with quantitative incorporation of oxygen from $H_2{}^{18}O_2$ (Figure 2; Table I), a finding that clearly points to a ferryl oxygen transfer mechanism. *cis*-Stilbene is likewise oxidized to *cis*-stilbene oxide with quantitative incorporation of oxygen from the labeled H_2O_2 (Figure 2; Table I) and is therefore also consistent with a ferryl oxygen transfer mechanism. However, a fraction of the *cis*-stilbene is oxidized to the *trans*-epoxide that, in contrast to the *cis*-epoxide, incorporates no trace of oxygen from the peroxide (Figure 2; Table I). Incorporation of oxygen from the peroxide into the product is thus inseparably linked with retention of the olefin stereochemistry, whereas failure to incorporate oxygen from the peroxide is similarly linked with isomerization to the thermodynamically more stable trans isomer. The epoxide oxygen in the latter reaction derives in part from O_2 and in part, although not explicitly demonstrated, from the medium. The fraction of the oxygen provided by the medium is not precisely known, but at least 34% of the isomerized product incorporates oxygen from O_2. The intervention of an intermediate that allows free rotation about the central carbon–carbon bond and the incorporation of molecular oxygen suggest that a cooxidation mechanism similar to that invoked earlier for the epoxidation of styrene is responsible for this fraction of the epoxidation. In this mechanism, addition of a protein peroxy radical to the olefin double bond produces a free radical species without barriers to rotation about the central carbon–carbon bond (Scheme I). The mechanism that results in the incorporation of oxygen from the medium, which clearly differs from the proposed cooxidation or oxygen-transfer pathways, is discussed below in the context of the epoxidation of *trans*-7,8,-dihydroxy-7,8-dihydrobenzo[*a*]pyrene.

Approximately 50% of the oxygen added to 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene by hemoglobin derives from H_2O_2. The remaining fraction of the oxygen in the tetrol product does not derive from molecular oxygen and therefore must be provided by the medium. Half of the epoxidation of 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene thus appears to proceed by a ferryl oxygen transfer mechanism and the other half by a process distinct from both ferryl oxygen transfer and protein-mediated cooxidation. The third epoxidation mechanism required to fully explain the epoxidation of 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene probably involves oxidation of the polycyclic aromatic hydrocarbon to a radical cation. The radical cation can then be converted to the epoxide by addition of water, loss of an electron, and epoxide ring closure or directly to the tetrol by a similar sequence that is terminated by addition of a second molecule of water rather than by epoxide ring closure (Scheme I). The data suggest that the latter is, at best, a minor pathway because addition of methanol to the incubation mixture after the reaction is terminated produces in good yield the methyl ether expected from reaction of methanol with the epoxide (Figure 4, insert). A similar mechanism may explain the incorporation of water into the *trans*-stilbene oxide produced from *cis*-stilbene. The probable intervention of a radical cation pathway in the case of 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene and stilbene, but not styrene, is compatible with the differences in their oxidation potentials ($E_{1/2}$ vs SCE): styrene, 2.1 V;[4] *cis*-stilbene, 1.54 V; *trans*-stilbene, 1.51 V; 7,8-dihydroxy-7,8-dihydrobenzo-[*a*]pyrene, estimated 0.9–1.16 V[5] (Cavalieri & Rogan, 1984; Heimbrook et al., 1986; Meites & Zuman, 1976). Evidence exists for oxidation of polycyclic aromatic hydrocarbons and stilbene to their radical cations by, respectively, horseradish peroxidase (Cavalieri & Rogan, 1984) and bleomycin (Heimbrook et al., 1986). The radical cation mechanism thus provides a reasonable mechanism for incorporation of oxygen from sources other than H_2O_2 or O_2 into the epoxide products. However, in the absence of independent evidence for hemoglobin-catalyzed one-electron oxidation of stilbene and 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene and of the incorporation of water into the products, the radical cation mechanism remains speculative.

An alternative explanation for incorporation of oxygen from the medium into the epoxides, exchange of the ferryl oxygen with water prior to reaction with the olefins, can be excluded. Exchange of the ferryl oxygen has been observed with metalloporphyrin models (Groves & Kruper, 1979; Groves et al., 1980) and horseradish peroxidase (Hashimoto et al., 1986). It has also been suggested to occur with cytochrome P-450 (Macdonald et al., 1982; Heimbrook et al., 1981) but this is probably not true [see discussion in Ortiz de Montellano (1986)]. Exchange of ferryl oxygen with the medium has been invoked to explain the incorporation of oxygen from water into the stilbene oxides produced by bleomycin (Murugesan & Hecht, 1985). However, the fact that hemoglobin and myoglobin oxidize *trans*- and *cis*-stilbene to the *trans*- and *cis*-stilbene oxides, respectively, with no incorporation of oxygen from the medium clearly argues that the ferryl oxygen does not undergo significant exchange within the 30-min time frame of the reaction. This is consistent with the report that reaction of myoglobin with $H_2{}^{18}O_2$ yields the ^{18}O-labeled (unexchanged) ferryl species (Sitter et al., 1985)

[4] Styrene was found by cyclic voltammetry in acetonitrile to have an oxidation potential of approximately 2.1 V vs SCE. Literature values for the $E_{1/2}$ of styrene in several solvents are 2.45 (dimethylformamide), 2.35 (dioxane), and 2.9 V (tetrahydrofuran) (Meites & Zuman, 1976).

[5] We have not found an experimental value for the oxidation potential of 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene but estimate that it lies between that of pyrene (1.16 V) and benzo[*a*]pyrene (0.94 V) (Pysh & Yang, 1963).

Our earlier study demonstrated that part of the styrene epoxide produced by hemoglobin and myoglobin incorporates oxygen from the peroxide. The results obtained with the stilbenes and 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene suggest that this reflects direct transfer of the ferryl oxygen to the olefin rather than, as first proposed, incorporation of oxygen produced from the peroxide by the catalatic action of the hemoproteins (Ortiz de Montellano & Catalano, 1985). This interpretation is supported by the observation that approximately 33% of the styrene oxide produced from *trans*-[1-^{2}H]styrene, as expected for a ferryl oxygen transfer, is formed with retention of stereochemistry (Ortiz de Montellano & Catalano, 1985).

The results suggest that direct transfer of the ferryl oxygen to the double bond accounts for the fraction of the epoxidation of styrene, *trans*- and *cis*-stilbene, and 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene characterized by retention of stereochemistry and incorporation of oxygen from the peroxide. The ferryl oxygen transfer thus resembles that catalyzed by cytochrome P-450 enzymes and model metalloporphyrins (McMurry & Groves, 1986; Ortiz de Montellano, 1986), although the two types of ferryl oxygen transfers probably differ in mechanistic details. The reactions of hemoglobin and myoglobin with H_2O_2 yield an [Fe(IV)=O]/protein radical rather than the [Fe(IV)=O]/porphyrin radical cation characteristic of metalloporphyrin models, horseradish peroxidase compound I (King & Winfield, 1963; Yonetani & Schleyer, 1967), and probably cytochrome P-450, although the placement of the oxidation equivalents in P-450 has not been defined. If the protein radical does not participate in the epoxidation reaction, ferryl oxygen transfer will terminate with hemoglobin or myoglobin in the ferrous state. Electron transfer from the ferrous heme to the protein radical would then quench the radical and return the prosthetic group to the ferric state. A second alternative is that the oxidation equivalent associated with the protein radical is returned to the prosthetic group *during* the epoxidation reaction. A third alternative is that the reaction with H_2O_2 produces a transient two-electron species analogous to that of horseradish peroxidase compound I that rapidly oxidizes the protein or, in a competitive manner, the olefin. This last mechanism is only viable if the olefin reacts with the ferryl complex very rapidly because no species other than the [Fe(IV)=O]/protein radical has been detected with hemoglobin or myoglobin. The available data do not allow a distinction to be made between these alternative ferryl oxygen transfer mechanisms, but the failure of carbon monoxide to inhibit the epoxidation of styrene clearly suggests that the reaction mechanism does not traverse the ferrous state (Ortiz de Montellano & Catalano, 1985).

The finding that the proposed cooxidation mechanism contributes to the epoxidation of styrene and *cis*-stilbene but not *trans*-stilbene or 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene is unexpected. Simple steric arguments suggest that the importance of ferryl oxygen transfer, which requires entry of the substrate into the sterically constrained heme crevice, should decrease in the order styrene > *cis*-stilbene > 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene > *trans*-stilbene. The converse order might be expected from the same steric arguments for the importance of cooxidation if the olefins are oxidized at the outer protein surface. Neither the ferryl oxygen transfer nor the cooxidation pathways, however, adhere to these expectations (Table I). It might be argued that the high lipophilicities of the stilbenes and 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene, which must be solubilized with Tween 20, disfavor binding to the protein surface, but the difference between the two stilbene isomers cannot be similarly rationalized. If cooxidation occurs outside the heme crevice, a shape-selective binding site must exist that controls access of the substrates to the peroxy radical. Structure specificity is expected, of course, if the cooxidation occurs primarily within the heme crevice.

Table III: Relative Importance of the Proposed Epoxidation Mechanisms[a]

	oxygen transfer (%)	cooxidation (%)	other (radical cation) (%)
hemoglobin			
styrene	46	38	16
trans-stilbene	100		
cis-stilbene	81	6	13
BAP-7,8-diol[a]	66	<5	29
myoglobin, horse			
styrene	16	78	6
trans-stilbene	100		
cis-stilbene	86	<1	13
BAP-7,8-diol[b]	50	<1	49

[a] The oxygen transfer value, based on incorporation of oxygen from H_2O_2, is firm. The cooxidation value, based on incorporation of oxygen from O_2, is likely to be low due to possible dilution of the labeled oxygen by unlabeled oxygen. The "other" (radical cation) value is the difference between 100% and the sum of the oxygen transfer and cooxidation values. It is therefore a maximum value because it includes the error in the cooxidation mechanism introduced by whatever contamination occurs with unlabeled oxygen. [b] BAP-7,8-diol is *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene.

Hemoglobin, as shown here, is able to catalyze the final step in the activation of benzo[*a*]pyrene to a carcinogenic species. Oxidation of the parent hydrocarbon to the diol by cytochrome P-450 could therefore be followed by hemoglobin- or myoglobin-catalyzed activation of the diol to the diol epoxide in remote target tissues. The toxicological significance of such a relay activation mechanism is difficult to evaluate with the available information. It is not known, for example, if the benzo[*a*]pyrene metabolites that are covalently bound to hemoglobin when the hydrocarbon is administered to rodents result from diffusion (Hsu et al., 1978) of preformed 7,8-diol 9,10-epoxide into the bloodstream or from activation of the 7,8-dihydrodiol by hemoglobin. The inefficiency of the hemoglobin- and myoglobin-catalyzed reactions (Table II) and the limited concentration of H_2O_2 under normal physiological conditions suggest that hemoglobin and myoglobin do not play a major role in the normal activation of polycyclic aromatic hydrocarbons. In view of the high local concentrations of hemoglobin and myoglobin, however, it is possible that these enzymes are significantly involved in the activation of hydrocarbons in some tissues or in situations where hydrogen peroxide concentrations are elevated. The report that stimulated polymorphonuclear leukocytes activate 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene to genotoxic metabolites (Thrush et al., 1985) is of interest in this context because the same system converts oxyhemoglobin to methemoglobin and elevates the hydrogen peroxide concentration in intact erythrocytes (Grisham et al., 1984; Weiss, 1982). The hemoglobin-supported oxidation of polycyclic aromatic hydrocarbons and other procarcinogens thus could contribute to inflammation-linked carcinogenesis.

In summary, the epoxidation of *cis*- and *trans*-stilbene and 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene by methemoglobin or metmyoglobin and H_2O_2 provides evidence for the existence of three oxidative mechanisms: (a) a ferryl oxygen transfer that may differ in detail from the analogous reactions catalyzed by metalloporphyrins and cytochrome P-450; (b) a cooxidation pathway probably mediated by protein residues on the internal

or external surface of the heme crevice; and (c) a third pathway for readily oxidized olefins that may involve radical cation intermediates. The balance between these reaction pathways, as summarized in Table III, depends on the olefin and, to some extent, on whether the reaction is catalyzed by hemoglobin or myoglobin.

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Registry No. BAP-7,8-diol, 57404-88-3; BAP-7,8,9,10-tetrol, 61490-66-2; H_2O_2, 7722-84-1; *trans*-stilbene, 103-30-0; *cis*-stilbene, 645-49-8; *trans*-stilbene oxide, 1439-07-2; *cis*-stilbene oxide, 1689-71-0.

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