

### [39] Oxidation of Olefins Catalyzed by Hemoglobin

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Besides its properties as an O<sub>2</sub> carrier, hemoglobin (Hb) has several enzymatic activities.<sup>1-3</sup> For example, it catalyzes the hydroxylation and the demethylation of various xenobiotics,<sup>4-6</sup> the binding of benzo[*a*]py-

<sup>1</sup> J. Everse, this volume [34].

<sup>2</sup> J. J. Mieyal and D. W. Starke, this volume [38].

<sup>3</sup> L. Zilletti, M. Cuiffi, S. Franchi-Micheli, F. Fusi, G. Gentilini, G. Maneti, and G. P. Sgaragli, this volume [37].

<sup>4</sup> B. L. Ferrarolo and J. J. Mieyal, *Mol. Pharmacol.* **21**, 1 (1982).

<sup>5</sup> D. W. Starke, K. S. Blisard, and J. J. Mieyal, *Mol. Pharmacol.* **1**, 25 (1984).

<sup>6</sup> O. Augusto, K. L. Kunze, and P. R. Ortiz de Montellano, *J. Biol. Chem.* **257**, 6231 (1982).

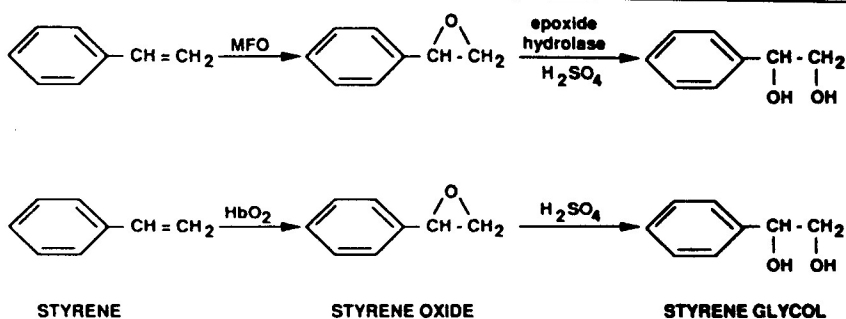


FIG. 1. Reaction of styrene catalyzed by mixed-function oxidases (MFO) and oxygenated hemoglobin ( $\text{HbO}_2$ ). The *in vivo* metabolism of styrene oxide includes conversion to styrene glycol catalyzed by epoxide hydrolase. The *in vitro* assay was performed measuring styrene glycol after quantitative hydrolysis with  $\text{H}_2\text{SO}_4$ .

rene,<sup>7</sup> the oxidation of *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene to anti-*trans*-7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene,<sup>8</sup> as well as the oxidation of olefins to olefin oxides.<sup>8-11</sup> Styrene is an olefin monomer used widely in the production of plastics, resins, and synthetic rubber. The first step of styrene metabolism in the organism, the conversion to styrene oxide by mixed-function oxidases (MFOs), occurs mainly in the liver and is followed by further conversion of styrene oxide to styrene glycol, which is catalyzed by epoxide hydrolase.<sup>12</sup> To characterize the properties of the various cytochrome *P*-450-dependent MFOs involved in the oxidation of styrene to styrene oxide, a method was developed to determine styrene oxide produced during incubation of styrene with rat liver microsomes in the form of styrene glycol (Fig. 1).<sup>13,14</sup>

In a study on the oxidation of styrene catalyzed by human lymphocyte MFOs, it was observed that the production of styrene oxide was greatly increased when cells were contaminated with blood.<sup>9</sup> This

<sup>7</sup> R. H. Rice, Y. M. Lee, and W. D. Brown, *Arch. Biochem. Biophys.* **221**, 417 (1983).

<sup>8</sup> C. E. Catalano and P. R. Ortiz de Montellano, *Biochemistry* **26**, 8373 (1987).

<sup>9</sup> G. Belvedere and F. Tursi, *Res. Commun. Chem. Pathol. Pharmacol.* **33**, 273 (1981).

<sup>10</sup> F. Tursi, M. Samaja, M. Salmona, and G. Belvedere, *Experientia* **39**, 593 (1983).

<sup>11</sup> P. R. Ortiz de Montellano and C. E. Catalano, *J. Biol. Chem.* **260**, 9265 (1985).

<sup>12</sup> K. C. Leibman and E. Ortiz, *J. Pharmacol. Exp. Ther.* **173**, 242 (1970).

<sup>13</sup> G. Belvedere, J. Pachecka, L. Cantoni, E. Mussini, and M. Salmona, *J. Chromatogr.* **118**, 387 (1976).

<sup>14</sup> M. Salmona, J. Pachecka, L. Cantoni, G. Belvedere, E. Mussini, and S. Garattini, *Xenobiotica* **6**, 585 (1976).

necessitated a characterization of the enzymatic reaction catalyzed by erythrocytes.

#### Styrene Oxidation to Styrene Oxide Catalyzed by Oxygenated Erythrocytes

##### *Methods*

*Preparation of Erythrocytes and Lysates.* Human venous blood suitable for transfusion [with 0.68% (w/v) citrate as anticoagulant] is obtained from local blood banks. Blood is centrifuged (100 *g*) for 10 min at room temperature. The buffy coat is removed and the erythrocyte pellet is resuspended in twice the volume of 154 mM NaCl and then centrifuged at 400 *g* for 20 min. Erythrocytes are then washed once with phosphate-buffered saline (PBS) containing no  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ , centrifuged, and counted with standard methods. The suspension is adjusted to  $500 \times 10^6$  cells/ml with PBS.

To obtain the lysate, washed erythrocytes are frozen rapidly in acetone/ $\text{CO}_2$  and thawed four times at room temperature; the resulting samples are then centrifuged at 10,000 *g* for 10 min and the supernatant is used for the experiments.

*Tonometry.* Tonometry is an operation aimed at equilibrating a liquid sample with a gas of known composition to establish a known partial pressure of a gas in a liquid. The gas usually contains  $\text{O}_2$  and  $\text{N}_2$  in various amounts to obtain the desired partial pressure of  $\text{O}_2$  ( $P_{\text{O}_2}$ ) in the liquid. The composition depends on temperature, the kind of preparation (erythrocytes or Hb), and the desired percentage of  $\text{HbO}_2$  ( $S_{\text{O}_2}$ ). For the purposes of this work,  $P_{\text{O}_2}$  values in the range 0–150 mm Hg are achieved.

Obtaining a given gas mixture from cylinders with the composition certified by the manufacturer is accurate but not practical because each desired mixture requires a separate cylinder. We therefore use two gas cylinders, containing either air or  $\text{N}_2$ . The gas flows are controlled by two high-precision flow meters (Sho-Rate Brooks, SIAD, Milano, Italy) and are then mixed in a chamber containing glass wool. Most of the gas is used for tonometry (see below), and roughly 50 ml/min is directed to a paramagnetic  $\text{O}_2$  analyzer (OA 273 Taylor Servomex, Crowborough, Sussex, England; nominal accuracy  $\pm 0.01\%$ ). We thus obtain virtually any gas repartition in the desired  $P_{\text{O}_2}$  range using two cylinders. High reproducibility is ensured by calibrating the flow meters in advance.

Tonometry is performed in either an open or a closed system. The first (IL237, Instrumentation Laboratory, Paderno Dugnano, Italy) is particularly suitable when complete deoxygenation of the sample is required.

Gas humidified at 37° (to avoid evaporation of the sample) flows over liquid that is stirred to provide maximal surface area for gas exchange, allowing full equilibration of up to 2-ml samples in 20–25 min.

When intermediate  $P_{O_2}$  values are required, the closed system is used. This system is based on anaerobically sealed flasks that are washed with gas of known composition. After this operation, a sample (0.2–0.7 ml) of erythrocytes or Hb is injected into the flask, which is placed into a temperature-controlled rotating device (7 rpm) for 20 min. The flask is designed so that when rotated at this speed the solution or suspension forms a thin layer of liquid, exposing the largest surface area to exchange gas. The advantages of this system are the small sample volume and the low stress to which the sample is exposed, with consequent low risk of Hb denaturation, metHb formation, and depletion of erythrocytic metabolic intermediates such as 2,3-diphosphoglycerate. On the other hand, the closed system cannot completely deoxygenate the sample (see below).

The final  $P_{O_2}$  is calculated theoretically from the fraction of  $O_2$  in the gas phase ( $x_{O_2}$ ), the barometric pressure ( $P_B$ ), and the water vapor pressure ( $P_{H_2O}$ ):

$$P_{O_2} = (P_B - P_{H_2O})x_{O_2}$$

This equation does not yield the true  $P_{O_2}$  in the closed system. First, the water vapor correction does not apply because the gas used to wash the flask is dry (humidified gas forms water condensation inside the flask). Another adjustment accounts for washing flasks at <37° with consequent increase of total pressure during tonometry:

$$P_{O_2}(t_{\text{ton}}) = P_{O_2}(t_w)[(273 + t_{\text{ton}})/(273 + t_w)]$$

where  $t_{\text{ton}}$  and  $t_w$  are temperatures (in degrees Celsius) of tonometry and of flask washing, respectively. Total pressure inside the flask is also increased for the addition of incompressible liquid into a finite volume:

$$P_{O_2} = P_{O_2}[V_f/(V_f - V_s)]$$

where  $V_f$  and  $V_s$  are flask and sample volumes, respectively. Finally, a third correction accounts for the increase of total  $O_2$  in the flask from the  $O_2$  already dissolved in the sample before tonometry. The following formula relates the final  $P_{O_2}$  with sample [Hb] (g/liter), its  $S_{O_2}$  before tonometry ( $S_{O_{2in}}$ ), final  $S_{O_2}$  ( $S_{O_{2fin}}$ ), tonometry temperature ( $t_{\text{ton}}$ ), volume of Hb or erythrocytes ( $V_s$ ), and barometric pressure ( $P_B$ ):

$$P_{O_2} = P_{O_2} + [(S_{O_{2in}} - S_{O_{2fin}})/S_{O_{2in}}][\text{Hb}] \times V_s P_B (273 + t_{\text{ton}})(1.213 \times 10^{-4})$$



Closed tonometry requires care when using samples with a heavy bacterial contamination or with an increased number of leukocytes, because these factors substantially increase the overall  $O_2$  consumption. Under normal conditions, the  $P_{O_2}$  decrease for this reason is no more than 0.09 mm Hg over a period of 30 min.<sup>15</sup> Temperature is critical because it influences the water vapor pressure,<sup>16</sup> the Hb  $O_2$  affinity,<sup>17</sup> and the solubility of  $O_2$  in blood or aqueous solutions,<sup>18</sup> and is to be controlled within  $\pm 0.1^\circ$ .

Measurement of  $S_{O_2}$ .  $S_{O_2}$  is defined as:

$$S_{O_2} = [\text{HbO}_2]/([\text{HbO}_2] + [\text{Hb}])$$

The classical procedures to determine  $S_{O_2}$  depend on spectrophotometric methods because the absorption spectra of the various Hb derivatives are well defined,<sup>19</sup> allowing determination of Hb $O_2$  and other Hb derivatives under practically any condition. The drawback of these methods in the absence of sophisticated devices, such as fiber optics equipment, is the need to get the sample from the test tube or the tonometer into the spectrophotometer cuvette without appreciable contamination from atmospheric air.

To avoid these problems, we used a simple and inexpensive yet accurate method to measure  $S_{O_2}$  suitable for small volumes of Hb or erythrocytes. This method is based on the fact that Hb becomes oxygenated quickly when diluted and placed in alkaline buffer and requires a 1-ml stoppered aluminum cuvette equipped with a Clark-type  $O_2$  electrode to continuously monitor the  $P_{O_2}$  of the buffer and a magnetically operated stirrer (Fig. 2). No temperature control is needed.

Figure 2 illustrates a practical example of this method. Assume we have to analyze a sample of erythrocytes with 50%  $S_{O_2}$  and that the cuvette contains 2.5 mM sodium tetraborate and 5 mM  $\text{Na}_2\text{HPO}_4$  buffer at pH 9.1, previously equilibrated with air ( $P_{O_2} = 152$  mm Hg). When Hb is packed into the erythrocyte, it has a relatively low affinity for  $O_2$ , and the  $P_{O_2}$  at which  $S_{O_2} = 50\%$  usually corresponds to roughly 26–28 mm Hg (Fig. 2A). When 10  $\mu\text{l}$  of the erythrocyte suspension is added to 1 ml of buffer, which also contains 1 ml/liter Sterox SE (Baker Chemicals B.V.,

<sup>15</sup> M. Samaja, A. Mosca, M. Luzzana, L. Rossi Bernardi, and R. M. Winslow, *Clin. Chem. (Winston-Salem, N.C.)* **27**, 1856 (1981).

<sup>16</sup> J. R. Hall and R. G. Brouillard, *J. Appl. Physiol.* **58**, 2090 (1985).

<sup>17</sup> M. Samaja, D. Melotti, E. Rovida, and L. Rossi Bernardi, *Clin. Chem. (Winston-Salem, N.C.)* **29**, 110 (1983).

<sup>18</sup> C. Christoforides and J. Hedley-White, *J. Appl. Physiol.* **27**, 592 (1969).

<sup>19</sup> O. W. Van Assendelft, in "Spectrophotometry of Haemoglobin Derivatives." Royal Vangorcum Ltd., Assen, The Netherlands, 1970.

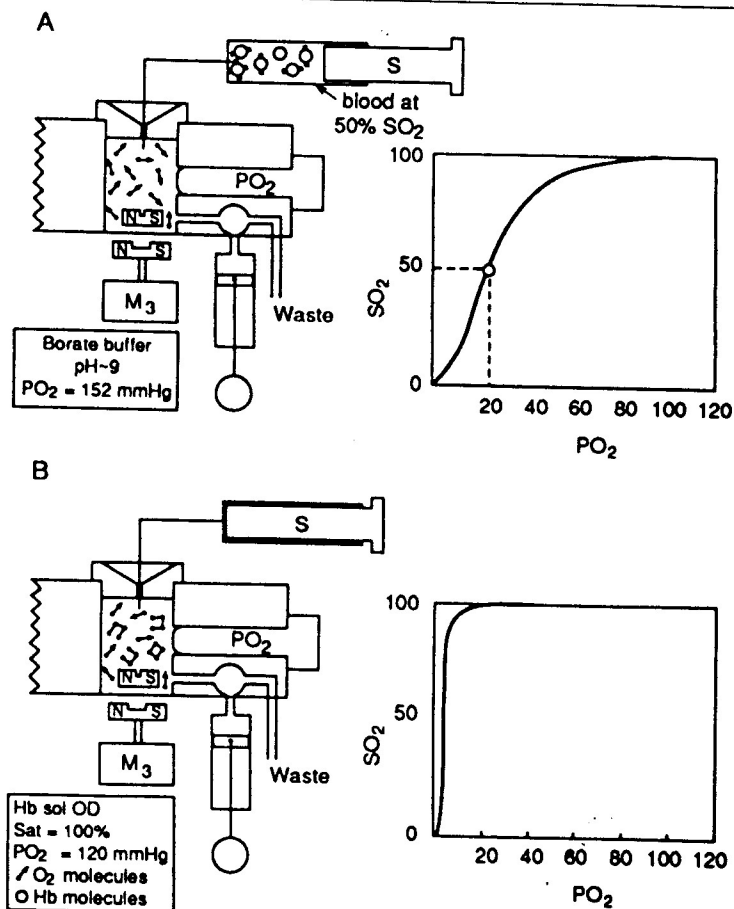


FIG. 2. Mixing of blood with  $S_{O_2} = 50\%$  with borate buffer at pH 9.1. Insets in A and B show the  $O_2$  dissociation curve of normal human blood or diluted normal human Hb, respectively.

The Netherlands) as hemolyzing agent, and 0.1 ml/liter silicone antifoam (Wacker Chemie, GMBH, Germany), the erythrocyte membrane is disrupted and Hb becomes diluted rapidly. Under these conditions of pH and protein concentration, the affinity of Hb for  $O_2$  increases greatly, and Hb becomes fully saturated with  $O_2$  at  $P_{O_2}$  values as low as 20 mm Hg (Fig. 2B). Because the cuvette in which the reaction occurs is sealed anaerobically, the total  $O_2$  content does not change. Therefore, the oxygenation process of deoxyHb, which usually occurs in less than 20 sec, causes

the  $P_{O_2}$  of the buffer to decrease in proportion to the amount of deoxyHb present in the original blood sample ( $\Delta P_{O_2}$ ). The exact amount of deoxyHb in the sample thus can be calculated from this decrease of  $P_{O_2}$ , the  $O_2$  solubility,<sup>20</sup> and the volumes of the sample and of the cuvette<sup>21</sup> (see below).

To calculate  $S_{O_2}$ , however, the total Hb concentration in the sample used to determine deoxyHb is required. This is done by two independent procedures:

1. By spectrophotometric methods with cuvettes equipped with the  $O_2$  electrode and a fixed-wavelength spectrophotometer (interference filters at 497, 565, and 620 nm) to determine the concentrations of HbO<sub>2</sub>, HbCO, and metHb.<sup>22</sup>
2. By the same  $P_{O_2}$  electrode, forcing HbO<sub>2</sub> to release  $O_2$  on oxidation by strong oxidant and measuring the increase of  $P_{O_2}$  ( $\Delta P_{O_2}$ ). For this purpose, 5  $\mu$ l of a solution containing 0.5 M  $K_3Fe(CN)_6$  in 0.7 M lactic acid is added because the more acidic pH induces faster (<20 sec) and complete (>99%) oxidation of HbO<sub>2</sub>.<sup>21</sup>

With the latter method, the two parameters needed to calculate  $S_{O_2}$  are measured simultaneously in the same sample.  $S_{O_2}$  is roughly estimated by the following equation:

$$S_{O_2} = [HbO_2]/([deoxyHb] + [HbO_2]) = (\Delta P_{O_2} - \Delta P_{O_2}')/\Delta P_{O_2}'$$

A more precise calculation takes into account factors such as the variation of the solubility for  $O_2$ , volume displacements, and contributions of dissolved  $O_2$  from addition of the reactants, and these are discussed in Samaja and Rovida.<sup>21</sup> The accuracy of this method is  $\pm 1\%$ . Figure 3 shows how a  $\pm 10\%$  error in one variable reflects on the final  $S_{O_2}$  measurement.

*Incubation of Erythrocytes with Styrene.* The incubation medium is composed of erythrocytes ( $500 \times 10^6$  cells/ml), 50  $\mu$ l/ml of 1 M styrene dissolved in acetonitrile, and PBS (pH 7.4) to yield a final Hb concentration of 0.25 mM heme. Blank samples without erythrocytes are also prepared. The incubation is carried out in a Dubnoff incubator for 30 min at 37°. Sealed flasks under  $N_2$  atmosphere are used for deoxygenated samples. The incubation is stopped by adding 0.4 ml of 0.6 N  $H_2SO_4$  per milliliter of incubation medium.

*Assay of Styrene Oxide.* The samples resulting from the above incubation (0.4 ml) are made alkaline with 0.8 ml of 0.6 N NaOH and then

<sup>20</sup> F. J. W. Rossington and J. W. Severinghaus, *J. Appl. Physiol.* **35**, 861 (1973).

<sup>21</sup> M. Samaja and E. Rovida, *J. Biochem. Biophys. Methods* **7**, 143 (1983).

<sup>22</sup> L. Rossi Bernardi, M. Perrella, M. Luzzana, M. Samaja, and I. Raffaele, *Clin. Chem. (Winston-Salem, N.C.)* **23**, 1215 (1977).

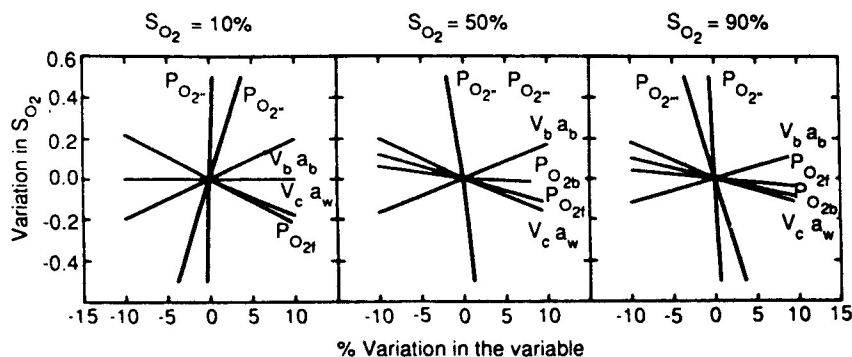


FIG. 3. Effects of the variation of a single variable ( $\pm 10\%$  max) on the value of  $S_{O_2}$  in three standard cases. Abbreviations:  $a_b$ ,  $O_2$  solubility coefficient in the erythrocyte sample;  $a_w$ ,  $O_2$  solubility coefficient in the aqueous buffer;  $P_{O_2^-}$ ,  $P_{O_2}$  of the buffer after addition of the erythrocyte sample;  $P_{O_2^-}$ ,  $P_{O_2}$  of the buffer after addition of the oxidant;  $P_{O_{2b}}$ ,  $P_{O_{2f}}$  of the erythrocyte sample before injection into the cuvette;  $P_{O_{2w}}$ ,  $P_{O_{2f}}$  of the ferricyanide solution before injection into the cuvette;  $V_b$ , volume of the erythrocyte sample;  $V_c$ , volume of the cuvette.

extracted twice with 3 ml of ethylacetate on a rotating shaker; 2.5 ml of this solution is transferred to conical test tubes, and the pooled extracts are dried under a gentle stream of  $N_2$  at  $37^\circ$ . The extraction residue is dissolved in 1 ml of toluene, to which 200  $\mu$ l of 0.05 M trimethylamine in toluene and 100  $\mu$ l of pure trifluoroacetic anhydride are added. Derivatization is carried out keeping the samples at  $60^\circ$  for 30 min.<sup>23</sup> Samples are cooled at room temperature and then 1 ml of distilled water and 1 ml of 5% ammonia solution (after 1 min) are added. The samples are shaken for 5 min and centrifuged at 3500 g. 1-Bromo-1-phenylethane (50  $\mu$ l; 28  $\mu$ g/ml in toluene) is used as a marker and is added to 50  $\mu$ l of the organic phase. Then 1  $\mu$ l of this solution is injected into a gas chromatograph (Carlo Erba Strumentazione, Milano, Italy) equipped with a  $^{63}Ni$  electron capture detector and a glass tube column (2 m  $\times$  4 mm i.d.) packed with 3% OV-17 on 100-120 mesh Gas-chrom Q (Supelco, Bellefonte, PA). The temperatures of the column, the injector port, and the detector are  $140^\circ C$ ,  $250^\circ C$ , and  $275^\circ C$ , respectively. The carrier gas is  $N_2$  at 30 ml/min flow rate.

The calibration curve was obtained using known amounts of styrene glycol (0.125, 0.25, 0.5, 0.75, 1, 1.5 and 2  $\mu$ g/ml PBS) processed as described above. The calibration curve is plotted by reporting on the X axis the amount of styrene glycol and on the Y axis the ratio of the area of

<sup>23</sup> G. Gazzotti, E. Garattini, and M. Salmona, *J. Chromatogr.* **188**, 400 (1980).

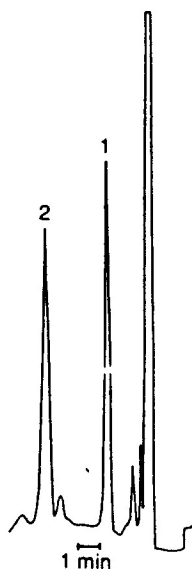


FIG. 4. Gas chromatogram of the styrene glycol trifluoroacetyl derivative. Peak 1 corresponds to the derivatized glycol and peak 2 to 1-bromo-2-phenylethane (marker). The amount of styrene oxide present in blank samples was subtracted from that found in those containing the erythrocytes.

the peak of styrene glycol to that of the marker 1-bromo-2-phenylethane (peaks 1 and 2 in Fig. 4).

### Results

Figure 4 is a typical chromatogram of the derivatized styrene glycol extracted from the reaction mixture of erythrocytes and styrene (peak 1) and its marker 1-bromo-2-phenylethane (peak 2). The sensitivity of the method was 100 ng/ml with a linearity range between 125 and 2000 ng/ml.<sup>23</sup>

The formation of styrene oxide at various styrene concentrations in intact erythrocytes and in lysates is shown in Fig. 5. With 50 mM styrene, a concentration that causes complete cell lysis within 5 min, the time courses overlapped (Fig. 5A). At a styrene concentration of 0.8 mM (Fig. 5B) which does not cause lysis, the erythrocytes were more active than the lysates. This was explained on the basis of higher Hb concentration inside the erythrocytes than in the lysate, although the total amount of Hb was the same in both samples.

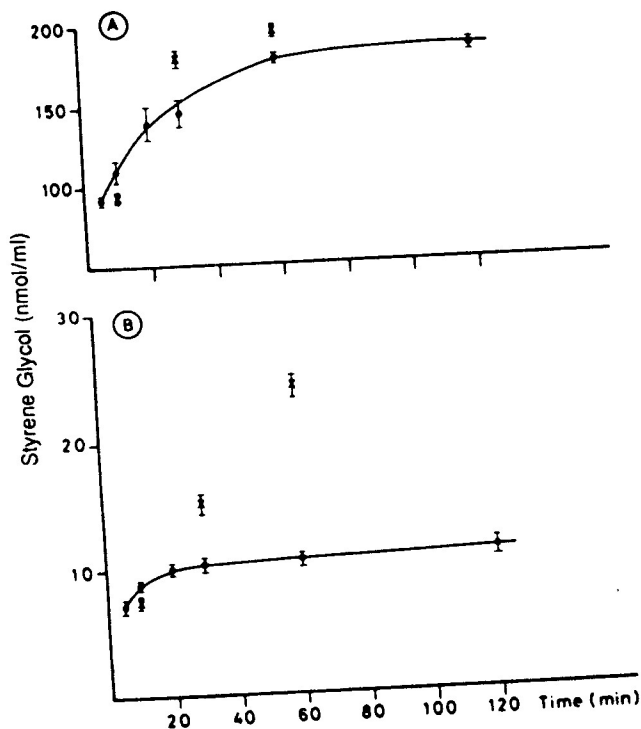


FIG. 5. Time course of styrene oxidation to styrene oxide in human erythrocytes (x) and cell lysate (●). Erythrocytes and lysate preparation were described in text (see Preparation of Erythrocytes and Lysates). Intact cells and lysates were incubated with 50 mM styrene (A) or 0.8 mM styrene (B). Mean  $\pm$  SEM.

Table I shows some characteristics of the enzymatic oxidation of styrene. The reaction was almost completely inhibited by CO, indicating the involvement of Fe<sup>II</sup> hemoprotein in the process.<sup>24</sup> In addition, it appears that the O<sub>2</sub>-derived free radicals such as O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, and OH· do not appear to be involved directly, although they originate from the autoxidation of Hb.<sup>25</sup> In fact, addition of various free radicals scavengers, i.e., superoxide dismutase (which converts O<sub>2</sub><sup>-</sup> to H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub>),<sup>25,26</sup> catalase (which re-

<sup>24</sup> I. Golly and P. Hlavica, *Biochim. Biophys. Acta* **760**, 69 (1983).

<sup>25</sup> H. P. Misra and I. Fridovich, *J. Biol. Chem.* **247**, 6960 (1972).

<sup>26</sup> J. M. McCord and I. Fridovich, *J. Biol. Chem.* **244**, 6049 (1969).

TABLE I  
STYRENE OXIDATION TO STYRENE OXIDE IN  
HUMAN ERYTHROCYTES<sup>a</sup>

System	Styrene glycol <sup>b</sup> (nmol/30 min/ml)
Erythrocytes	130.0 ± 6.0
+ CO <sup>c</sup>	29.2 ± 1.0
+ Superoxide dismutase (50 units/ml)	117.0 ± 1.4
+ Catalase (1750 units/ml)	158.3 ± 16.2
+ Tryptophan (2 mM)	104.0 ± 5.2
+ Mannitol (20 mM)	126.0 ± 6.3
+ Dimethyl sulfoxide (280 mM)	150.0 ± 8.0

<sup>a</sup> Styrene concentration in the incubation mixture was 50 mM. Data are mean ± SEM.

<sup>b</sup> The styrene oxide formed enzymatically was chemically converted to styrene glycol (see text, Incubation with Styrene).

<sup>c</sup> CO was bubbled into the incubation mixture for 1 min before addition of styrene.

moves H<sub>2</sub>O<sub>2</sub>),<sup>27</sup> and mannitol, dimethyl sulfoxide,<sup>28,29</sup> and tryptophan<sup>28</sup> (which are scavengers of the hydroxyl radical, OH·) did not inhibit styrene oxide formation. Some superoxide dismutase and catalase are contained in the erythrocyte.<sup>25,26</sup> However, it is possible that hemolysis dilutes these enzymes, thus preventing their protective action; therefore, they were added to the medium in saturating amounts.

Based on these experiments, the formation of styrene oxide was attributed to the O<sub>2</sub> bound to Hb. This O<sub>2</sub> may be in a partially reactive form in HbO<sub>2</sub>.<sup>30,31</sup> The involvement of HbO<sub>2</sub> in the oxidation of styrene was confirmed by the linear relationship between the fraction of HbO<sub>2</sub> and the amount of styrene oxide produced (Fig. 6).

#### Oxidation of Olefins to Olefin Oxides Catalyzed by metHb and H<sub>2</sub>O<sub>2</sub>

Ortiz de Montellano and Catalano have studied extensively the oxidation of styrene and *cis*- and *trans*-stilbene catalyzed by bovine metHb and hydrogen peroxide.<sup>8,11</sup>

<sup>27</sup> G. E. Gaetan, G. Galiano, L. Canepa, A. M. Ferraris, and H. N. Kirkman, *Blood* **73**, 134 (1979).

<sup>28</sup> R. C. Armstrong, and A. J. Swallow, *Radiat. Res.* **40**, 563 (1969).

<sup>29</sup> J. E. Spinc, J. L. Eaton, M. W. Anders, J. R. Hoidal, and R. B. Fox, *J. Clin. Invest.* **64**, 1642 (1975).

<sup>30</sup> J. Weiss, *Nature (London)* **202**, 83 (1964).

<sup>31</sup> S. Peisach, W. L. Blumberg, B. A. Wittenberg, and J. B. Wittenberg, *J. Biol. Chem.* **243**, 671 (1968).

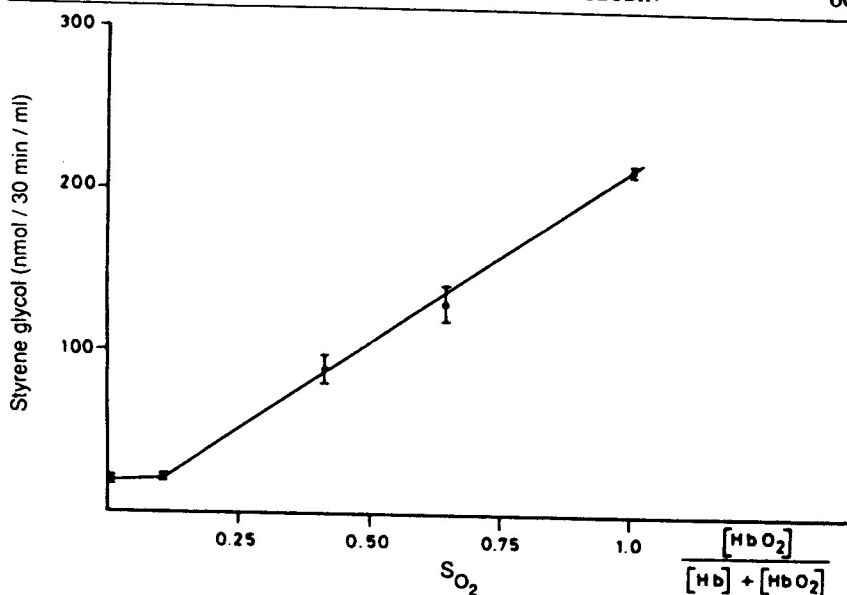


FIG. 6. Relationship between the fraction of  $HbO_2$  and styrene oxidation to styrene oxide in human erythrocytes. Preparation of partially deoxygenated erythrocytes is described in text (see Tonometry).

### Methods

**Incubations of Hb with Styrene.** Duplicate 10-ml incubations, containing bovine Hb (40  $\mu M$  heme), styrene (10 mM) or *cis*- or *trans*-stilbene (50  $\mu M$ ) in 0.2 M phosphate buffer, pH 7.4, are cooled to 0° for 15 min. An acetonitrile solution of styrene and stilbenes is employed in the preparation of this mixture, but the acetonitrile concentration in the final volume did not exceed 1%. A precooled solution of  $H_2O_2$ , to minimize decomposition of the hemoprotein, in 0.2 M phosphate buffer (0.6 mM final concentration) is then added to start the reaction, and the mixtures are incubated at 0° with occasional stirring for 90 min. 2-Undecanone (approximately 5  $\mu g/ml$ ) is added as an internal standard at the end of the incubation to quantitate product formation. For the analysis of stilbene oxides, 1  $\mu g$  of diphenylmethane is added to the incubation mixture. The samples are then extracted with diethyl ether (2  $\times$  5 ml) and the combined extracts are washed with saturated NaCl solution. The organic phase is filtered to remove precipitated protein and then dried over anhydrous  $K_2CO_3$ .

The ether solutions are reduced to volumes less than 0.5 ml and the concentrations of styrene metabolites are determined by gas chromatogra-



phy on a Varian 2100 flame ionization instrument fitted with a 6-ft glass column packed with 3% OV-225 on 100/200 mesh Supelcoport. The injector and detector are held at 250° while the oven temperature is programmed to rise from 80° to 140° at 15°/min. The *cis*- and *trans*-stilbene oxides are analyzed by gas chromatography on a Hewlett-Packard 5890A instrument equipped with a 0.5 mm × 30 m DB-5 capillary column at an isothermal temperature of 180°.

A set of eight incubations to which acetonitrile but not styrene is added are carried through the incubation procedure, and authentic styrene oxide or benzaldehyde (0, 10, 20, 40, 80, and 160 μg) is added before the 2-undecanone standard. For the analysis of stilbene oxides a standard curve is plotted by substituting known amounts (0.1, 0.5, 1, and 5 μg) of the *cis*- and *trans*-stilbene oxides for the stilbenes.

*Identification of the Metabolites of Styrene.* The ether extracts from 100-ml incubations are freed of styrene by low-pressure liquid chromatography on a Lichroprep Si-60 size B silica gel column (Merck) with 40% acetonitrile/water as the elution solvent. The eluent is monitored at 265 nm with a Hitachi Model 100 variable-wavelength detector. Metabolites are analyzed by high-performance liquid chromatography on an Altech Spherisorb S-5-ODS 5-μm C<sub>18</sub> reversed-phase column with 40% acetonitrile/water as the elution solvent. The eluent is monitored at 265 nm with the Hitachi Model 100 variable-wavelength detector. High-performance liquid chromatography is employed to avoid the formation of phenylacetaldehyde generated by decomposition of styrene oxide in the gas chromatograph. The amount of phenylacetaldehyde generated this way depends on the history of the chromatographic column. The least decomposition is observed when a gas chromatography column is freshly packed and baked at 200° for 30 min prior to use.

The structures of the metabolites detected in the incubations are confirmed by coinjection and mass spectrometric comparison with authentic samples.

Electron-impact (70-eV) mass spectra are obtained on a Kratos AEI-MS 25 mass spectrometer coupled to a Varian 3700 gas chromatograph fitted with a 30-μm SE 52 column programmed to rise from 50° to 150° at 5°/min.

*Incubations with <sup>18</sup>O<sub>2</sub>.* Styrene (100 μl of a 1 M solution in acetonitrile) is added to a solution of metHb (6.5 mg, 0.1 μmol) in 8.9 ml of 0.2 M phosphate buffer (pH 7.4). The mixture is cooled in an ice bath and is taken six times through a cycle in which the flask is first evacuated to <1 Torr and then brought to atmospheric pressure with nitrogen. In a final cycle 100 ml of <sup>18</sup>O<sub>2</sub> is introduced into the evacuated flask before nitrogen is used to bring the system to slightly above atmospheric pressure (a

balloon attached to the flask is used as a pressure indicator and reservoir). A N<sub>2</sub>-flushed syringe is then used to transfer 1 ml of a 6 mM H<sub>2</sub>O<sub>2</sub> solution that had been similarly cooled and deoxygenated. After a 90-min incubation at 0°, 50 ml of diethyl ether is added by syringe. The mixture is then worked up normally, and the styrene oxide and benzaldehyde obtained are analyzed by gas chromatography-mass spectrometry.

*Incubations with H<sub>2</sub><sup>18</sup>O<sub>2</sub>.* Labeled hydrogen peroxide is prepared from <sup>18</sup>O<sub>2</sub> as described by Sawaki and Foote.<sup>32</sup> The hydrogen peroxide concentration is determined by iodometric assay.<sup>33</sup> The <sup>18</sup>O content of the peroxide is determined by a new procedure based on alkaline oxidation of menadione to the corresponding epoxide by hydrogen peroxide<sup>34</sup> followed by mass spectrometric analysis. In brief, 35 mg of sodium carbonate is added to 50 μl of the peroxide in 1 ml of double glass-distilled water. A warm solution of menadione (430 μg, 2.5 μmol) in 1 ml of ethanol is then added, and 5 min later the ethanol is removed by rotary evaporation. Ether extraction of the residual mixture after addition of brine, followed by drying over CaCO<sub>3</sub>, yields a solution that is analyzed by gas chromatography-mass spectrometry. The sample used in the present studies contained 42% labeled O<sub>2</sub>, as shown by 42% incorporation of label into menadione epoxide. The incubation of labeled peroxide with styrene and the hemoprotein (40 μM heme concentration) is carried out as already described.

*O<sub>2</sub> Evolution.* The free O<sub>2</sub> concentration is measured by polarographic methods with the Clark O<sub>2</sub> electrode in a jacketed cell maintained at 0°. The electrode was calibrated against air-saturated water at the same temperature. The appropriate amount of Hb in 0.2 M phosphate buffer (pH 7.4) is mixed with 15-fold excess H<sub>2</sub>O<sub>2</sub> (heme basis) into the cell, and O<sub>2</sub> evolution is monitored for 90 min.

*Purification and Chemical Epoxidation of Cis-Stilbene.* Commercial preparations of *cis*-stilbene are usually contaminated with 2–3% *trans*-stilbene that must be eliminated to avoid interferences. *cis*-Stilbene is purified by low-pressure chromatography on a Lichroprep Si-60 size B silica gel column (Merck). The column is eluted with 10% diethyl ether/hexane at 6 ml/min. The retention times of *cis*- and *trans*-stilbene are 18.1 and 20.4 min, respectively. The collected 5-ml fractions are examined by isothermal (180°) gas-liquid chromatography (Hewlett-Packard 5890A) equipped with 0.5 × 30 mm DB-5 coated column. The retention times of

<sup>32</sup> Y. Sawaki and C. Foote, *J. Am. Chem. Soc.* **101**, 6292 (1979).

<sup>33</sup> M. Pesez and J. Bartos, in "Colorimetric and Fluorimetric Analysis of Organic Compounds and Drugs" (M. K. Schwartz, ed.), p. 329. Dekker, New York, 1974.

<sup>34</sup> L. Fieser and M. Fieser, "Reagents for Organic Synthesis," p. 466. Wiley, New York, 1967.

the *cis* and *trans* isomers under these conditions are 9.3 and 15.3 min, respectively. Fractions with low content of the *trans* isomer are pooled and concentrated on a rotary evaporator. The *cis*-stilbene product after this procedure is contaminated with less than 0.2% of the *trans* isomer.

Authentic *cis*-stilbene oxide is prepared by vigorously stirring 1 mmol *cis*-stilbene with 2 mmol *m*-chloroperbenzoic acid that has been washed previously with 0.2 M phosphate buffer and dried overnight under vacuum in 10 ml of  $\text{CH}_2\text{Cl}_2$ . The organic layer is washed with 1 N NaOH, water, and saturated NaCl, and is dried over  $\text{K}_2\text{CO}_3$ . The residue gives 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° at 12°/min.

### Results

*Oxidation of Styrene by metHb and  $\text{H}_2\text{O}_2$ .* Addition of  $\text{H}_2\text{O}_2$  to a solution of styrene and Hb at 0° causes an immediate shift of the Soret band peak from 408 to 412 nm, a position characteristic of the ferryl ( $\text{Fe}^{\text{IV}}$ ) complex. The shift is followed by a decrease in absorbance at 412 nm (Fig. 7), presumably an index of time-dependent irreversible loss of the heme.<sup>11</sup> The loss is independent of  $\text{H}_2\text{O}_2$  concentration for  $\text{H}_2\text{O}_2$ /heme ratios ranging from 5 to 40, but when the  $\text{H}_2\text{O}_2$ /heme ratio approaches unity the Soret band shifts back from 412 to 408 nm after 60 min, presumably because of exhaustion of  $\text{H}_2\text{O}_2$  in the medium and metHb regeneration. The formation of metabolites is not dependent on temperature, although the Soret band is lost more rapidly at 37° than at 0°. Therefore incubations were carried out at 0°.

Two styrene metabolites were detected by gas chromatography after incubation of styrene with metHb and  $\text{H}_2\text{O}_2$  (Fig. 7, Table II). These metabolites were identified as styrene oxide and benzaldehyde. Benzaldehyde, however, is not a product of secondary oxidation of styrene oxide or phenylethylene glycol because these compounds did not yield benzaldehyde when incubated with Hb and  $\text{H}_2\text{O}_2$ . Unlike styrene oxide, the formation of benzaldehyde is not significant before 30 min of incubation (Fig. 7).

The production of styrene oxide from styrene and metHb was characterized using various scavengers of the reactive  $\text{O}_2$  intermediates and anaerobic conditions. The low inhibition by CO suggests that iron does not cycle extensively through the ferrous state because it is not trapped as complex with CO.<sup>24</sup>

Product formation decreased in incubations carried out anaerobically but was never completely suppressed despite extensive efforts to ensure

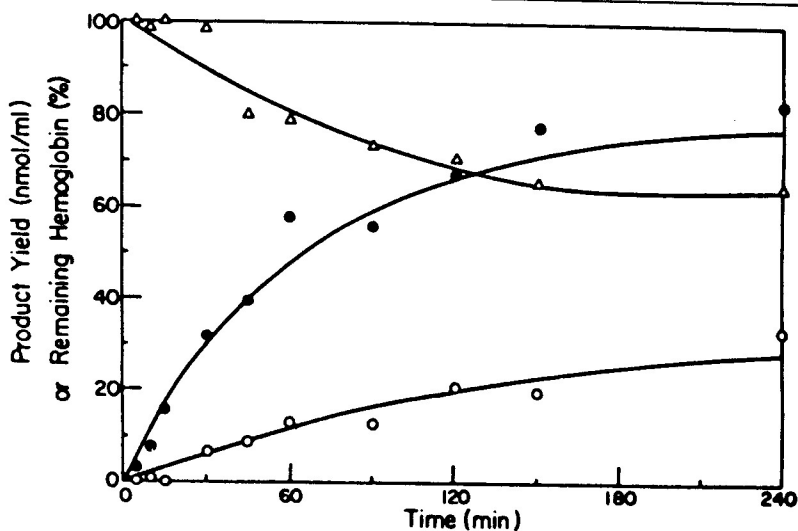


FIG. 7. Loss of heme and formation of styrene oxide and benzaldehyde as a function of time. The absorbance loss at 408 nm in standard incubations of styrene (10 mM) with metHb (10  $\mu$ M) and  $H_2O_2$  (0.6 mM) at 0°, as a percentage of the absorbance immediately after addition of the peroxide ( $\Delta$ ), is plotted as a function of time of incubation. The yields of benzaldehyde (O) and styrene oxide (●) in the same incubations are also plotted as a function of time.

complete removal of  $O_2$  before addition of  $H_2O_2$ . This is because the reaction of metHb with  $H_2O_2$  results in formation of  $O_2$  (Fig. 8). The amount of  $O_2$  produced per mole of heme in 90 min rises linearly with Hb concentration from 2 mol/mol to 5.5 mol/mol as heme concentration increases from 20 to 160  $\mu$ M. Approximately 2–3 mol of  $O_2$  are thus produced per mole of heme in standard incubations with 40  $\mu$ M heme. The  $O_2$  concentration, therefore, can reach values in the range of 100  $\mu$ M in incubations that are virtually anaerobic.

The  $H_2O_2$ -supported, metHb-catalyzed oxidation of styrene under an atmosphere of  $^{18}O_2$  resulted in incorporation of the label into 38% of the styrene oxide. The incubation of metHb and  $^{18}O_2$  was repeated three times to ensure that the fractional incorporation of label did not result from incomplete removal of unlabeled  $O_2$  from the incubation system. However, the result was approximately the same in all three experiments. It is, therefore, clear that a fraction of styrene oxide is formed in an  $O_2$ -dependent process. These results require a parallel oxidative mechanism to incorporate  $O_2$  from a source other than molecular  $O_2$  into styrene oxide. The finding that a fraction of the styrene oxide (70%), roughly complemen-

TABLE II  
FACTORS AFFECTING FORMATION OF STYRENE OXIDE  
AND BENZALDEHYDE<sup>a</sup>

Incubation	Styrene oxide	Benzaldehyde
Normal incubation <sup>b</sup>	100	100
- H <sub>2</sub> O <sub>2</sub>	8	6
- Hb	1	5
+ KCN (50 mM)	15	14
+ Mannitol (50 mM)	104	114
+ Ascorbic acid (50 mM)	3	28
+ POBN (50 mM)	57	101
+ BHT (50 mM)	74	70
+ DABCO (50 mM)	125	60
+ Catalase (60 units/ml)	ND <sup>c</sup>	16
+ Superoxide dismutase (60 units/ml)	107	100
+ CO (1:1 with O <sub>2</sub> )	95	85
+ Argon atmosphere	82	88

<sup>a</sup> Data expressed as percentage of controls. POBN,  $\alpha$ -(4-Pyridyl-1-oxide)-*N*-*tert*-butyl nitron; BHT, butylated hydroxytoluene; DABCO, diazabicyclo[2.2.1]octane.

<sup>b</sup> The results are averages of duplicate incubations except for the original controls and the values in the absence of O<sub>2</sub>, which are averages of six incubations. Styrene oxide and benzaldehyde formations in the control incubations were 54 and 29 nmol/ml, respectively.

<sup>c</sup> ND, Not detectable.

tary to the fraction labeled by molecular O<sub>2</sub> (38%), was labeled in incubations with <sup>18</sup>O<sub>2</sub>-labeled H<sub>2</sub>O<sub>2</sub> helps to identify the source of O<sub>2</sub> in this second mechanism. The sum of O<sub>2</sub>- and H<sub>2</sub>O<sub>2</sub>-derived product is more than 100%, but this discrepancy is readily accounted for by the fact that O<sub>2</sub> is generated from H<sub>2</sub>O<sub>2</sub> by the hemoprotein (Fig. 8). The incorporation of label from molecular O<sub>2</sub> into benzaldehyde in the reaction supported by metHb was also examined, but the 17% incorporation observed cannot be taken as a measure of the actual incorporation because the aldehyde O<sub>2</sub> exchanges readily with the medium. Mannitol, an OH· scavenger, had no effect on the reaction but butylated hydroxytoluene (BHT) and  $\alpha$ -(pyridyl-1-oxide)-*N*-*tert*-butyl nitron (POBN), two radical scavengers of relatively low specificity, partially inhibited metabolite formation (Table II). Ascorbic acid markedly inhibited metabolite formation, but this could reflect the reduction of radical intermediates as the direct reduction of metHb. The superoxide radical is ruled out as the oxidant because superoxide dismutase stimulated rather than inhibited metabolite formation;

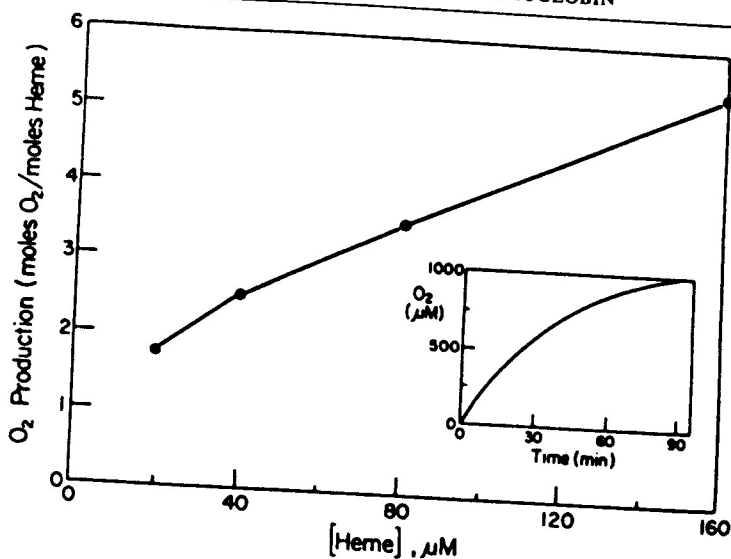


FIG. 8. Oxygen evolution in incubations of metHb with  $H_2O_2$  after 90 min. The  $O_2$  that evolved in standard incubations of metHb with  $H_2O_2$  in a closed cell at  $0^\circ$  is plotted against the hemoprotein concentration. The response of the Clark electrode, expressed as  $O_2$  evolved, is given as a function of time in the inset for the incubation with  $160 \mu M$  heme ( $40 \mu M$  metHb). No  $O_2$  is evolved in the absence of either component of the reaction mixture.

diazabicyclo[2.2.1]octane (DABCO), a singlet  $O_2$  trap, stimulated styrene oxide formation but somewhat inhibited benzaldehyde formation.

*Oxidation of cis- and trans-Stilbene to Stilbene Oxides Catalyzed by metHb and  $H_2O_2$ .* Incubation of *trans*-stilbene with bovine metHb 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 (Fig. 9). This product was identified as *trans*-stilbene oxide by cochromatography with authentic material and by the identity of its mass spectrum with a reference sample. A minor product with a retention time of 12.0 min is also detected by gas chromatography, but its identity is unknown (Fig. 9). The formation of both *trans*-stilbene oxide and the unidentified product is strictly dependent on the presence of Hb and  $H_2O_2$ . Incubation of *trans*-stilbene with metHb and  $H_2^{18}O_2$  (77 atom %) yields *trans*-stilbene oxide in which the  $O_2$  derives quantitatively from the peroxide.

Two major products are found by gas-liquid chromatography in incubations of *cis*-stilbene with Hb and  $H_2O_2$  (Fig. 10, peaks 1 and 3). These were identified by retention time and mass spectrometric comparison with

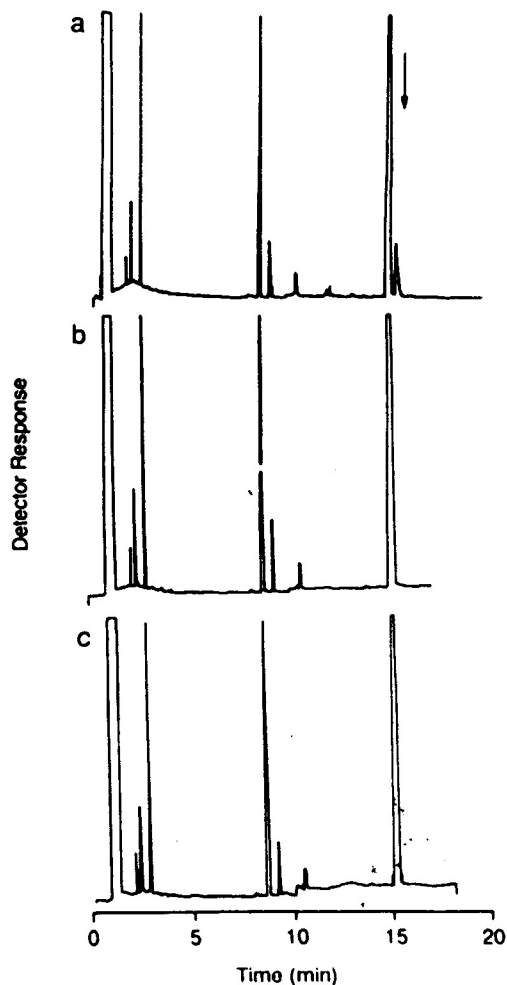


FIG. 9. Gas chromatographic analysis of the products from oxidation of *trans*-stilbene by the Hb/H<sub>2</sub>O<sub>2</sub> system: (a) complete incubation system (the arrow marks the position of the major metabolite); (b) incubation with Hb omitted; (c) incubation with H<sub>2</sub>O<sub>2</sub> omitted. The incubation and chromatographic conditions are discussed in text (see Incubation of Hb with Styrene). Reprinted with permission from Catalano and Ortiz de Montellano.<sup>8</sup> Copyright 1987 American Chemical Society.

authentic standards as *cis*- and *trans*-stilbene oxide. The *cis*- and *trans*-stilbene oxides are formed in a 3 : 1 ratio (Table III), but neither product is formed in the absence of Hb or H<sub>2</sub>O<sub>2</sub> (Fig. 10). As with *trans*-stilbene, a minor unidentified product (retention time 12.1 min) is also formed.

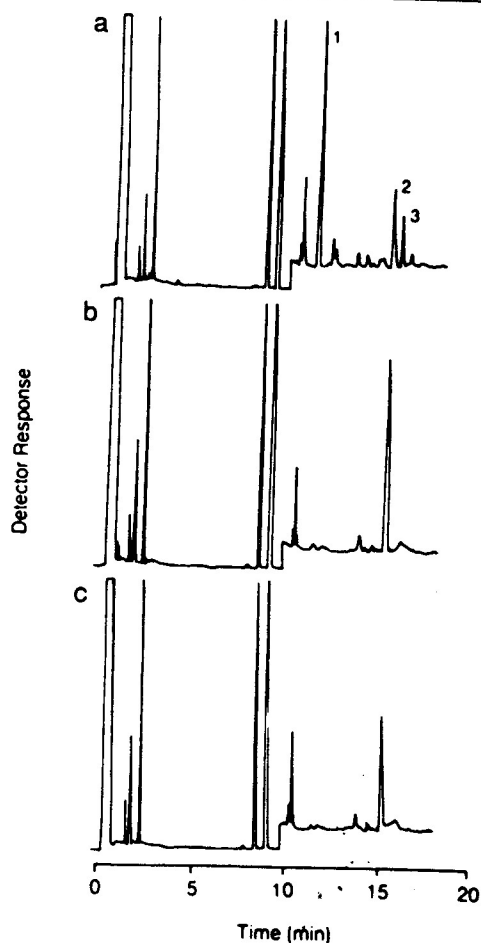


FIG. 10. Gas chromatographic analysis of the products from oxidation of *cis*-stilbene by the Hb/H<sub>2</sub>O<sub>2</sub> system: (a) complete incubation system (peaks 1, 2, and 3, respectively, are *cis*-stilbene oxide, *trans*-stilbene, and *trans*-stilbene oxide); (b) incubation with Hb omitted; (c) incubation with H<sub>2</sub>O<sub>2</sub> omitted. The attenuation of the detector was decreased at approximately 10 min. The incubation and chromatographic conditions are discussed in text (see Incubations of Hb with Styrene). Reprinted with permission from Catalano and Ortiz de Montellano.<sup>8</sup> Copyright 1987 American Chemical Society.

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 Hb and H<sub>2</sub>O<sub>2</sub>.



TABLE III  
 PRODUCTS AND YIELDS IN H<sub>2</sub>O<sub>2</sub>-DEPENDENT  
 REACTIONS CATALYZED BY Hb<sup>a</sup>

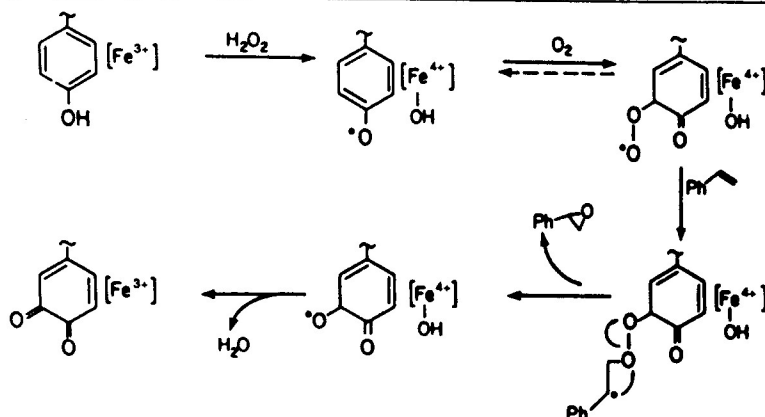
Substrate	Product	Yield nmol/ml
Styrene	Styrene oxide	35
<i>trans</i> -Stilbene	<i>trans</i> -Stilbene oxide	1.0
<i>cis</i> -Stilbene	<i>cis</i> -Stilbene oxide	0.7
	<i>trans</i> -Stilbene oxide	0.2

<sup>a</sup> Adapted with permission from Catalano and Ortiz de Montellano.<sup>8</sup> Copyright 1987 American Chemical Society.

The *cis*- and *trans*-stilbene oxides obtained in incubations of *cis*-stilbene with Hb and H<sub>2</sub><sup>18</sup>O<sub>2</sub> (77 atom %) were analyzed by gas chromatography-mass spectrometry. The <sup>18</sup>O<sub>2</sub> content of the *cis*-stilbene oxide (77%) is essentially identical with that of the H<sub>2</sub>O<sub>2</sub> employed in the incubations. The O<sub>2</sub> in the *cis* epoxide thus derives exclusively from the peroxide. In contrast, no <sup>18</sup>O<sub>2</sub> is detected in the *trans*-stilbene oxide produced from *cis*-stilbene by Hb, so the O<sub>2</sub> in this isomer derives from a source other than the peroxide. When *cis*-stilbene is incubated under <sup>18</sup>O<sub>2</sub>, approximately 32% of the *trans*-stilbene oxide product is labeled. It has not been possible to carry out the incubation in H<sub>2</sub><sup>18</sup>O to see whether O<sub>2</sub> 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 O<sub>2</sub> from the atmosphere and probably from water but not from the peroxide.

### Discussion

To investigate the oxidation of styrene catalyzed by Hb, we have used washed human erythrocytes and metHb plus H<sub>2</sub>O<sub>2</sub>. In both systems, the O<sub>2</sub>-derived free radicals do not appear involved in styrene oxidation. Indeed, OH· and O<sub>2</sub><sup>-</sup> scavengers have no effect. However, the mechanism of styrene oxide formation does not appear to be the same in the two systems because the presence of H<sub>2</sub>O<sub>2</sub> is required in the system containing metHb but not in the system with erythrocytes. The strong inhibition by CO in the latter system and the poor effect in the former can be correlated with the occurrence of the Fe<sup>II</sup> and Fe<sup>III</sup> states in the two systems, respectively.



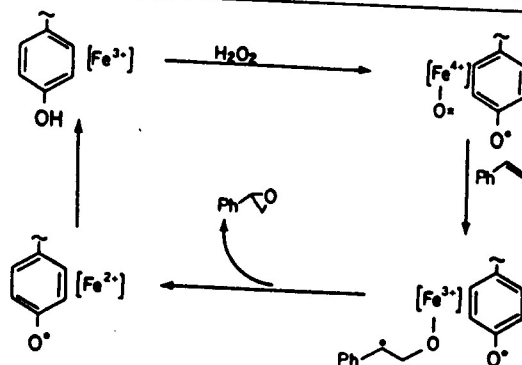
SCHEME I. Proposed mechanism for the incorporation of molecular O<sub>2</sub> into styrene oxide in the H<sub>2</sub>O<sub>2</sub>-dependent oxidation of styrene by metHb. The prosthetic heme, represented by the brackets around the iron, and the putative action site Tyr are shown. Ph, Phenyl ring in styrene. Alternative fates for the hydroxylated Tyr radical at the end of the sequence can be envisioned.

Strong experimental evidence indicates that in the system with metHb and H<sub>2</sub>O<sub>2</sub>, the formation of the ferryl complex is intimately connected with epoxidation of styrene. This evidence includes (1) the requirement of H<sub>2</sub>O<sub>2</sub>, (2) the persistence of metHb in the ferryl state throughout the reaction, (3) the time dependence of the heme loss, and (4) the strong inhibition by cyanide. However this is in contrast with the observation that the ferryl O<sub>2</sub> is found in only 38% of the styrene oxide produced in the presence of <sup>18</sup>O<sub>2</sub>. The inability of mannitol and superoxide dismutase to inhibit the reaction (Table II) suggests that neither O<sub>2</sub><sup>-</sup> nor OH• acts as oxidant in that reaction, although the modest inhibition by POBN and BHT does not prevent some minor participation of other unidentified radicals. Our data thus point to an oxidative mechanism triggered by a reaction with H<sub>2</sub>O<sub>2</sub> but primarily mediated by species other than the ferryl complex or a diffusible O<sub>2</sub> species.

Removal of one electron from Fe<sup>III</sup> and the protein in the reaction of metHb with H<sub>2</sub>O<sub>2</sub> generates the ferryl complex and a protein radical likely centered on a Phe or Tyr residue, as suggested by EPR studies.<sup>35</sup> A computer graphic analysis showed that the Tyr-42 residue is very close to the heme vinyl groups in bovine metHb.<sup>36</sup> This residue is part of the

<sup>35</sup> T. Yonetani and H. Schleyer, *J. Biol. Chem.* **242**, 1979 (1967).

<sup>36</sup> R. E. Dickerson and I. Geis, "Hemoglobin: Structure, Function, Evolution, and Pathology." Benjamin-Cummings, Menlo Park, CA, 1983.



SCHEME II. Mechanism for the incorporation of  $O_2$  from  $H_2O_2$  into styrene oxide in the oxidation of styrene by methHb. The active site of methHb is abbreviated as in Scheme I. Ph, Phenyl ring in styrene. An alternative mechanism employs molecular  $O_2$  generated from  $H_2O_2$  in a sequence similar to that shown in Scheme I.

outer surface of the hemoproteins and thus is in contact with the surrounding medium containing styrene and the heme.

A proposed mechanism (Scheme I) for the activation of molecular  $O_2$  is based on the oxidation of a Tyr residue or another amino acid and is similar to the epoxidation of olefins by lipid peroxy radicals.<sup>37</sup> The peroxy radical generated by the reaction of the protein radical with molecular  $O_2$  would be expected to react with the double bond of styrene to give a transient species with the impaired electron adjacent to the aromatic ring. Rupture of the peroxide bond in a second step would yield styrene oxide and a protein-bound alkoxy radical that could be quenched by electron transfer to or from the heme. This mechanism is consistent with the incorporation of an atom of molecular  $O_2$  into the styrene oxide.

A prerequisite for the above mechanism is that the protein radical involved in styrene oxidation is readily accessible to molecular  $O_2$  and to styrene. Phenylhydrazines, substrates comparable in size to styrene, do bind the heme iron atom and, therefore, can enter the heme pocket.<sup>38,39</sup> However, their motion inside the heme pocket is severely restricted as shown by NMR studies.<sup>40</sup> The discrepancy between the need for an unconstrained reaction environment and the congested nature of the heme binding sites is readily resolved by the proposed mechanism because the

<sup>37</sup> G. A. Reed, E. A. Brooks, and T. E. Eling, *J. Biol. Chem.* **259**, 5591 (1984).

<sup>38</sup> K. L. Kunze and P. R. Ortiz de Montellano, *J. Am. Chem. Soc.* **105**, 1380 (1983).

<sup>39</sup> D. Ringo, G. A. Petsko, D. E. Kerr, and P. R. Ortiz de Montellano, *Biochemistry* **23**, 2 (1984).

<sup>40</sup> P. R. Ortiz de Montellano and D. E. Kerr, *Biochemistry* **24**, 1147 (1985).

reaction with styrene is expected to occur outside the heme pocket. The Tyr radical on the outer protein surface may, therefore, react with  $O_2$  and styrene outside the protein. The Tyr residue can even pick up  $O_2$  inside the heme pocket and deliver it, by a rotation, to styrene outside the protein. This latter mechanism can rationalize preferential incorporation of  $O_2$  from  $H_2O_2$  into styrene oxide.

The incorporation of  $O_2$  from  $H_2O_2$  may stem from a direct reaction of the ferryl  $O_2$  with styrene in the active site (Scheme II), but it is likely that the fraction of  $O_2$  that derives from  $H_2O_2$  and that is incorporated into the epoxide reflects an initial conversion of  $H_2O_2$  to molecular  $O_2$ , which is then utilized by the protein radical as in Scheme I to oxidize styrene. The possibility that benzaldehyde arises from reaction of styrene with singlet  $O_2$  generated *in situ* is supported by the partial inhibition caused by DABCO (Table II). The aldehyde could, alternatively, arise from a reaction of the peroxy styrene radical intermediate with a second molecule of  $O_2$ .

Styrene is better substrate than *trans*- and *cis*-stilbene in the  $H_2O_2$ -dependent oxidation to olefins oxides catalyzed by Hb (Table III). Experiments with  $H_2^{18}O_2$  and  $^{18}O_2$  have shown that  $O_2$  produced from *trans*- and *cis*-stilbene derives exclusively from  $H_2O_2$ . On the other side, in styrene oxide and in *trans*-stilbene oxide produced from *cis*-stilbene,  $O_2$  derives partly from  $^{18}O_2$ .

These experiments indicate that, as far as the oxidation of olefins to olefins oxides is concerned, the behavior of Hb is much like that of an enzyme. This reaction may be relevant *in vivo* in the activation of styrene to its toxic metabolite styrene oxide, as shown by experiments with oxygenated erythrocytes. It was in fact observed that more styrene oxide was formed when perfusing rat livers in the presence of erythrocytes than in a physiological medium.<sup>41</sup> Moreover, sister chromatid exchanges, one of the toxic effects of styrene, were more frequent in cultures of human lymphocytes contaminated with erythrocytes.<sup>42</sup> Although the liver seems able to detoxify to styrene glycol all of the styrene oxide produced by erythrocytes and the liver,<sup>41</sup> the metabolic activation of styrene and possibly other olefins to reactive metabolites by blood may have some relevance in the production of toxic effects.

<sup>41</sup> G. Belvedere, E. Elovaara, and H. Vainio, *Toxicol. Lett.* **23**, 157 (1984).

<sup>42</sup> H. Norppa, H. Vainio, M. Sorsa and G. Belvedere, *Arch. Toxicol., Suppl.* **7**, 286 (1984).