A series of \(p\)- and \(m\)-substituted phenols were examined for their effect on the cyclooxygenase activity of prostaglandin \(H\) synthase in 0.1 M phosphate buffer at pH 8.0 and 25.0 ± 0.1 °C. A biphasic response was observed. At low concentrations phenols stimulate, but at higher concentrations inhibit, cyclooxygenase activity. Both enhancement and inhibition are increased by phenolic substituents which are electron-donating, quantified by Hammett \(\sigma\) constants, and hydrophobic, quantified by Hansch \(\pi\) constants. The same series of substituted phenols was also reacted with compound II of prostaglandin \(H\) synthase at 4.0 ± 0.5 °C. The compound II data fit the Hammett \(p\sigma\) equation; no hydrophobic factors are required. Phenols inhibit cyclooxygenase activity by interfering with the binding of arachidonic acid to compound I and by competing directly with arachidonic acid as reducing substrates for compound I. Phenols stimulate cyclooxygenase activity by acting as reducing substrates for compound II, thereby accelerating the peroxidatic cycle. Phenols also protect the enzyme from self-catalyzed inactivation, most likely by removing the free radical of prostaglandin \(G_2\) by reducing it to prostaglandin \(G_2\). Kinetic parameters \(K_m\) and \(k_{cat}\) for cyclooxygenase activity were determined in the presence of phenols. Identical values of \(K_m\) (15.3 ± 0.5 mm) and \(k_{cat}\) (89 ± 2 s\(^{-1}\)) were obtained regardless of which phenol was employed. Therefore these represent the true \(K_m\) and \(k_{cat}\) values for cyclooxygenase activity.

Prostaglandin \(H\) (PG\(H\))\(^1\) synthase is a key enzyme in the biosynthesis of prostaglandins, prostacyclins, and thromboxanes. It is two enzymes in one: a cyclooxygenase and a peroxidase (1–3). The cyclooxygenase component converts arachidonic acid to a hydroperoxy endoperoxide (PG\(G_2\)), and the peroxidase component reduces the endoperoxide to the corresponding alcohol (PG\(H_2\)) (4–6), the precursor of all prostaglandins.

The relationship between these two activities has attracted considerable attention but is not yet clearly defined. The cyclooxygenase activity requires hydroperoxide for the initial activation (7–10), whereas the peroxidase activity removes hydroperoxide. This would be paradoxical if, as originally believed, cyclooxygenase and peroxidase activities were independent. However, a synergistic interaction was found between these two activities present in the same molecule (11). Both cyclooxygenase and peroxidase activities require heme (12–15), but only one heme is present per subunit for full catalytic activity (16). Therefore, it was proposed that the cyclooxygenase reaction shares some common enzyme intermediates with the peroxidase reaction (11). A tyrosyl radical has been detected in the reaction of PGHS with hydroperoxides or with arachidonic acid and \(O_2\) (17, 18). It appears to be formed by an intramolecular electron transfer from a tyrosine residue on the protein to the oxidized heme, and it has been suggested that it is the tyrosyl radical which is involved in the initiation step of the cyclooxygenase reaction (17, 18).

The self-catalyzed inactivation of cyclooxygenase activity (7, 19, 20) can be diminished substantially by some peroxidase-reducing cosubstrates (20–22). On the other hand, some excellent reducing substrates are inhibitors of the cyclooxygenase reaction under certain conditions (23–25). Do both the stimulatory and inhibitory effects of reducing substrates on cyclooxygenase activity result from their reducing capability? If so, then a linear free energy relationship with \(\sigma\), the Hammett electronic substituent constant, might be expected in both cases. In addition, these two relationships might be expected to be parallel to the correlation of the peroxidatic rate constants for the same series of reducing substrates with \(\sigma\). Therefore, we attempted to investigate the quantitative structure-activity relationships for both the effect of reducing substrates on cyclooxygenase activity and their reducing efficiency in the peroxidase cycle.

Phenols are known as antioxidants and have been widely used as drugs or antimicrobial agents. Their peroxidatic oxidation plays an important role in chemical toxicity and chemical carcinogenesis (26–28). Therefore we selected monosubstituted phenols as cosubstrates to perform the investigations on PGHS. We found that the same phenols could both stimulate and inhibit cyclooxygenase activity; at lower concentrations, stimulation was observed, and at higher concentrations, inhibition. We have defined and measured their stimulatory ability in terms of \(A_{50}\) the concentration of phenol required to produce one-half of the maximum observed stimulation in the rate of oxygen consumption; and inhibitory ability in terms of \(I_{50}\), the concentration of phenol required to reduce the rate of oxygen consumption to one-half its maximum. In addition we measured the second order rate constants for reactions of the phenols with PGHS-II. The correlations of \(A_{50}\) and \(I_{50}\) for the cyclooxygenase reaction with substituent constants were obtained and compared with the Hammett \(p\sigma\) equation determined for the peroxidase reaction. We found that in the reactions with PGHS-II, only electronic effects...
are observed, whereas in the cyclooxygenase reaction both electronic and hydrophobic effects of phenol substituents are observed.

**MATERIALS AND METHODS**

PGHS was isolated and purified from ram seminal vesicles using a published procedure (29) as modified (30). In this procedure DDC is always added as a stabilizing agent during enzyme purification and storage. The purified enzyme obtained is roughly a 50/50 mixture of apo- and holoenzyme and had a specific cyclooxygenase activity of 42-45 μmol of arachidonic acid oxidized per mg of protein/min in the presence of 1 μM hematin and 1 mM phenol. The enzyme preparations showed a single band in sodium dodecyl sulfate-gel electrophoresis. These features did not vary significantly from batch to batch. For all of our kinetic measurements enzyme preparations were used without reconstitution with hematin.

Arachidonic acid with a purity of 99% was purchased from Sigma and treated with borohydride immobilized on Amberlite IRA-400 (Aldrich) to remove the contaminating hydroperoxide. The immobilized borohydride, prepared as described previously (31), was added to a stock solution of arachidonic acid in benzene. The mixture was allowed to stand at room temperature for 5-10 min with occasional stirring and was then kept at -20 °C. The working solution was prepared just before use as follows. An aliquot of benzene stock solution was dried under a stream of nitrogen, and then absolute ethanol was added to the residue. The resultant ethanolic solution was kept on ice, and small aliquots were removed for the subsequent experiments. The efficiency of immobilized borohydride to remove trace hydroperoxide was checked by measuring the effect of its treatment on the ability of hydroperoxide solution to initiate cyclooxygenase activity of PGHS (31).

All other reagents were of the highest grade available. PPHP was purchased from Oxford Biochemical Research. Analytical reagent grade phenol was obtained from British Drug House; p-chloro-, p-methoxy-, p-ethoxyphenol (99%) and p-hydroxybenzoic (Aldrich) to remove the contaminating hydroperoxide. The immobilized borohydride was prepared as described previously (31). The working solution was prepared just before use as follows. An aliquot of benzene stock solution was dried under a stream of nitrogen, and then absolute ethanol was added to the residue. The resultant ethanolic solution was kept on ice, and small aliquots were removed for the subsequent experiments. The efficiency of immobilized borohydride to remove trace hydroperoxide was checked by measuring the effect of its treatment on the ability of hydroperoxide solution to initiate cyclooxygenase activity of PGHS (31).

All other reagents were of the highest grade available. PPHP was purchased from Oxford Biochemical Research. Analytical reagent grade phenol was obtained from British Drug House; p-chloro-, p-methoxy-, p-ethoxyphenol (99%) and p-hydroxybenzoic acid (99%), from Aldrich; and m-ethoxyphenol, from Eastman Kodak.

Stock solutions of all phenolic compounds were prepared just before use in deionized water, except m-ethoxyphenol, which was prepared in a mixture of ethanol with water. The amount of ethanol in the final reaction mixture had no effect on the rate of the cyclooxygenase reaction. All phenolic compound stock solutions were kept on ice and protected from light.

Enzyme concentrations were determined spectrophotometrically at 410 nm using an extinction coefficient of 123 mM cm⁻¹ (16). Optical absorption measurements were made on a Cary 215 recording spectrophotometer.

**Cyclooxygenase Reaction**—The cyclooxygenase reaction was followed by measuring O₂ consumption by arachidonic acid at 25.0 ± 0.1 °C in 0.1 M phosphate buffer, pH 8.0, using a Yellow Spring Instrument model 53 oxygen monitor. For experiments to determine the kinetic parameters Kₚ and kₗ, the reaction mixture contained 53 nM PGHS and phenolic compound at a fixed concentration. The concentration of arachidonic acid was varied from 0 to 110 μM. However, for experiments to examine the effect of phenols, their concentration was varied, whereas the arachidonic acid concentration was held constant at 30 μM. The reaction rate was determined from the maximum slope of the recorded O₂ consumption curve.

**Reduction of PGHS-II by Substituted Phenols**—The reactions of PGHS-II with phenols were investigated at 4.0 ± 0.5 °C by transient state kinetics, using rapid scan and stopped-flow techniques, performed with a P hotol (formerly Union Giken) rapid reaction analyzer model 601. One reservoir contained enzyme with DDC in buffer, the other reservoir contained PPHP and phenolic compound in the same buffer. No reaction was found between PPHP and phenolic compound in independent experiments. The reaction was monitored by measuring the absorbance change at 410 nm, the wavelength of maximum absorbance of the native enzyme. Pseudo-first order rate constants were obtained by a nonlinear least squares analysis carried out by computer.

**RESULTS**

**Experimental Measurements**

**Cyclooxygenase Activity**—The rate of the cyclooxygenase reaction was determined by measuring the amount of oxygen consumed as a function of time. A typical result is shown in Fig. 1. An induction period is observed followed by a burst in oxygen consumption which levels off as the reaction nears completion. We have defined the rate of oxygen consumption for each experiment in terms of the slope at the inflection point, as illustrated in Fig. 1; this is the quantitative parameter we have used to correlate all of our results on cyclooxygenase activity.

**Reduction of PGHS-II**—Both PGHS-I and PGHS-II have a very short life. Therefore the reactions were studied by starting with native PGHS. The reaction starts from oxidation of PGHS by PPHP to produce PGHS-I. PGHS-I is then reduced by phenols to PGHS-II which is subsequently reduced back to the native enzyme. Examples of the kinetic traces are shown in Fig. 2, which were obtained by monitoring the
absorbance change at 410 nm. The initial rapid decrease in absorbance corresponds to PGHS-I formation followed by its reduction to PGHS-II, whereas the later increase in absorbance corresponds to the reduction of PGHS-II to the native state. The pseudo-first order rate constants $k_{obs}$ for PGHS-II converting back to native enzyme were obtained by computer exponential curve fitting to the latter part of the trace.

A rate equation for the conversion of PGHS-II to native enzyme under pseudo-first order conditions was derived previously for phenol and hydroquinone (32) and is applicable here.

$$v = k_{obs}[\text{PGHS-II}]$$  \hspace{1cm} (1)

where $k_{obs}$ is given by the following.

$$k_{obs} = k_p[\text{phenolic compound}] + k_0[\text{DDC}] + k_u$$  \hspace{1cm} (2)

In Equation 2 $k_p$ is the rate constant for the reaction of PGHS-II with a substituted phenol, $k_0$ is the rate constant for reaction of PGHS-II with DDC, the necessary stabilizing agent present in the enzyme preparations, and $k_u$ is the rate constant for spontaneous decay of PGHS-II to the native state (33). The value of $k_p$ can be obtained from one set of experiments in which DDC concentration is fixed, but the phenolic compound concentration is varied. An example of a plot of $k_{obs}$ versus concentration of substituted phenol is shown in the inset to Fig. 2a.

**Stimulation of Cyclooxygenase Activity by Lower Concentrations of Phenols**

At fixed concentrations of arachidonic acid and PGHS the $O_2$ consumption rate increased with increasing phenol concentration until a plateau value was reached as shown in Fig. 3. A similar result has been reported (7) illustrating protection of the enzyme by phenol from self-catalyzed inactivation. The difference denoted as $\Delta(dO_2/\Delta t)$ between the maximum rate and the rate in the absence of phenol increased with the concentration of arachidonic acid (Fig. 3, inset); so does the concentration of phenol at which the maximum rate of oxygen consumption is attained (data not shown). Based on the concentrations of phenol determined for the optimum rate of $O_2$ consumption at different arachidonic acid concentrations up to 110 $\mu$M, the concentration of 1 mM for phenol was found suitable to be employed for protecting the enzyme in the cyclooxygenase reaction system. The change of the rate of $O_2$ consumption with arachidonic acid concentration in the presence of 1 mM phenol is plotted in Fig. 4 as closed circles. The double reciprocal plot (Fig. 5a) showed a deviation from linearity at concentrations of arachidonic acid above 40 $\mu$M. It is likely that 1 mM phenol cannot completely protect 53 nM PGHS from suicide inactivation at higher concentrations of arachidonic acid.

Similar stimulatory behavior was observed when phenol was replaced by substituted phenols. However, the efficiency of phenols protecting PGHS was different; the higher the electron donating ability of the substituent the larger the protection by the substituted phenol. The saturation curves of rate of oxygen consumption by arachidonic acid in the presence of hydroquinone or $p$-hydroxybenzoic acid are presented in Fig. 4 together with the saturation curve in the presence of phenol. The double reciprocal plot (Fig. 5b) shows that with 0.2 mM hydroquinone, 53 nM enzyme can be completely protected at concentrations of arachidonic acid up to 100 $\mu$M. However, 12 mM $p$-hydroxybenzoic acid can protect the same amount enzyme only at arachidonic acid concentrations below 40 $\mu$M (Fig. 5c).

Kinetic parameters $K_m$ and $k_{cat}$ for cyclooxygenase activity were estimated in the presence of phenol, hydroquinone, or $p$-hydroxybenzoic acid by nonlinear least squares fitting of $O_2$ consumption rates to the Michaelis-Menten equation (Equation 3).

$$v = \frac{k_{cat}}{[E_0] + k_m/[\text{AA}]}$$  \hspace{1cm} (3)

where $v = -d[O_2]/dt$ as defined by inflection points as illustrated in Fig. 1, $[E_0]$ is the concentration of native PGHS as determined by spectrophotometry, and AA is arachidonic acid. Only data points corresponding to the linear portions of the double reciprocal plots shown in Fig. 5 were used; results at higher concentrations of arachidonic acid were not included. Results are given in Table I along with those reported in the literature (34, 35). It is difficult to compare these values obtained under different conditions. However, the values of $K_m$ and $k_{cat}$ obtained in this study are identical to one another within experimental error regardless of which substituted...
Prostaglandin H Synthase Kinetics

1.0 -

0

0.1 0.2

[AA], μM

FIG. 5. Double reciprocal plot of O₂ consumption rate versus arachidonic acid (AA) concentration in the presence of a, 1 mM phenol; b, 0.2 mM hydroquinone; c, 12 mM p-hydroxybenzoic acid. Reaction conditions are described in Fig. 4.

TABLE I

Kinetic parameters \( K_\text{m} \) and \( k_{\text{cat}} \) for cyclooxygenase activity of PGH synthase reacting with arachidonic acid in the presence of cosubstrate in 0.1 M phosphate buffer, pH 8.0, at 25.0 ± 0.1 °C

<table>
<thead>
<tr>
<th>Cosubstrate</th>
<th>( K_\text{m} ) (μM)</th>
<th>( k_{\text{cat}} ) (μM s⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol (1.0 mM)</td>
<td>14.6 ± 0.3</td>
<td>87 ± 2</td>
<td>Present work</td>
</tr>
<tr>
<td>Hydroquinone (0.2 mM)</td>
<td>15.9 ± 0.3</td>
<td>89 ± 2</td>
<td>Present work</td>
</tr>
<tr>
<td>p-Hydroxybenzoic acid (12 mM)</td>
<td>15.4 ± 0.6</td>
<td>91 ± 4</td>
<td>Present work</td>
</tr>
<tr>
<td>Phenol (1 mM)</td>
<td>2*</td>
<td>31</td>
<td>34</td>
</tr>
<tr>
<td>and glutathione (1 mM)</td>
<td></td>
<td></td>
<td>31.5 35†</td>
</tr>
</tbody>
</table>

*In 0.1 M phosphate buffer, pH 7.2, containing 1 mM phenol and 1 mM glutathione, at 30 °C.

†No experimental conditions were provided.

phenol is present. This might indicate that the enzyme was completely protected from suicide inactivation, at least in the early stages of the reaction, under these conditions. Thus, \( K_\text{m} \) and \( k_{\text{cat}} \) values reported here appear to be the intrinsic values for the cyclooxygenase reaction.

Inhibition of Cyclooxygenase Activity by Higher Concentrations of Phenols

Above we have shown that phenols have a stimulatory effect on cyclooxygenase activity. However, when the concentration of phenol is increased above a certain value, an inhibitory effect is observed. Examples are shown in Fig. 6. This biphasic response is common for all substituted phenols which were investigated except for p-hydroxybenzoic acid. No inhibition effect was observed on the reaction of 53 nM PGHS with 30 μM arachidonic acid for concentrations of p-hydroxybenzoic acid up to 26 mM (Fig. 7). Detailed experiments were performed to measure the change in the rate of O₂ consumption with the variation in concentrations of phenols at various fixed concentrations of arachidonic acid and PGHS. Results (not shown) indicate that the concentration range of phenols at which stimulation or inhibition occurred depended on the
feature of substituent, the concentration of arachidonic acid, and the concentration of PGHS. To have readily comparable results for different phenols we fixed the concentration of PGHS at 53 nM and of arachidonic acid at 30 μM.

The abilities of substituted phenols to stimulate and to inhibit 53 nM PGHS reacting with 30 μM arachidonic acid were estimated as $A_{50}$ and $I_{50}$ values from the curves of $O_2$ consumption rate plotted against the concentrations of phenols. Values of $A_{50}$ and $I_{50}$ determined for various substituted phenols are listed in Table II. Comparisons among these compounds reveal that not only the electronic effect but also the hydrophobic property of the substituent determine the activity of phenols. Both stimulatory and inhibitory effects are increased by substituents which are electron-donating or which are hydrophilic, but they are decreased by substituents which are electron-withdrawing or hydrophobic. This is well illustrated in the comparisons of phenol with p-methoxyphenol and p-methylphenol, of p-ethoxyphenol with m-ethoxyphenol, and of p-ethoxyphenol with methoxyphenol.

Quantitative analysis of these structural effects was conducted by using Equations 4 and 5 which are based on a simple model for electronic and hydrophobic characteristics of the substituents.

$$\log \frac{1}{A_{50}} = a_i \sigma + b_i \pi + c_i \tag{4}$$

$$\log \frac{1}{I_{50}} = a_i \sigma + b_i \pi + c_i \tag{5}$$

Multiple linear regression of the experimental data yielded the following equations.

$$\log \frac{1}{A_{50}} = -2.2\sigma + 0.72\pi - 1.7 \tag{6}$$

$$\log \frac{1}{I_{50}} = -2.6\sigma + 1.1\pi - 0.48 \tag{7}$$

Figs. 8 and 9 show logarithmic plots of the calculated values of $A_{50}$ and $I_{50}$ (Table II) by Equations 6 and 7 against their measured values. The standard deviation for the plot of log $A_{50}$ is 0.13 and for log $I_{50}$ is 0.18.

**Hammett Equation for Substituted Phenols Reacting with PGHS-II**

Phenols serve as good reducing substrates for the peroxidase activity of PGHS via one-electron processes (22, 32). What is the mechanism underneath the stimulatory and inhibitory action of phenols on cyclooxygenase activity? Are these effects connected with the peroxidase reactions? To help answer these questions, the substituent effects on the ability of phenols to reduce PGHS-II, one intermediate in the peroxidase cycle, were investigated. The rate constants for phenol and hydroquinone reacting with PGHS-II have been determined in our previous work (32). In the present study we measured the rate constants for $p$-methoxy, $p$-ethoxy-, $p$-methyl-, $p$-chloro-, and $m$-ethoxy-substituted phenols and $p$-hydroxybenzoic acid. The experimental procedures and data analyses to obtain rate constants are similar to those described previously (32, 38). Results reported here show that in the peroxidase reaction, the electronic effect of the substituent is predominant in determining the reactivity of phenols as reducing substrates, whereas the hydrophobic property of the substituent has little or no effect. This is in contrast to the results for cyclooxygenase activation and inhibition. Thus, $p$-methoxyphenol and $p$-ethoxyphenol, which have very similar $\sigma$ values (-0.27 and -0.24), give the same rate constant within experimental error for their reaction with PGHS-II ($1.7 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$), although their $A_{50}$ and $I_{50}$ values are significantly different because of their different hydrophilicities $\pi$. Fig. 10 is the Hammett $\rho\sigma$ plot for the reduction of PGHS-II by substituted phenols according to Equation 8.

$$\log \frac{k_i}{k_0} = \rho\sigma \tag{8}$$

where $k_x$ and $k_y$ are the rate constants for substituted and unsubstituted phenols reacting with PGHS-II, $\sigma$ is the Hammett electronic substituent constant, and the slope $\rho$ is a measure of the sensitivity of the reaction to $\sigma$. From this plot a $\rho$ value of $-2.0 \pm 0.1 (4^\circ C)$ was obtained, compared with the values of $-2.2$ and $-2.6 (25^\circ C)$ obtained in cyclooxygenase stimulation and inhibition (Equations 6 and 7).

**Inactivation of PGH Synthase during the Cyclooxygenase Reaction in the Absence of Phenols**

Spectral evidence for the suicide inactivation of the cyclooxygenase activity of PGHS during its catalysis is shown in Fig. 11. In the presence of arachidonic acid and DDC but in the absence of a protective phenol inactivation occurs, and the original spectrum of native PGHS is not recovered. This behavior is in marked contrast to that observed in the peroxidase cycle of PGHS with PPHP and reducing substrates (32, 38).

**DISCUSSION**

**Selection of Investigative Method**—The enzymatic reactions of PGHS might be simplified as follows.

<table>
<thead>
<tr>
<th>Table II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Effects of substituents on the ability of phenols to act as stimulators ($A_{50}$) or inhibitors ($I_{50}$) of cyclooxygenase activity of PGH synthase in 0.1 M phosphate buffer, pH 8.0, at 25.0 ± 0.1 °C</strong></td>
</tr>
<tr>
<td>Substituent</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>p-OH</td>
</tr>
<tr>
<td>p-OCH₃</td>
</tr>
<tr>
<td>p-OC₆H₄⁺</td>
</tr>
<tr>
<td>p-CH₃</td>
</tr>
<tr>
<td>H</td>
</tr>
<tr>
<td>m-OC₆H₄⁺</td>
</tr>
<tr>
<td>p-Cl</td>
</tr>
<tr>
<td>p-COO⁻</td>
</tr>
</tbody>
</table>

*a $\sigma$ values are taken from Ref. 36, except values for p-CH₃ and p-COO⁻, which are quoted in Ref. 37.

*b $\pi$ values are from Ref. 36.

*c For definitions of $A_{50}$ and $I_{50}$, see text.

*d $A_{50}$ and $I_{50}$ are calculated values from Equations 4 and 5.
that of other peroxidases (39). The peroxidatic cycle of PGHS (Equations 10-12) is similar to cyclooxygenase activity to a hydroperoxide PGG2, which is subsequently reduced by peroxidase activity to PGH2. The value for p-hydroxybenzoic acid does not fit the plot.

According to this scheme arachidonic acid at first is converted by cyclooxygenase activity to hydroperoxide PGG2, which is subsequently reduced by peroxidase activity to PGH2. The peroxidatic cycle of PGHS (Equations 10-12) is similar to that of other peroxidases (39).

An intrinsic property of this enzyme is the suicide inactivation of cyclooxygenase activity (7, 15, 19, 20). In addition, both cyclooxygenase and peroxidase activities are inactivated by hydroperoxides (21, 22, 24, 40), and so the product PGG2 is a feedback inhibitor. This complicates a steady-state kinetic study of the cyclooxygenase reaction. To avoid these complications, it would be ideal to study the cyclooxygenase reaction by transient-state kinetics. Since compound I formation (Equation 10) and its reaction with reducing substrate (Equation 11) e.g. DDC (the stabilizing agent present in enzyme preparations) are fast and the rate constant for these two steps have been determined (33), it might be possible to employ rapid scan and stopped-flow techniques to follow the reaction of PGHS with arachidonic acid by monitoring PGHS-II formation if the step of PGHS-II reduction to the native state is sufficiently slow compared with the other steps (Equation 12).

However, our results showed the following. (i) The rate constant of the overall conversion of arachidonic acid to PGG2 is about $4 \times 10^4$ M$^{-1}$ s$^{-1}$ at 25.0 °C (data not shown), which is even slower than the rate constant of $2 \times 10^4$ M$^{-1}$ s$^{-1}$ at 4 °C for the conversion of PGHS-II by DDC to the native state (33). Thus, the step of PGHS-II reduction to native enzyme cannot be neglected in the kinetic study of cyclooxygenase reaction, resulting in a very complicated rate equation. (ii) Rapid scan spectra (Fig. 11) and kinetic traces under different conditions demonstrated that the enzyme inactivates even in the transient phase when the arachidonic acid concentration is in 15-fold molar excess over that of the enzyme. This limited the accessible concentration range of arachidonic acid. (iii) To protect the enzyme from self-catalyzed destruction at higher arachidonic acid concentrations, phenol had to be present which speeded up the conversion of PGHS-II to the native enzyme. For all of these reasons we decided to employ rapid scan spectra of 1.16 μM PGHS during its reaction with 52.7 μM arachidonic acid in the presence of 20 μM DDC, in 0.1 M phosphate buffer, pH 8.0, at 25.0 ± 0.1 °C. a, spectra 0.46–3.32 s after mixing. From 2.22 to 3.32 s the spectrum remained the same. b, spectra 3.35–8.30 s after mixing. During this time period, the maximum absorbance shifted back toward 410 nm, but the intensity of the absorbance did not increase back to the original value (about 24% loss of the initial absorbance) indicating bleaching of the enzyme. The spectrum of the original native enzyme is shown for reference. Broad arrows indicate the direction of the absorbance change with increasing time.
We propose that there are dual effects of phenols, both in the enhancement of the cyclooxygenase reaction (small phenol concentrations) and inhibition of it (large phenol concentrations). The enhancement of the rate of arachidonic acid conversion to PGG_2, as measured by maximum rate of oxygen consumption, is partly caused by reduction of PGHS-II by phenols. PGHS-II is not directly required for arachidonic acid conversion; it is a dead end. However, if it is not reduced to the native enzyme then PGHS-I formation ceases.

The other enhancement effect of phenols is to act as hydrogen atom donors for the free radical of PGG_2, reducing it to PGG_1. Without this reduction, the free radical of PGG_2 could abstract a hydrogen atom from the enzyme, leading to enzyme inactivation. Thus the phenols act as free radical scavengers, the well established role for another phenol, α-tocopherol. A phenolic binding site near the arachidonic acid binding site may be required for the free radical scavenging but not for PGHS-II reduction, thus explaining the presence of the hydrophobic effect in enhancement of cyclooxygenase activity.
but the absence of the hydrophobic effect in PGHS-II reduction.

The inhibition of cyclooxygenase activity is explained readily in terms of dual competing effects by phenolic compound and arachidonic acid: (i) competition for a binding site, in which a hydrophobic substituent would be expected to enhance binding of the phenolic compound and (ii) competition for reaction with PGHS-I, in which an electron-donating substituent would be expected to make the phenolic compound more reactive.

Failure to observe the inhibition of cyclooxygenase by p-hydroxybenzoic acid can be explained by very strong hydrophilicity of the carboxyl group, which has a π constant of −4.36. The calculated \( \dot{I}_D \) value from Equation 7 would be 1.5 \( \times 10^7 \) M, and thus lack of inhibition by p-hydroxybenzoic acid under our experimental conditions is not surprising.

The \( \rho \) values for horseradish peroxidases I and II reacting with phenols are −6.9 (37) and −4.6 (45). The larger value of the \( \sigma \) coefficient, −2.6, for inhibition of cyclooxygenase activity by \( \rho \) hydroperoxides or with arachidonic acid but in the presence of various reducing substrates (32, 33, 38) also demonstrates that the cyclooxygenase reaction is 65% initiated by the reducing substrate (32, 50). In this model there are two forms of PGHS-I, a conventional iron(IV) porphyrin \( \pi \) cation radical, and an iron(IV) tyrosyl radical. The latter form can be detected when PGHS is reacted with various hydroperoxides or with arachidonic acid but in the absence of reducing substrates. When reducing substrates are present the ESR signal disappears (50). We could not obtain any transient state optical spectral evidence for tyrosyl radical formation when phenol is present as reducing substrate (32).

The rate constant for the conversion of the porphyrin \( \pi \) cation radical to the tyrosyl cation radical is 65 s\(^{-1}\) (18). Comparison of this rate constant with our rate constants for the spontaneous decay of PGHS-I (33) and its conversion to PGHS-II by different reducing substrates (32, 33, 38) also demonstrates that formation of the tyrosyl radical is unlikely when reducing substrate is present. Furthermore the tyrosyl radical could not be detected in manganese-substituted PGHS (56). One possibility is that the tyrosyl radical form of PGHS-I is an artifact, formed only in the absence of reducing substrate.

At the present stage of our knowledge, direct hydrogen atom abstraction from arachidonic acid by the ferriyl group of PGHS-I is a viable alternative to the tyrosyl radical abstraction. Abstraction of \( \cdot H \) from a C-H bond to form Fe-O-H and -C occurs in a cytochrome P-450 reaction. However, in the cytochrome P-450 reaction the carbon radical remains internally bound in the active site. \( \cdot \) OH addition occurs stereospecifically to the -C radical, and the active enzyme intermediate is converted directly to its ferric state (51–54). In our scheme for PGHS, shown in Fig. 12, the abstracted hydrogen atom from arachidonic acid is transferred to a distal group of PGHS-I as in a normal reduction of a peroxidase compound I to compound II (55–57).

Also shown in Fig. 12 are the mechanisms of inhibition caused by a large excess of phenol. The phenol competes for, or interferes with, the binding site of arachidonic acid on the same enzyme monomer. It also competes directly with arachidonic acid as \( \varepsilon \) reducing substrate for PGHS-I and can shut down PGG\(_2\) formation. Fig. 13 shows the mechanisms of enhancement of the cyclooxygenase reaction by a small excess of phenol. PGHS-II would be a dead end if arachidonic acid were the only reducing agent present. In the presence of phenol it is reduced to native enzyme leading to enzyme recycling. Phenol can also scavenge -PGG\(_2\) which otherwise could inactivate the enzyme by abstracting a hydrogen atom from the active site. Phenol scavenging of -PGG\(_2\) on the enzyme surface could account for the necessary hydrogen peroxide or superoxide formation for enzyme stimulation.

Formation of PGG\(_2\) is a precursor to PGH\(_2\) and PGHS-I formation. The phenol radicals can be inactivated by self-reaction or attack on excess phenol (55, 56).

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Prostaglandin H Synthase Kinetics